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Zamalou Gisèle Ouandaogo, Nelly Frydman, Laetitia Hesters, Said Assou, Delphine Haouzi, et al.. 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.. *Human Reproduction*, 2012, 27 (8), pp.2438-47. 10.1093/humrep/des172 . inserm-00726681

HAL Id: inserm-00726681

<https://inserm.hal.science/inserm-00726681>

Submitted on 1 May 2013

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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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Submitted on January 30, 2012; resubmitted on April 3, 2012; accepted on April 17, 2012

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 (Tsfaye *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 Béclert hospital of Clamart. The ages of the patients (mean \pm SEM) were 33.7 ± 4.7 , 32.3 ± 3.3 and 33.8 ± 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 (CC_{GV}), MI (CC_{MI}) and MII (CC_{MI}) stages. To differentiate samples isolated from *in vitro* (patients with PCOS) or *in vivo* (normal responders) matured COCs, the samples from patients with PCOS were labelled as CC_{GV-IVM}, CC_{MI-IVM} and CC_{MI-IVM}, while the samples from normal responders were marked as CC_{GV}, CC_{MI} and CC_{MI}. Samples were then regrouped into three classes for transcriptomic comparison: Class I (CC_{GV} versus CC_{GV-IVM}), Class II (CC_{MI} versus CC_{MI-IVM}) and Class III (CC_{MI} versus CC_{MI-IVM}).

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* (CC_{MI} PCOS *in vivo*) was added as a control for CC_{MI-IVM}. Thus, another comparison was made between CC_{MI} PCOS *in vivo* and CC_{MI-IVM}.

Protocol for *in vivo* 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^{FTM}, LuverisTM, 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 (Chorionique 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 (MediCult, 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 1). After removal, CCs were put immediately in RLT lysis buffer (RNeasy 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 ConsoleTM software and normalization was performed with the MASS.0 algorithm to obtain the signal intensity and the detection call

Table I Samples used for microarray and RT-qPCR analyses.

Analysis	Maturation condition	Patients type (number of patients)	Oocyte maturation stage	Number of CC samples	
Microarray processing	<i>In vivo</i>	Normal responders (<i>n</i> = 16)	CC _{GV}	8	
			CC _{MI}	8	
			CC _{MII}	8	
	<i>In vitro</i>	PCOS (<i>n</i> = 9)	CC _{MII}	9	
			PCOS (<i>n</i> = 8)	CC _{GV-IVM}	8
				CC _{MI-IVM}	6
RT-qPCR	<i>In vivo</i>	Normal responders (<i>n</i> = 13)	CC _{GV}	8	
			CC _{MII}	8	
	<i>In vitro</i>	PCOS (<i>n</i> = 10)	CC _{GV-IVM}	8	
			CC _{MII-IVM}	8	
Total number of CC samples				88	

CC, cumulus cell; PCOS, polycystic ovary syndrome.

CC_{GV}, CC_{MI} and CC_{MII} 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.

(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).

Microarray data analysis

MAS5 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* > 7. Class II (MI stage) samples were filtered with *P* > 5. The cut-off was *P* > 8 for the first sorting of the CC_{MII} PCOS *in vivo* and CC_{MII-IVM} samples. Then, a second filtering was performed using the coefficient of variation (CV ≥ 40%) which is the ratio, expressed in percentage, between standard deviation (SD) and the mean of the signal intensity of each probe set between samples (second lists). The filtering choices were selected arbitrarily. After selection, the list of genes obtained from each class was also used for unsupervised hierarchical clustering. The Tree View software was used to display the cluster (Eisen et al., 1998).

