Human Reproduction, Vol.25, No.5 pp. 1259–1270, 2010
Advanced Access publication on March 13, 2010 doi:10.1093/humrep/deq049

human reproduction

ORIGINAL ARTICLE *Reproductive biology*

Cumulus cell gene expression is associated with oocyte developmental quality and influenced by patient and treatment characteristics

T. Adriaenssens[1,*], S. Wathlet[1], I. Segers[1], G. Verheyen[2], A. De Vos[2], J. Van der Elst[2], W. Coucke[3], P. Devroey[2], and J. Smitz[1]

[1]Follicle Biology Laboratory, Vrije Universiteit Brussel (VUB), Laarbeeklaan 101, B 1090 Brussels, Belgium [2]Center for Reproductive Medicine, Universitair Ziekenhuis Brussel – Vrije Universiteit Brussel, Laarbeeklaan 101, 1090 Brussels, Belgium [3]Scientific institute of Public health, Department of Clinical Biology, J. Wytsmanstraat 14, 1050 Brussels, Belgium

*Correspondence address. Tel: +32-2-477-50-50; Fax: +32-2-477-50-60; E-mail: tom.adriaenssens@uzbrussel.be

Submitted on June 23, 2009; resubmitted on November 3, 2009; accepted on February 4, 2010

BACKGROUND: Gene expression of cumulus cells (CC) could predict oocyte developmental quality. Knowledge of the genes involved in determining oocyte quality is scanty. The aim was to correlate clinical and biological characteristics during ovarian stimulation with the expression of 10 selected genes in CC.

METHODS: Sixty-three ICSI patients were stimulated with GnRH-agonist plus highly purified hMG ($n = 35$) or recombinant FSH ($n = 28$). Thirteen variables were analyzed: Age, BMI, duration of stimulation, serum concentrations of progesterone, 17β-estradiol, FSH and LH on day of hCG, Ovarian Response, Oocyte Maturity, 2 pronuclei and three embryo morphology related variables: ≥7 cells, Low Fragmentation, Good Quality Embryos score. Expression of HAS2, VCAN, SDC4, ALCAM, GREM1, PTGS1, PTGS2, DUSP16, SPROUTY4 and RPS6KA2 was analyzed in pooled CC using quantitative PCR, and the relationship to the 13 variables was evaluated by multivariable analysis.

RESULTS: All 10 genes are expressed at oocyte retrieval, with PTGS1, SPROUTY4, DUSP16 and RPS6KA2 described in human ovary for the first time. The three variables that correlated most often with differential expression were Age, BMI and serum FSH level. Significant correlation was found with Oocyte Maturity (VCAN, $P < 0.005$), Low Fragmentation (RPS6KA2, $P < 0.05$), Embryos with ≥7 cells (ALCAM and GREM1, $P < 0.05$). The expression of the other genes was also correlated to oocyte developmental quality but to a less extent. SDC4, VCAN, GREM1, SPROUTY4 and RPS6KA2 showed gonadotrophin preparation-dependent expression and/or interactions (all $P < 0.05$).

CONCLUSION: The expression of ovulation related genes in CC is associated with patient and treatment characteristics, oocyte developmental potential and differs with the type of gonadotrophin used.

Key words: cumulus cells / ovulation / gene expression / gonadotrophins / oocyte quality

Introduction

The intrafollicular signaling processes and the molecular events driving ovulation are still largely unknown. This process, initiated by a rise in LH or by hCG administration, enables the release of competent oocytes (Espey and Richards, 2002).

The complex inter- and intracellular communication that coordinates follicle differentiation and oocyte maturation involves the 4 cell types of the ovarian follicle: theca, mural granulosa, cumulus cells (CC) and oocyte. The reciprocal cumulus–oocyte communication in particular deserves further scrutiny, because its preservation contributes to oocyte competence up to fertilization (Albertini *et al.*, 2001). Expression analysis by microarray confirmed that CC represent a distinct population, in as much as these cells not only respond to ovulation with mucification and expansion, but also express neuronal and immune response-related genes in reaction to ovulation to ensure a stable and protective environment for the oocyte (Hernandez-Gonzalez *et al.*, 2006).

