Human Reproduction Vol.22, No.12 pp. 3069–3077, 2007 doi:10.1093/humrep/dem336
Advance Access publication on October 19, 2007

Gene expression in human cumulus cells: one approach to oocyte competence

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BACKGROUND: Dialogue between the oocyte and cumulus cells is essential for oocyte maturation. A prospective laboratory research project was designed to evaluate transcription of specific genes in cumulus cells harvested before intracytoplasmic sperm injection from pre-ovulatory follicles, according to individual oocyte nuclear maturity and developmental competence. Genes were chosen because their expression was induced by the LH peak [Steroidogenic Acute Regulatory protein (*STAR*), Cyclooxygenase 2 (*COX2* or *PTGS2*), Amphiregulin (*AREG*)] or because they were involved in oocyte lipidic metabolism [Stearoyl-Coenzyme A Desaturase 1 and 5 (*SCD1* and *SCD5*)] or in gap-junctions [Connexin 43 (*CX43* or *GJA1*)]. METHODS: mRNA levels in cumulus cells were assessed by real-time PCR. RESULTS: Expression levels of all genes investigated, except Cx43, were increased after resumption of meiosis. Nuclear maturation was thus associated with increased expression of *STAR*, *COX2*, *AREG*, *SCD1* and *SCD5* by cumulus cells. When considering only cumulus associated with metaphase II oocytes, gene expression was independent of morphological status at Day 2. In contrast, transcript levels were lower and distributed over a narrower range in cumulus enclosing oocytes achieving blastocyst development at Day 5/6 than in cumulus enclosing oocytes unable to develop beyond the embryo stage. CONCLUSION: Further developmental potential from embryo to blastocyst stage was associated with lower expression in a narrow range for these genes.

Keywords: oocyte maturation; human cumulus cells; gene expression; developmental competence; resumption of meiosis

Introduction

Single embryo transfer is increasingly used in assisted reproduction in order to avoid adverse outcomes related to multiple pregnancies, and selecting embryos with high implantation potential for this purpose remains one of the major goals in the field of assisted reproduction. Evaluation of the morphological criteria of early embryo development has to date been the most widely used method to select embryos with high development ability. Zygote scoring, early cleavage and embryo morphology at Day 2 or 3 are related to both development and implantation potential, although their contribution to accurate prediction of such potential is quite limited (Guerif *et al*., 2007). In addition to such criteria of early embryo development, defining oocyte quality remains one of the most difficult challenges (Wang and Sun, 2007). Follicular growth and maturation are prerequisites to oocyte fertilization and subsequent early embryo development. Among all the events involved in this process, those taking place precisely at the pre-ovulatory stage within the cumulus-oocyte-complex (COC) might offer new criteria for choosing embryos with the best development ability (Russell and Robker, 2007).

The mechanisms acting in the coordination of maturation of the follicle and its enclosed oocyte are not yet fully understood. During its maturation, the oocyte successively acquires first meiotic competence (nuclear maturation), then developmental competence (cytoplasmic maturation), while being interdependent on surrounding somatic cells (Tanghe *et al*., 2002). *In vitro* maturation experiments with immature oocytes have clearly reported the impact of oocyte–cumulus cell relationships on both maturation and fertilizability (Cross and Brinster, 1970; Goud *et al*., 1998).

Such maturational processes also involve somatic cells, particularly thecal and granulosa cells. Two major functionally distinct phenotypes of granulosa cells develop in antral follicles. Granulosa cells line the follicle wall, whereas cumulus cells form the COC, while maintaining intimate contact with the oocyte. Paracrine factors and low molecular weight factors allow an actual dialogue to be established between oocyte and cumulus cells through gap junctions (Canipari, 2000; Feuerstein *et al*., 2006). The distinct phenotype of cumulus cells is established and maintained by gonadotrophins as well as paracrine factors from oocytes (Eppig *et al*., 2002;

Diaz *et al*., 2007). These factors promote cumulus cell differentiation (Eppig, 2001) and affect the pattern of gene expression and protein synthesis (Hernandez-Gonzalez *et al*., 2006). Moreover, metabolic co-operation between oocyte and cumulus cells has already been established as a determining factor in oocyte maturation (Sutton *et al*., 2003; Sugiura and Eppig, 2005).

Studies in humans do not allow direct access to the oocyte, except in totally unphysiological conditions (immature oocytes after HCG injection, abnormal fertilization) but cumulus cells are easily available since they are always discarded before ICSI procedures. In view of concept of the beneficial effect of surrounding cells on oocyte maturation, and the need for greater understanding of the mechanisms involved in late folliculogenesis and oogenesis, we focused on various target genes in cumulus cells which share the ability to be affected (expression or product) by the pre-ovulatory LH surge, i.e. Steroidogenic Acute Regulatory protein (STAR), Cyclooxygenase 2 (COX2), an epidermal growth factor (EGF)-like protein [Amphiregulin (AREG)], two Stearoyl-Coenzyme A Desaturases (SCD1 and SCD5) and Connexin 43 (CX43).