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: CC_{GV} versus CC_{GV-IVM}, CC_{MI} versus CC_{MI-IVM} and CC_{MII} versus CC_{MII-IVM}. SAM was also used to compare CC_{MII-IVM} and CC_{MII} 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 (CC_{GV} versus CC_{GV-IVM}), II (CC_{MI} versus CC_{MI-IVM}) and III (CC_{MII} versus CC_{MII-IVM}) CC samples with the Babelomics ([\[.bioinfo.cipf.es/\]\(http://babelomics3\)\) 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.](http://babelomics3</p>
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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 CC_{GV} (*n* = 8 samples), CC_{GV-IVM} (*n* = 8), CC_{MII} (*n* = 8) and CC_{MII-IVM} (*n* = 8; Table I). Validation of the CC_{MI} 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 CC_{GV} and CC_{GV-IVM} lists, respectively, and 6/each for the CC_{MII} and CC_{MII-IVM} lists). The primer sequences (Sigma) are listed in Supplementary data, Table S1. 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

formula: $E^{\Delta Ct} = E^{\Delta Ct_{\text{tested primer}} - \Delta Ct_{\text{GAPDH}}}$ ($E = 10^{-1/\text{slope}}$), where $\Delta Ct = Ct_{\text{control}} - Ct_{\text{unknown}}$ and control = one CC sample of CCs.

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 MASS5 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 SII-MII](#).