For this reason, analyzing CC gene expression in assisted reproduction treatment (ART) cycles is an attractive non-invasive approach to evaluate the oocytes' developmental competence. Full transcriptome analysis in human CC indicated that CC expression correlates to

oocyte developmental potential (Hamel et al., 2008; van Montfoort et al., 2008). Replication of these data in fresh biological samples, however, indicated that a maximum of 40% of the results could be confirmed by array (Hamel et al., 2008) or 33% by quantitative PCR (Q-PCR; van Montfoort et al., 2008). The apparent 'loss' of differentially expressed genes could be attributed to technical differences, but could in part also be a result of the patient's specific treatment regime and differences in genetic background. This inter-patient variation could result from differences in patient characteristics with known effects in reproduction, including age, BMI and ovarian response to the type of gonadotrophin preparation used, the latter also influencing serum steroid levels and follicle fluid cytokine profiles (Smitz et al., 2007, Foster et al., 2009).

In ART, CC become available after follicle aspiration, before completion of ovulation. For this reason, we analyzed a panel of 10 genes (Table I), which have been found to be regulated in CC using micro array analysis in relation to oocyte competence (unpublished data) and are related to the ovulatory LH response. For these genes the importance of patient-to-patient variation is investigated.

The aim of this study was to identify the patient and treatment cycle related variables that significantly influence gene expression in the cumulus oocyte complexes (COC) of 63 patients undergoing controlled ovarian stimulation (COS) with highly purified (HP) hMG or recombinant FSH (rFSH). A total of 13 patient and treatment related variables were tested for their possible interaction with the expression levels of the ten selected genes. For each gene, the three strongest variables and their positive or negative correlation to the expression are discussed in relation to the type of gonadotrophin used.

Materials and Methods

Patient population and ovarian stimulation

This study was approved by the Ethics Committee of UZ-VUB Brussel. Over a period of 6 months, the patients attending the fertility clinic at UZ-VUB Brussel for ICSI, but without severe male factor, and stimulated with HP-hMG or rFSH, were considered for inclusion. Participation in the study involved CC gene expression analysis and collection of patients' stimulation and oocyte-development related characteristics. Sixty-three patients undergoing COS with ICSI were enrolled into the study. COS consisted of the administration of HP-hMG (Menopur, Ferring Pharmaceuticals, Copenhagen, Denmark; $n = 35$) or rFSH (Gonal-f, Serono, Geneva, Switzerland; $n = 15$ or Puregon, Organon, Holland; $n = 13$) following down-regulation using a GnRH-agonist in a long protocol. Daily doses ranged from 121 to 406 IU/day for rFSH and from 134 to 375 IU/day for HP-hMG. GnRH agonist was administered for at least 14 days before starting ovarian stimulation whereas gonadotrophins were administered in incremental doses for 7–17 days. Follicular development was monitored by pelvic ultrasound. The endocrine profile was evaluated by analysis of serum 17β-estradiol (E_2), progesterone, FSH and LH on two to four consecutive days. We used only the final dosage, on day of hCG, as a variable in the study. About 10 000 IU of hCG were administered to induce final follicular maturation when at least three follicles of at least 17 mm diameter were observed by transvaginal ultrasound, and oocytes were aspirated 36 h later. ICSI was performed as described previously (Van Landuyt et al., 2005).

Patient characteristics, biological variables and embryo development were recorded and were found to be comparable in the two treatment groups (Table II). The study population consisted of ICSI patients with male factor subfertility as the most frequent indication for ART (69% in the HP-hMG group and 82% in the rFSH group). In a minority of couples, a female factor of subfertility was identified (7 and 6% in the HP-hMG and rFSH group, respectively). In 10% of couples in the HP-hMG group and 6% in the rFSH group, the indication for ART was idiopathic subfertility. A combination of male and female (tubal pathology) factors was observed in 14 and 6% in HP-hMG and rFSH patients, respectively.