Our aim was therefore to evaluate the expression of these genes in cumulus cells according to stages in oocyte maturity (nuclear maturity and developmental competence) to establish whether meiotic or developmental competence of the oocyte may be related to a specific pattern of expression of these genes.

Materials and Methods

Patients and IVF treatment

The study participants were 47 women undergoing IVF using intracytoplasmic sperm injection (ICSI). Average patient age was 32 years (range 23–40 years). The ovarian stimulation protocol, the ICSI procedure used and embryo culture with sequential media have already been described elsewhere (Guerif *et al*., 2004). The mean number of oocytes retrieved per patient was 7 (range 3–13 oocytes). Distribution of the collected COC is depicted in Fig. 1.

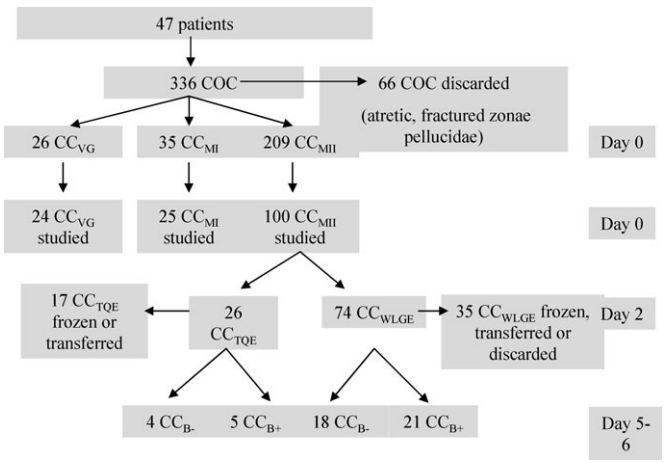

Figure 1: Distribution tree of COC involved in the study for all 47 patientsCOC, cumulus–oocyte-complex; CC_{GV}, cumulus cells from GV oocyte; CC_{MI}, cumulus cells from MI oocyte; CC_{MII}, cumulus cells from MII oocyte; CC_{WLGE}, cumulus cells from oocytes yielding a weak or low grade embryo at Day 2; CC_{TQE}, cumulus cells from oocytes yielding a top quality embryo at Day 2; CC_{B-}, cumulus cells from an oocyte which stopped developing at the embryo stage at Day 6; CC_{B+}, cumulus cells from an oocyte yielding a blastocyst after 5/6 days of *in vitro* culture

Retrieval of cumulus cells

Shortly before ICSI, individual COC were subjected to dissociation. Cumulus cells were separated from the oocyte with strippers after brief exposure to hyaluronidase (80 UI/ml, SynVitro Hyadase®, Medicult, Jyllinge, Denmark) at 37°C. After oocyte recovery, cumulus cells were washed in cold phosphate buffer saline (Dubelcco's medium) (Gibco, Invitrogen, Paris, France) then centrifuged at 200 g for 10 min. The supernatant was removed and the pellet was resuspended in cell lysis buffer of the Absolutely RNA® Nanoprep kit (Stratagene Europe, Amsterdam, The Netherlands) before storage at −80°C. Labelling allowed individual follow-up of the whole process.

Assessment of oocyte and embryo quality

Follow-up of the morphological characteristics of the oocyte and embryo were recorded on an individual basis. At the time of ICSI (Day 0), the oocytes were first classified into three categories based on nuclear status: (i) mature oocyte with the first polar body (metaphase II, MII), (ii) immature oocyte at the germinal vesicle (GV) stage, and (iii) immature oocyte without first polar body or GV, arbitrarily called metaphase I (MI). On Day 1, fertilization was investigated under a microscope and oocytes with abnormal fertilization (triploidy or presence of micronuclei) were discarded. On Day 2 (44–46 h post-ICSI), individually cultured embryos were evaluated for the number of blastomeres, fragmentation rate and presence of multinucleated blastomeres. The degree of fragmentation was expressed as a percentage of the total oocyte volume occupied by anucleate cytoplasmic fragments. Embryos with one or several multinucleated blastomeres were excluded. Embryos with four regular blastomeres, <20% fragmentation and no multinucleated blastomeres were classified as 'top quality' (Guerif *et al*., 2007).

Both the outcome of extended embryo culture and day of blastocyst development (Day 5/6) were recorded for each individually cultured embryo. The blastocyst assessment score was based on the expansion of the blastocoele cavity and the number and cohesiveness of the inner cell mass and trophectodermal cells (Gardner and Schoolcraft, 1999).