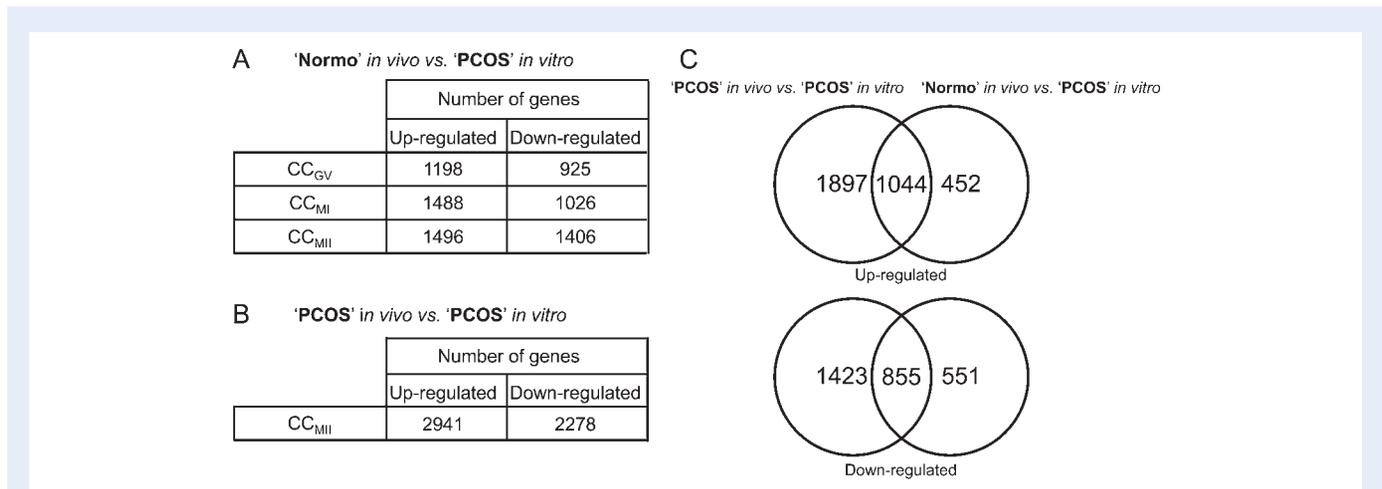
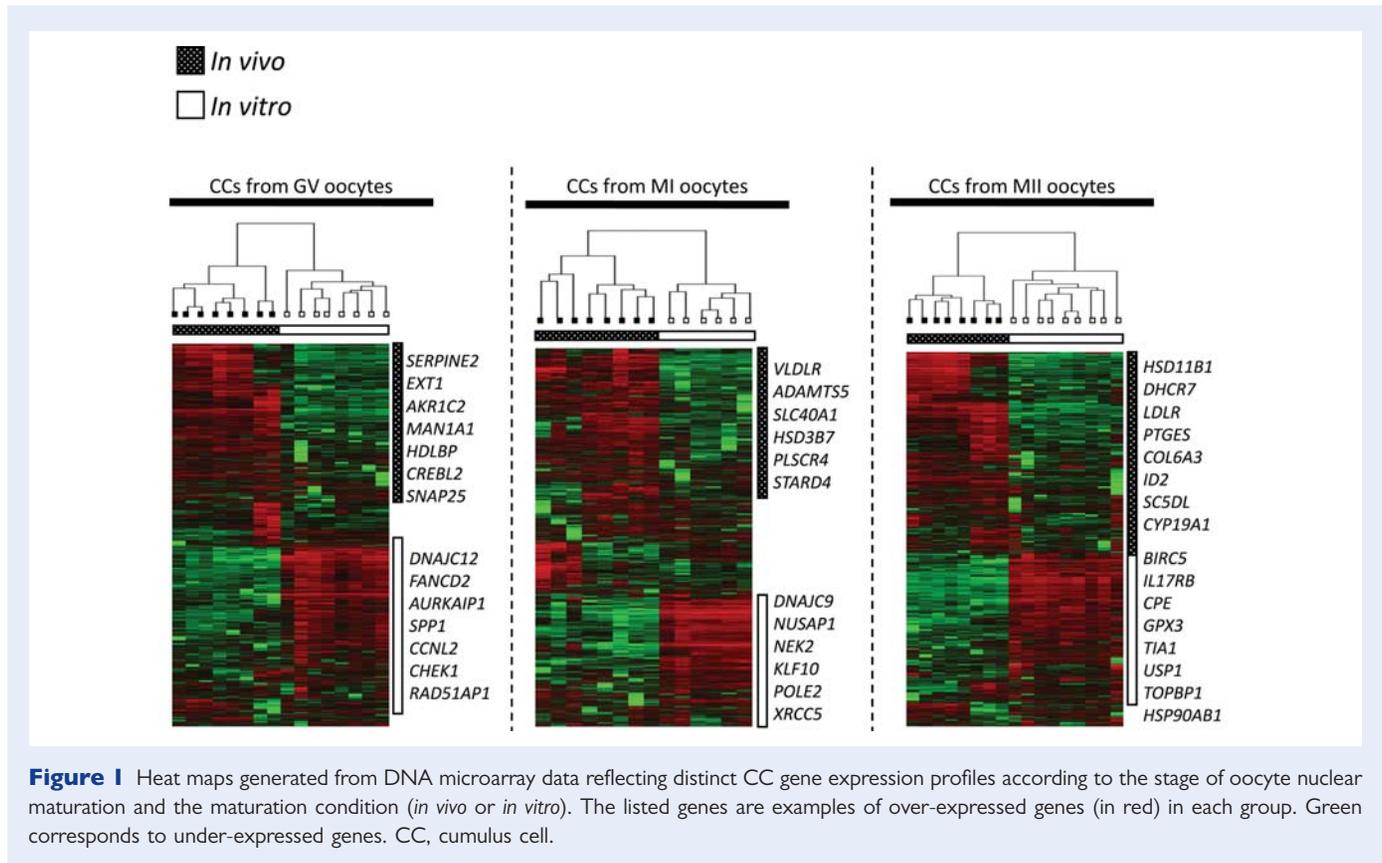
Among the up-regulated transcripts, the gene encoding *VLDLR*, a low-density lipoprotein member, was over-expressed in CC_{MI} (versus CC_{MI-IVM} ; FC = 6.8), whereas the genes encoding the heat shock proteins (*HSP*) *HSP90AB1* (FC = 2.9) and *DNAJC9* (FC = 4.2) were over-expressed in CC_{MI-IVM} (compared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