Evaluation parameters

Three categories of variables were evaluated for their influence on the gene expression observed in the CC at oocyte retrieval: patient characteristics, ovarian stimulation dependent variables and oocyte developmental competence related variables. These three categories, yielding a total of 13 variables, are enumerated in Table II. The variables related to oocyte developmental competence comprised: (i) oocyte maturation rate, i.e. the number of metaphase II (MII) oocytes calculated over the total number of COCs retrieved (Oocyte Maturity); (ii) fertilization rate, i.e. the proportion of 2 pronuclei (PN) observed at 16–18 h after insemination over the number of intact oocytes after ICSI (2PN); (iii) embryo developmental rate, i.e. the proportion of embryos at least at the 7-cell stage on Day 3 post-ICSI over the number of 2PN (≥7-cell Day 3); (iv) degree of fragmentation in embryos, calculated as the proportion of embryos with less than 10% fragmentation on Day 3 over the number of 2PN (Low Fragmentation); (v) proportion of good quality embryos, i.e. embryos containing at least 7 cells and <10% fragmentation on Day 3 post-insemination, calculated over the total number of 2PN (Good Quality Embryos).

Handling of human CC

COCs were retrieved at 36 h post-hCG and washed in multiple dishes with human tubal fluid (HTF, Lonza, Verviers, Belgium) supplemented with serum substitute supplement (SSS, Irvine Scientific, CA, USA) to free them of any remaining floating granulosa cells (GC), blood, cell debris and large (mural) cell clumps. COCs were incubated in fertilization medium (Universal IVF medium, Medicult, Denmark) covered with paraffin oil (Irvine Scientific, CA, USA) until denudation. The time interval between oocyte retrieval and denudation was 2–3 h. Denudation was performed in a 100 μl droplet of HTF-SS containing 80 IU of recombinant cumulase (Halozyme Therapeutics, CA, USA) for 30 s at most, and sequentially in two washing droplets without enzyme. MII oocytes were used for ICSI and the CC were pooled per patient and snap frozen immediately after denudation. All manipulations were performed under sterile conditions on a heated-stage microscope (37°C).

RNA extraction and RT reaction

Total RNA was extracted using the RNeasy Micro kit (Qiagen, Westburg, Leusden, The Netherlands) on the Qiacube (Qiagen, Westburg, Leusden, The Netherlands) using the RNeasy DNase digestion extraction protocol. Isolated RNA was eluted with 14 μl of RNase-free water and was subsequently treated with RQ1 RNase-Free DNase (Promega, Leiden, Netherlands) according to the manufacturer's protocol in a total volume of 20 μl and stored at −80°C. Subsequently, the human total RNA was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Gent, Belgium) according to the manufacturer's instructions using the blend of oligo(dT) and random hexamers in a total volume of 20 μl which was ultimately diluted to 80 μl. Negative controls were generated by omitting the RNA or the reverse transcription enzyme. All resulting cDNA was frozen at −80°C and used for PCR within 2 weeks.

Table I Known function and suggested relevance for ARTs of the studied genes.