RNA extraction and reverse transcription

A total of 149 cumulus were analysed individually. Total RNA extraction was carried out using the Absolutely RNA® Nanoprep kit (Stratagene Stratagene Europe), and a DNase digestion step was included in the kit to remove residual genomic DNA contamination. Total RNA was quantified using a NanoDrop® ND-1000 spectrophotometer (Nyxor Biotech, Paris, France). RNA from each sample was used to generate cDNA using the iScript™ cDNA Synthesis kit (Bio-Rad Laboratories, Marnes-la-Coquette, France) using a blend of oligo(dT) and random hexamer primers to provide complete RNA sequence representation.

Real-time PCR

Real-time PCR amplification reactions were carried out using a Light Cycler apparatus with the iQ detection system and the iQ™ SYBR® Green Supermix kit (Bio-Rad Laboratories). Real-time PCR was used to quantify the mRNA transcripts levels of *STAR*, *COX2*, *CX43*, *AREG*, *SCD1* and *SCD5*, with 18S rRNA and Ribosomal Protein L19 (RPL19) mRNA transcripts as endogenous references.

Table I. Oligonucleotide primer sequences used for real-time PCR in this study.

Gene name	Gene Bank no.	Primer 5′-3′	Product size (bp)
18S	X03205		130
Sense		CGGCGATGCGGCGGCGTTATTCC	
Antisense		CTCCTGGTGGTGCCCTTCCGTCAATTCC	
RPL19	NM_000981		94
Sense		TGAGACCAATGAAATCGCCAATGC	
Antisense		ATGGACCGTCACAGGCTTGC	
STAR	BC010550		76
Sense		GAGCAGAAGGGTGTCATCAGG	
Antisense		TAGAGGGACTTCCAGCCAACG	
COX2	NM_000963		58
Sense		ACTGTACGGGGTTTGTGACTGG	
Antisense		GAAAGGGCATTAATTAGAATGGGAACG	
CX43	NM_000165		158
Sense		GGAGAGGGAGGGGATAAGAGAGC	
Antisense		CCGCTCATTCACATACACAGAACC	
AREG	NM_001657		251
Sense		TGGACCTCAATGACACCTACTCTG	
Antisense		GGGCTTAACTACCTGTTCAACTCTG	
SCD1	NM_005063		58
Sense		TGGAGGATTATCAGTATCACGATTTGC	
Antisense		GTTTCCAGAATGAAGCCCAGAAGG	
SCD5	AF389338		167
Sense		TGACCTGCTTGCTGATCCTGTG	
Antisense		TGAGTGAGATGGTATAGCGGAGAATAG	

Primers (Table I) were designed using the Beacon Designer version 2.0 software (Bio-Rad Laboratories). RPL19 and STAR primers were designed to amplify a specific fragment spanning 2 exons. The PCR products for STAR and RPL19 from all the samples were evaluated by agarose gel electrophoresis to verify the absence of contaminating genomic DNA. The PCR thermal cycling conditions were 95°C for 3 min for polymerase activation and the initial denaturation step, followed by 40 cycles with denaturation at 95°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 s. A melting curve analysis was recorded at the end of the amplification to evaluate the absence of contaminants or primer dimers.

All samples were run in triplicate in 96-well plates with two ranges of standards, negative controls using water as substrate and two control samples included in all plates. The standard curves were deduced from fourfold serial dilutions of cDNA derived from pooled human cumulus cells. For each sample, contents of target genes 18S and RPL19 cDNA were quantified. For each sample, detection was normalized for the mean of each triplicate to the endogenous reference. Depending on the transcript targeted, cumulus samples that did not reveal any expression data were excluded from the analysis.

Table II. STAR, COX2, CX43, AREG, SCD1, SCD5 mRNA relative expression, using real-time RT–PCR with RPL19 as an endogeneous reference, in individual human cumulus cells as a function of embryo status at Day 2 following fertilization.

Target gene	CC_{WLGE} ($n = 71–74$)	CC_{TQE} ($n = 26$)
STAR	9.2 ± 1.3	8.8 ± 1.7
COX2	4.6 ± 0.9	6.9 ± 1.9
CX43	2.8 ± 0.2	3.2 ± 0.5
AREG	4.8 ± 0.6	7.1 ± 2.2
SCD1	9.5 ± 1.5	10.5 ± 2.9
SCD5	3.6 ± 0.4	4.0 ± 0.9

Values are presented as means ± SEM.
CC_{WLGE}, Cumulus cells from oocytes yielding a weak or low grade embryo at Day 2; CC_{TQE}, Cumulus cells from oocytes yielding a top quality embryo at Day 2.

Results were expressed for each group as mean ± standard error (Table II and Results section) or box plots with median and 10th, 25th, 75th and 90th percentiles (Figs. 3 and 4).

Statistical analysis

Statistical analysis was performed using analysis of variance (Anova, Statview 4.1®, Abacus Concept, Berkeley, USA), followed by *post hoc* comparison using the Games–Howell test. Statistical significance was defined as $P < 0.05$.