Moreover, the luteinizing hormone/choriogonadotrophin receptor (*LHCGR*) gene was over-expressed in CC_{MI} (compared with CC_{MI-IVM} ; FC = 20.2) and CC_{MII} (compared with $CC_{MII-IVM}$; 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-IVM} ; FC = 6.0, 4.5, 4.1, respectively), CC_{MI} (compared with CC_{MI-IVM} ; FC = 10.3, 3.6, 4.3, respectively) and CC_{MII} (compared with $CC_{MII-IVM}$; FC = 17.8, 2.8, 7.1, respectively). Other cumulus expansion-related genes, such as pentraxin 3 (*PTX3*), amphiregulin (*AREG*), prostaglandin-endoperoxide synthase 2 (*PTGS2*) and follistatin (*FST*) were also over-expressed in CC_{GV} (compared with CC_{GV-IVM} ; FC = 11.1, 5.3, 3.1, 3.0, respectively), CC_{MI} (compared with CC_{MI-IVM} ; FC = 2.5, 11.1, 5.3, 2.8, respectively) and CC_{MII} (compared with $CC_{MII-IVM}$; 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-IVM} (compared with CC_{GV} ; FC = 12.6, 7.2, 4.0; 5.8, 5.6, respectively), CC_{MI-IVM} (compared with CC_{MI} ; FC = 16.3, 12.8, 4.5, 4.4, 4.9, respectively) and $CC_{MII-IVM}$ (compared with CC_{MII} ; 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_{MII} versus $CC_{MII-IVM}$) was compared with the one obtained by comparing the transcriptome of the $CC_{MII-IVM}$ group with that of CCs that were isolated from MII oocytes of patients with PCOS following *in vivo* maturation (CC_{MII} 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 CC_{MII} PCOS *in vivo* $CC_{MII-IVM}$ 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

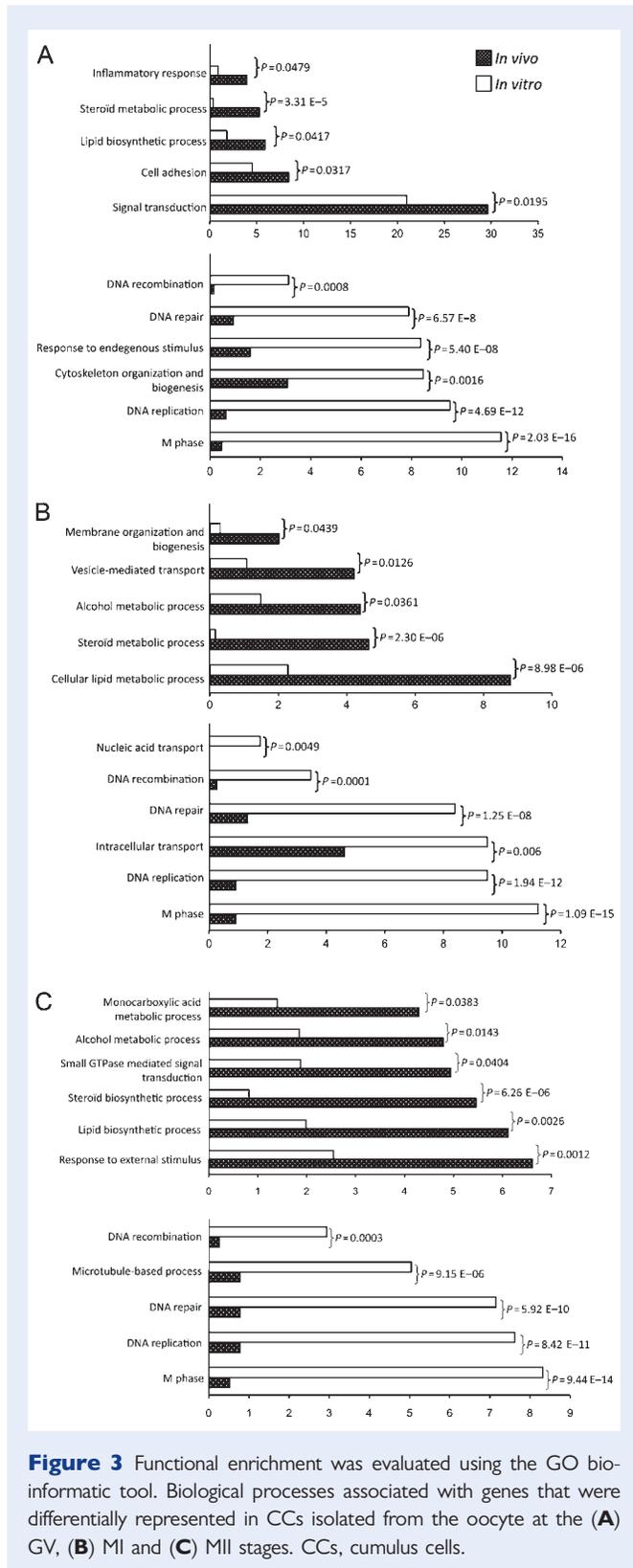
FatiGO was used to assess functional enrichment for specific genes in the three CC classes (GV, MI and MII) following *in vivo* or *in vitro* oocyte maturation. Only significant functions (adjusted *P*-value $< 5\%$) were reported. The comparison of the CC_{GV} and CC_{GV-IVM} gene expression profiles 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-IVM} 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 $CC_{MII-IVM}$; 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 $CC_{MII-IVM}$ 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 signalling pathway was specifically activated in $CC_{MII-IVM}$ and the BRCA1 signalling cascade in CC_{GV-IVM} (Supplementary data, Fig. S1A 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* ($CC_{MII-IVM}$) and *in vivo* (CC_{MII} PCOS *in vivo*) matured COCs at MII. The expression profile of $CC_{MII-IVM}$ was strongly altered in comparison with that of CC_{MII} PCOS *in vivo* and also of CC_{MII} (normal responders; Fig. 5). Specifically, the $CC_{MII-IVM}$ 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 embryo stage.