Gene symbol (Name)	Identified in CC		Known function	Relevance for ART	References
	Human	Rodents			
HAS2 (Hyaluronan synthase)	Yes	Yes	• extracellular matrix (ECM) formation	• eCM-(re)modeling is essential for ovulation and fertilization • expression in cumulus cells (CC) correlated with embryonic development	Russell *et al.* (2003a), Russell and Robker (2007), McKenzie *et al.* (2004)
VCAN (Versican)	Yes	Yes	• secreted proteoglycan that can bind hyaluronic acid • component of the extracellular matrix involved in cell adhesion, proliferation, migration and angiogenesis • plays a central role in tissue morphogenesis and maintenance	ECM-(re)modeling is essential for ovulation and fertilization	Russell *et al.* (2003b), van Montfoort *et al.* (2008)
SDC4 (Syndecan 4)	No	Yes	• can modulates signaling of transforming growth factors beta(TGFB), fibroblast growth factor (FGF) or vascular endothelial growth factor (VEGF) • favors attachment of anticoagulant heparane sulfate chains (aHSPG)	(Co)receptor for TGFB aHSPG are hormonally induced and control proteolysis and fibrin formation at ovulation regulated in mouse mural granulosa cells during follicular growth and atresia Increased in CC with final differentiation during ovulation response	Beauvais and Rapraeger (2004), Principalle *et al.* (2001), Ishiguro *et al.* (1999)
ALCAM (Activated leukocyte cell adhesion molecule)	No	Yes	• hematopoetic stem cells • immune response	• described in endometrial epithelium and blastocysts • possibly involved in blastocyst implantation	Fujiwara *et al.* (2003), Hernandez-Gonzalez *et al.* (2006)
GREM1 (Gremlin 1)	Yes	Yes	• antagonist of bone morphogenetic protein and TGFB signaling	• regulating the GDF9 and BMP15 balance • CC expression correlated with embryonic development	Hsu *et al.* (1998), Pangas *et al.* (2004), McKenzie *et al.* (2004)
PTGS1 (Prostaglandin-endoperoxide synthase 1)	No	No	• prostaglandin biosynthesis • PTGS1 is often constitutively expressed • regulates angiogenesis in endoth. cells • presumably involved in cell–cell signaling and maintaining tissue homeostasis • expression is regulated by cytokines and growth factors.	• involved in tissue repair after ovulation • isozyme of PTGS2 so might share or contribute to some of the functions of PTGS2	
PTGS2 (Prostaglandin-endoperoxide synthase 2)	Yes	Yes	• inducible isozyme of PTGS1 • regulated by specific stimulatory events • prostaglandin biosynthesis • involved in inflammation and mitogenesis	• expansion, ovulation • expression in the CC correlated with oocyte maturity and embryonic development	Davis *et al.* (1999), Hizaki *et al.* (1999), McKenzie *et al.* (2004), Feuerstein *et al.* (2007)
DUSP16 (Dual specificity phosphatase 16)	No	No	• negative regulator of mitogen-activated protein kinase-pathway	• regulator of epidermal growth factor (EGF) signaling (regulating the balance between the LH and EGF signaling in favor of the EGF pathway)	
SPROUTY4 (Sprouty homolog 4)	No	No	• antagonist to growth factors (EGF, FGF, VEGF) • supporting EGF signaling	• regulator of EGF signaling	Cabrita and Christofori (2008)
RPS6KA2 (Ribosomal protein S6 kinase, 90 kDa, polypeptide 2)	No	No	• intermediate in the EGF signaling	• involved in the EGF signaling cascade at ovulation • has been detected in bovine oocytes	Pelech *et al.* (2008), Dalbies-Tran and Mermillod (2003)

Table II Overview of the patient and treatment variables in the two gonadotrophin treatments.

	Variable	Unit	HP-hMG			rFSH			t-Test result
			Average	SD	n	Average	SD	n	
Group 1	Age	Year	33	6	35	34	5	28	ns
	BMI	kg/m^2	23	5	32	22	3	27	ns
Group 2	Days of stimulation	#	11	2	35	11	2	28	ns
	Gonadotrophin dose*	U/day	232	67	35	233	71	28	ns
	FSH[a]	U/l	16	6	24	18	9	18	ns
	LH[a]	U/l	1.26	0.67	25	1.44	0.88	18	ns
	Relative E2 = E2[a]/COC retrieved	ng/l	263	85	28	225	199	19	ns
	Progesterone[a]	μg/l	1.08	0.61	27	1.41	0.69	18	ns
	COC retrieved at pick up*	#	10	6	35	13	7	28	ns
	Ovarian Response = COC retrieved/Gonadotrophin dose	#	5	4	35	6	5	28	ns
Group 3	Oocyte Maturity = #MII/COC retrieved	%	81	17	35	79	21	28	ns
	2PN = proportion of 2PN/intact oocytes after ISCI	%	74	23	35	70	26	28	ns
	≥7cell Day3 = proportion of embryos with at least 7 cells on Day 3 post-ICSI/2PN	%	40	34	35	32	29	28	ns
	Low Fragmentation = proportion of embryos with <10% fragmentation on Day 3/2PN	%	56	38	35	45	35	28	ns
	Good Quality Embryos = proportion of embryos on Day 3 with <10% fragmentation and at least 7 cells/2PN	%	38	34	35	26	29	28	ns

PN, pronuclei; COC, cumulus–oocyte complex; E_2, estradiol 17-B; MII, metaphase II.
Group 1 = Patient characteristics.
Group 2 = Stimulation-dependent biological variables.
Group 3 = Development-related variables.
*This value was used for calculation but was not used as a characteristic for developing the models.
[a]Serum values as measured on day of hCG adminstration.

Table III The primer sequences (5′–3′) used in Q-PCR.