Results

The biological material studied was cells from individual human cumulus recovered at the time of oocyte collection. The average number of cells was 11 500 cells per cumulus (range 3500–22 600 cells) and an average of 93 ng of total RNA (range 6–581 ng) was purified from each cumulus.

Detection of mRNA in human cumulus cells

All real-time PCR amplicons (six target genes and two reference genes) were of the expected size (Fig. 2). Interestingly,

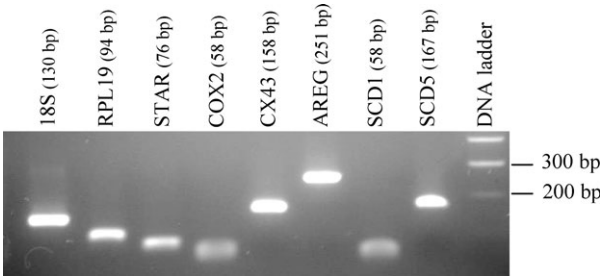

Figure 2: Real-time RT–PCR analysis of expression of target gene mRNA using migration by agarose gel 2.5% electrophoresis
Real-time RT–PCR for 18S and RPL19 was performed as an endogeneous reference

SCD1 and SCD5 were observed for the first time in human cumulus cells. The detection levels of the expression of the target genes were optimized for dilutions ranging from 1/100 to 1/1000 of a single cumulus.

Transcripts levels of target genes according to nuclear maturity of the oocyte

Transcripts levels of target genes in individual cumulus were evaluated according to the maturity of the oocyte nucleus (Fig. 3). Three distinct populations were analysed: (i) cumulus cells from an immature oocyte at the GV stage (CC_{GV}), (ii) cumulus cells from a mature oocyte, i.e. MII (CC_{MII}) and (iii) cumulus cells from an immature oocyte at MI stage (CC_{MI}). The results were normalized using RPL19 as an endogeneous reference.

Mean transcripts levels of all target genes, except *CX43*, were significantly higher in CC_{MII} as compared with CC_{GV}, with 7.6-, 6.5-, 5.8-, 3- and 2.4-fold increases for *STAR* (9.1 ± 1 versus 1.2 ± 0.1), *COX2* (5.2 ± 0.8 versus 0.8 ± 0.2), *SCD1* (9.8 ± 1.3 versus 1.7 ± 0.2), *AREG* (5.4 ± 0.7 versus 1.8 ± 0.3) and *SCD5* (3.7 ± 0.4 versus 1.5 ± 0.3), respectively. Expression levels in CC_{MI} were in between those in CC_{GV} and CC_{MII}. Expression levels of *COX2* and *SCD5* in CC_{MI} were closer to those in CC_{GV}. On the other hand, expression levels in CC_{MI} were closer to those in CC_{MII} for *STAR*, *AREG* and *SCD1*.

Transcripts levels of target genes according to oocyte development outcome

Transcripts levels of target genes were then evaluated according to follow-up of the oocyte after fertilization, first after