Conversely following IVM of COCs from patients with PCOS ($n = 277$ samples), 15.52% CCs were isolated from GV oocytes, 11.19% from MI oocytes and 63.18% from MII oocytes (P value = 10^{-7} when compared with the MII percentage from *in vivo* matured oocytes); 10.11% of *in vitro* matured samples were atretic or with empty zona pellucida. Moreover, only 65.71% of mature oocytes (MII) reached the embryo stage (P value = 10^{-6} when compared with the percentage from *in vivo* matured oocytes).

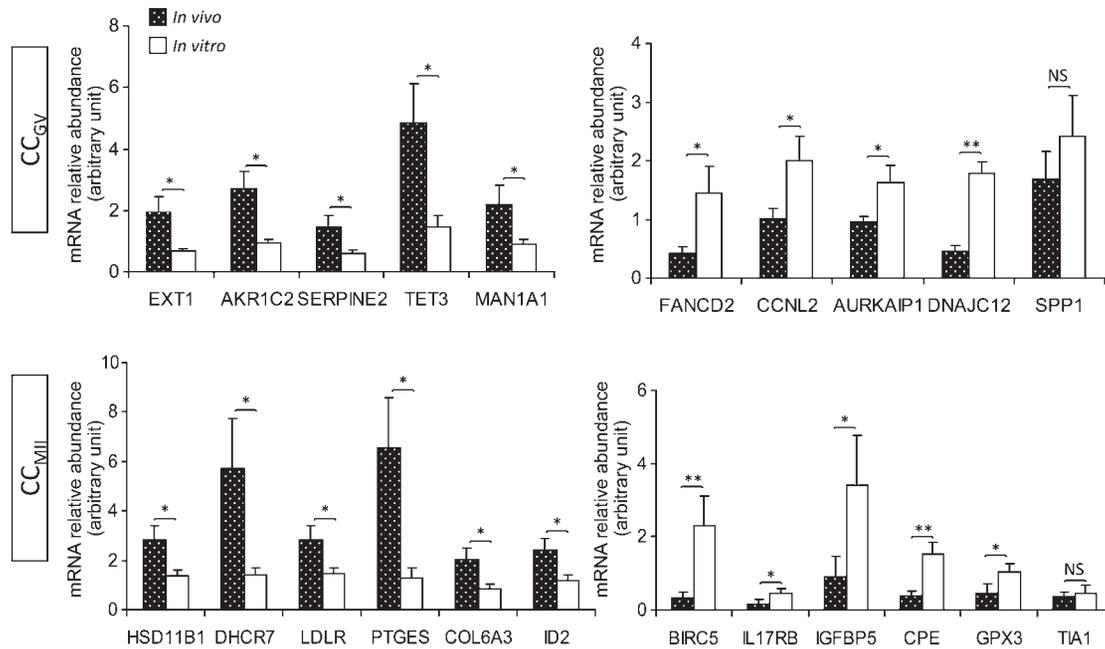


Figure 4 Relative mRNA levels of genes that were differentially regulated in CCs from oocytes at the germinal vesicle (GV) and metaphase II (MII) stages were assessed by RT-qPCR. Graphs show the differential expression of selected transcripts in CCs following *in vivo* and IVM conditions. Data are plotted as the mean \pm SEM. ** $P < 0.01$; * $P < 0.05$; NS, not significant; CCs, cumulus cells.

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

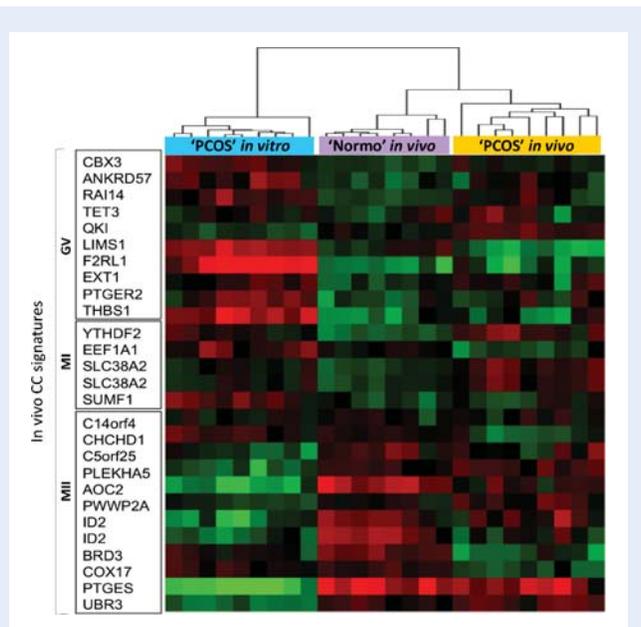


Figure 5 The molecular signature of CCs isolated from *in vitro* matured oocytes is different from the signature of CCs from *in vivo* matured oocytes. More precisely, the molecular signature of the CC_{MI-IVM} group was characterized by the over-expression of the CC_{GV} and CC_{MI} signatures, whereas genes belonging to the CC_{MI} *in vivo* signature were not detected or down-regulated. CCs, cumulus cells; PCOS, polycystic ovary syndrome.

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 (Teskaye *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; Sturmey 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 *FANCD2* and *BIRC5* (Taniguchi *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 *FANCD2* is essential for the S-phase checkpoint (Garcia-Higuera *et al.*, 2001; Joenje and Patel, 2001; Taniguchi *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; Lánská *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_{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 (Dozier *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_{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 MII stage is the consequences of the IVM conditions rather than the PCOS profile. These findings can help understand the less favourable 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/>.

Acknowledgements

This work was supported by Ferring Pharmaceutical, Genevrier and Vitrolife companies. We thank the University Hospital of Montpellier and Paris XI for their support and the ART/PGD teams for providing the samples.

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.

Funding

This work was partially supported by a grant from Ferring and Genevrier Pharmaceutical Companies.

Conflict of interest

None declared.

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