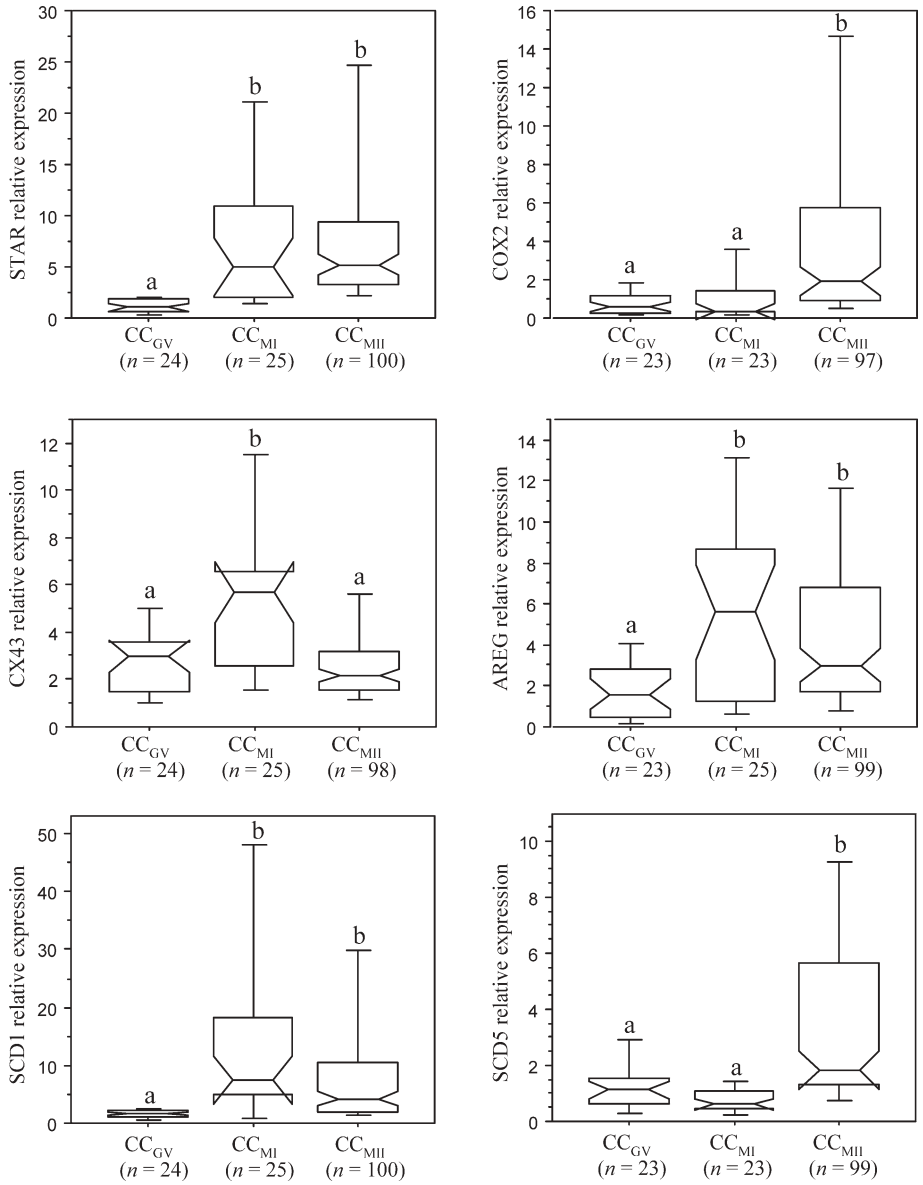

Figure 3: Relative STAR, COX2, CX43, AREG, SCD1, SCD5 mRNA expression using real-time RT–PCR with RPL19 as an endogeneous reference in individual human cumulus cells according to oocyte nucleus maturity
Data are box plotted with median and 10th, 25th, 75th and 90th percentiles. Values with different superscripts refer to significant differences ($P < 0.05$)

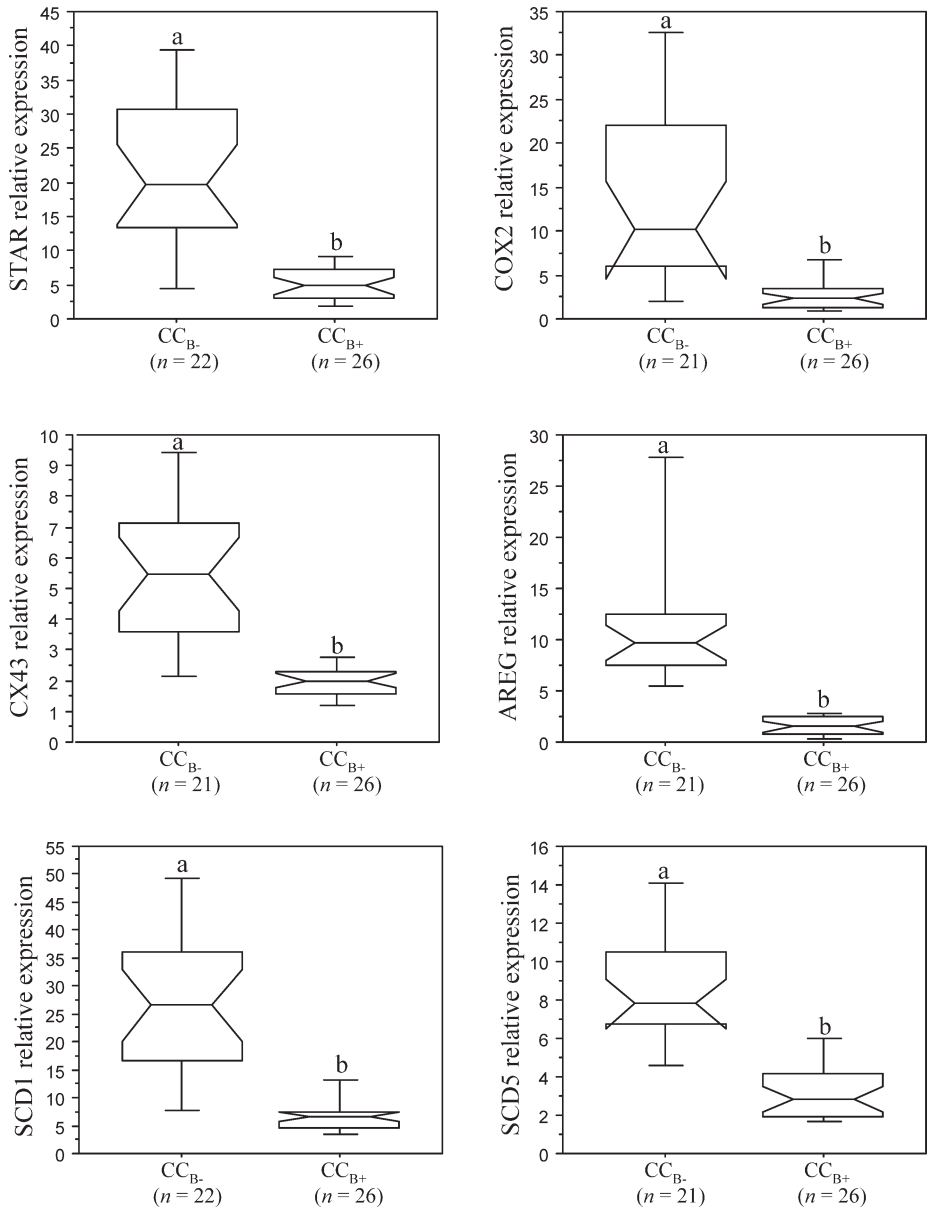

Figure 4: Relative STAR, COX2, CX43, AREG, SCD1, SCD5 mRNA expression using real-time RT–PCR with RPL19 as an endogeneous reference in individual human cumulus cells according oocyte developmental ability after fertilization
Data are box plotted with median and 10th, 25th, 75th and 90th percentiles. Values with different superscripts refer to significant differences ($P < 0.05$)

2 days, and then after 5–6 days of *in vitro* culture, when available. Cumulus cells from MII oocytes were therefore divided into alternate populations: (i) cumulus cells from oocytes yielding a top quality embryo at Day 2 or (ii) cumulus cells from oocytes yielding a weak or low grade embryo at Day 2 and (iii) cumulus cells from oocytes yielding a blastocyst after 5–6 days of *in vitro* culture (CC_{B+}) or (iv) cumulus cells from oocytes which did not develop into a blastocyst at Days 5–6 (CC_{B-}). Mean expression levels of target genes in cumulus cells were not significantly different between those from oocytes yielding top quality embryos and those from oocytes yielding weak or low grade embryos (Table II) in terms of early embryo development.

In contrast, evaluation of transcript expression levels according to the outcome of the fertilized oocytes after 5–6 days of culture demonstrated lower expression of these transcripts (Fig. 4). Mean expression levels for all genes studied were lower in CC_{B+} than in CC_{B-} [*STAR* (5.5 ± 0.6 versus 22.0 ± 3.1), *COX2* (3.4 ± 0.7 versus 14.5 ± 2.6), *CX43* (2.0 ± 0.1 versus 5.6 ± 0.6), *AREG* (1.7 ± 0.3 versus 12.4 ± 1.9), *SCD1* (7.0 ± 0.8 versus 27.4 ± 3.5) and *SCD5* (3.4 ± 0.4 versus 8.9 ± 0.9)].

Discussion

Since cumulus cells can be obtained non-invasively during ICSI and in view of the physiological relevance of the oocyte-cumulus dialogue during the oocyte maturation process, we investigated the expression of various target genes (*STAR*, *COX2*, *CX43*, *AREG*, *SCD1* and *SCD5*) in human cumulus

cells according to individually recorded nuclear or cytoplasmic maturation of the corresponding oocytes. We observed that the expression of these genes increased with the degree of oocyte nucleus maturity. Moreover, their expression in cumulus from MII oocytes was inversely related to their *in vitro* developmental potential.

Our target genes were chosen because their expression in cumulus cells were known to be gonadotrophin dependent.

STAR is a critical enzyme in the steroidogenic activity of the ovary, where it acts as a regulator of cholesterol transport into the inner mitochondrial membrane. *STAR* is expressed in granulosa and cumulus cells after the LH surge (Rimon *et al*., 2004; Sasson *et al*., 2004), and its expression is regulated by various transcription factors (Sugawara *et al*., 1997; Tajima *et al*., 2003). StAR-deficient mice display incomplete follicular maturation and anovulation (Hasegawa *et al*., 2000).

COX2, also known as prostaglandin synthase 2 (PTGS2), is involved in prostaglandin E_2 (i.e. PGE_2) synthesis. PGE_2 synthesis has been reported to increase a few hours before ovulation and to induce cumulus expansion while being essential for maintenance of the structure of the cumulus in the mouse (Davis *et al*., 1999). Infertility in Cox2-deficient mice was found to be related to impaired meiosis resumption, defective ovulation and oocyte fertilization failure (Lim *et al*., 1997).

The EGF-like proteins, Amphiregulin (AREG), Epiregulin and Betacellulin, act as mediators of LH and bind EGF-receptors (EGFR) present on the surface of oocyte and cumulus cells. EGFR activation by EGF-like proteins has been reported to induce cumulus expansion and oocyte nuclear maturation in humans (Freimann *et al*., 2004) and mice (Park *et al*., 2004; Jamnongjit *et al*., 2005).

EGF signalling through EGFR in the mouse was reported to promote both steroidogenesis and oocyte maturation in various models of oocyte–granulosa cell complexes (Jamnongjit *et al*., 2005; Downs and Chen, 2007). Moreover, LH-induced EGF-like proteins in the mouse were reported to induce *Cox2* expression in granulosa cells, leading to PGE_2 production through both autocrine and paracrine mechanisms, with a regulation loop leading to increased expression of EGF-like proteins in cumulus cells (Shimada *et al*., 2006). This mediating effect of PGE_2 on LH induction of EGF-like proteins has been also reported in humans (Ben-Ami *et al*., 2006b).

SCD, also known as delta-9 desaturase, is a microsomal rate-limiting enzyme in the biosynthesis of monounsaturated fatty acids from saturated fatty acids. Among various SCD genes described in different species, two Scd were observed in rat, Scd1 and Scd2. The latter was reported to be expressed in the rat ovary, particularly in cumulus cells where it may be involved in providing unsaturated fatty acids to the oocyte which express few if any Scd (Moreau *et al*., 2006). Two SCD have been identified in humans. The first, SCD1, is the homologue of rodent Scd1 (Zhang *et al*., 1999). The second human SCD does not share any homology with SCD described in any other species except primates and is called SCD5 (Wang *et al*., 2005).

CX43, also known as Gap Junction α1 (GJA1), is one of the major proteins of the gap-junctions expressed in granulosa cells. This protein has been detected at every stage of folliculogenesis in the mouse, mainly in large antral and pre-ovulatory follicles in granulosa cells, and reported to participate in cumulus TransZona Projections (Albertini *et al*., 2001; Teilmann, 2005). Its presence is required for follicular growth and expansion of the cumulus (Ackert *et al*., 2001). These channels not only permit the transfer of metabolites for growth and development (Sugiura and Eppig, 2005) but also play an important role in the maintenance of meiotic arrest of the oocyte (Edry *et al*., 2006).

From a methodological point of view, a major asset of real-time PCR is its sensitivity, allowing us to detect several target transcripts from a single cumulus, whereas a minimum of five cumulus were required for protein detection by Western blot. We therefore chose to analyse levels of mRNA expression of genes in human cumulus cells using real-time PCR to allow individual follow-up.

There were wide variations in the amount of purified RNA attributed to the number of cumulus cells. However, these variations were not related to the degree of oocyte maturity or the level of gene expression. The absence of genomic DNA contamination was deduced, first from the systematic use of DNase included in the kit during RNA extraction, then from the control using PCR products for STAR and RPL19 from all samples to migrate on agarose gel electrophoresis (data not shown), since experimental conditions (iTaq, 30 s, 72°C) should have allowed the detection of such genomic DNA contamination.

Two housekeeping genes were evaluated for this study of cumulus cells, i.e. *18S* and *RPL19*. Expression did not vary for either of these genes depending on oocyte maturity, thus allowing their use as reference genes. However, due to the abundance of 18S RNA, we chose *RPL19* as reference gene, since the abundance of its mRNA was in a range similar to the target mRNAs.

We first analysed transcripts levels in the cumulus of oocytes with increasing degrees of nuclear maturity (GV, MI and MII). Two patterns were observed. First, no significant change in CX43 mRNA level was observed between CC_{GV} and CC_{MII}, although some increase was observed in CC_{MI}. Then an increase in expression level was observed for all other target genes, with two orders of magnitude: 2-fold increase for *SCD5* and *AREG*, and more than 5-fold increase for *STAR*, *COX2* and *SCD1*. Contradictory results have previously been reported concerning whether COX2 level associates with oocyte maturity. The first study in humans using logistic regression analysis and receiver operating curves did not find any correlation [area under the curve, AUC 0.49 (0.34–0.64)] (McKenzie *et al*., 2004), whereas another study in mice reported a clear relationship between *Cox2* expression and nuclear maturation with some insights into the signalling pathways involved in this process (Takahashi *et al*., 2006). We have no clear explanation regarding the heterogeneity of expression values for CC_{MI} compared with other nucleus maturity stages, except that these situations corresponded to a wide-range of stages of meiosis, ranging from oocytes just after GVBD to oocytes just before expulsion of the first polar body. Regarding the higher level of CX43 expression observed in CC_{MI} compared with CC_{GV} and CC_{MII}, a decrease in Cx43 transcripts has already been reported

in bovine COC after 6–24 h of *in vitro* maturation following addition of FSH and LH (Calder *et al*., 2003).

In the next part of our study, we evaluated the expression of target genes in cumulus cells according to the *in vitro* oocyte development potential, as judged by early and late preimplantation development ability. Regarding development at Day 2, only slight and non-significant changes in levels of expression of the six target genes were observed when the embryos were classified on the basis of their morphological characteristics. Interestingly, it has previously been reported in humans (McKenzie *et al*., 2004) that *COX2* may have an acceptable discrimination value for embryo status at Day 3 [AUC 0.76 (0.67–0.86)]. We cannot say whether this difference may be explained by methodological factors, including time of observation (developmental stage at Day 3 rather than at Day 2) and real-time PCR procedure. Progesterone receptor expression in human cumulus cells was recently reported to decrease at the time of ovulation, and this decrease was related to embryo quality at Day 3 (Hasegawa *et al*., 2005). This might be considered similar to our results.

In contrast with development at Day 2, late development at Days 5–6 was related to fairly low levels of transcript expression. Indeed, whereas expression values were high (as already discussed) compared with cumulus cells surrounding GV oocytes, expression levels were in the lower range of the distribution related to CC_{MII}. Such results suggest that the expression of the genes investigated might vary in a precise chronological pattern to allow full developmental competence of oocytes. Furthermore, the biphasic evolution of gene expression might argue for both precise expression of these genes and 'consumption' of their transcripts. Thus late expression or inadequate use of such mRNA might reflect a deleterious effect on oocyte competence.

To the best of our knowledge, little information is available on cumulus gene expression and late *in vitro* embryo development. Greater developmental competence as judged by blastocyst achievement has been reported after *in vitro* maturation of human COC, with intermediate compared to greater numbers of cell layers (Sato *et al*., 2007). The authors hypothesized that an optimal window of hormone profile (estradiol and progesterone production) was required for the best developmental competence. Kinetics studies have been published related to LH peak or HCG administration. Transient expression of *Areg* and *StAR* was reported in rat granulosa cells within 6 h post-HCG injection, followed by a decrease (Jo *et al*., 2004). Similarly, human primary granulosa cells displayed increased expression of *AREG* 2–8 h after LH stimulation, followed by a decrease (Ben-Ami *et al*., 2006a). COX2 mRNA was reported to increase 30–33 h after HCG in equine granulosa cells then to decrease at 36–39 h (Boerboom and Sirois, 1998). In bovine COC, COX2 protein expression increased during *in vitro* maturation between 6 and 24 h. This increase was associated with cumulus expansion and oocyte nucleus maturation (Nuttinck *et al*., 2002). The same group reported similar results regarding the kinetics of expression of COX2 in bovine granulosa cells while characterizing the role of early growth response factor-1 and pituitary adenylate cyclase-activating polypeptide (Sayasith *et al*., 2006; Sayasith *et al*., 2007). Another model using rat pre-ovulatory follicles in culture showed similar biphasic expression of *Cox2* following LH or Ereg stimulation (Tsafriri *et al*., 2005). In contrast, results in primate granulosa cells in culture using semi-quantitative PCR or real-time PCR did not support this biphasic pattern of expression for *COX2*, although a decrease was observed 48 h after addition of HCG (Duffy and Stouffer, 2001; Duffy *et al*., 2005). Short term up-regulation was reported in expression of *StAR*, *Areg* and *Cox2* 0–8 h following HCG injection in mice, without any consistent decrease thereafter, but this study was based on a threshold of 4-fold change in expression values (Hernandez-Gonzalez *et al*., 2006).

Bearing in mind all these results related to the kinetics of expression of genes modulated by LH peak or HCG, we can justify their use in this study in humans, and our findings indicate a possible link between some gene expression patterns and developmental ability. Bearing in mind the data available on the metabolic co-operativity between cumulus cells and the oocyte (Eppig *et al*., 2005) and the expressed factors which might influence oocyte maturation (Progesterone receptors, Hasegawa *et al*., 2005; EGF-like factors (Park *et al*., 2004; Freimann *et al*., 2004), it is tempting to speculate on the importance for such mechanisms to take place in COC at the right moment. Insufficient or late co-operativity might definitely alter the developmental competence of the oocyte, whereas early steps could appear quite normal. In other words, a coordinated dialogue between the oocyte and cumulus cells might involve a specific pattern of gene expression, with precise timing at the level of cumulus cells to sustain or reflect further embryo development. Whether the genes involved in this study were the best suited for such assessment or whether additional genes need to be identified is currently under study in our laboratory.

Acknowledgements

We thank O. Gasnier, J. Poindron and M.H. Saussereau for their help in cumulus–oocyte-complex collection, embryo culture and observation and the clinicians who referred the couples. We thank D. Raine for editing our English.

Funding

This work was supported by a project grant from INSERM (Réseau de Recherche 'Reproduction Humaine-AMP', Contrat No. 4REO3H). P.F. holds an INRA/Ferring SA fellowship.

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Submitted on July 11, 2007; resubmitted on August 17, 2007; accepted on September 12, 2007