Gene symbol	Accession nr	Forward primer	Reversed primer	Fragment length
ALCAM	NM_001627.2	GGCAGTGGAAGCGTCATAA	CATTCTCTTCAGGGGAAATGA	91
PTGS1	NM_080591.1	CACCCATGGGAACCAAAG	TGGGGGTCAGGTATGAACTT	94
PTGS2	NM_000963.1	GCTTTATGCTGAAGCCCTATGA	TCCAACTCTGCAGACATTTCC	70
HAS2	NM_005328.1	TTGGGGGAGATGTCCAGA	CCTTTCTATATTAAAAGCCATCCAA	92
VCAN	NM_004385.2	GCACCTGTGTGCCAGGATA	CAGGGATTAGAGTGACATTCATCA	70
SDC4	NM_002999.2	GGCAGGAATCTGATGACTTTG	GGCCGATCATGGAGTCTTC	72
GREM1	NM_013372.5	Assay on demand ref: hs00171951_m1		80
SPROUTY4	NM_030964.2	CCCCGGCTTCAGGATTTA	CTGCAAACCGCTCAATACAG	85
RPS6KA2	NM_001006932.1	CGAGTGAGATCGAAGATGGAG	AGCTTTCCTTCCGTCTGAAA	95
DUSP16	NM_030640.1	CAGCGAGATGTCCTCAACAA	GCTGGCATTTAACACATAACCA	66

Quantitative PCR

PCR primer design for Q-PCR was performed using the Universal Probe Library (UPL) software (Roche Diagnostics, Roche Applied Science, Mannheim, Germany). Primers were selected to be RNA specific and complementary to the human sequence of Hyaluronan synthase (HAS2), Versican (VCAN), Syndecan 4 (SDC4), Activated leukocyte cell adhesion molecule (ALCAM), Prostaglandin-endoperoxide synthase 1 (PTGS1), Prostaglandin-endoperoxide synthase 2 (PTGS2), Dual specificity phosphatise 16 (DUSP16), Sprouty homolog 4 (SPROUTY4) and Ribosomal protein S6 kinase, 90 kDa, polypeptide 2 (RPS6KA2) (Table III). The expression in the human CC was normalized using the ALU-J primers (kindly provided by Prof. J. Vandesompele, CMG UZGent, Belgium). Q-PCR was performed on the LightCycler 480 (Roche Diagnostics, Mannheim, Germany) with the LC480 SYBR Green I Master (Roche Diagnostics, Mannheim, Germany) containing 2 μl of cDNA and 0.6 μM of primers in a total volume of 15 μl. After 10 min of activation at 95°C, cycling conditions were 10 s at 95°C, 30 s at 60°C and 1 s at 72°C for 40–55 cycles for the specific genes, and 15 s at 95°C, 60 s at 60°C for 40 cycles for ALU-J, the endogenous control. For Gremlin1 (GREM1), a commercial assay was used (Taqman Gene expression assay Hs00171951_m1, Applied Biosystems, Lennik, Belgium) on the LC480 in a total volume of 15 μl. A log6 dilution series of a synthetic oligonucleotide corresponding to the amplicon

sequences and PCR no-template controls were simultaneously run with the samples. For all genes except GREM1, the specificity of the PCR products was confirmed by melting curve analysis performed at the end of the amplification, and once by gel electrophoresis and sequencing. Each sample was tested in triplicate and all values fell within the exponential range as determined by the specific standard curve run with each gene. All negative controls were as expected and all results mentioned later are the normalized values.

Statistical analysis

Gene expression ratios were log transformed and proportions were arcsin transformed to ensure Gaussian distribution. The patient and treatment related variables in the two stimulation groups were compared using the two-tailed *t*-test GraphPad Prism 4.01 (GraphPad Software, San Diego, USA).

Since gene expression values most likely resulted from a combination of patient and treatment related variables, a linear regression model was established on the model of $y = a + bx + cz + dt$ with the gene expression data as response variables. X, z and t were the patient and treatment related variables, with b, c, d as their respective indexes and a as the intercept.

Multivariable data analysis is more powerful than 2-variable analysis and also allows a more exploratory, hypothesis generating form of research. The ensuing regression model contained the variables with the highest influence on the expression of the specific genes. The patient and treatment related variables were chosen progressively, in three steps, with the best fitting variable from one of the three categories defined earlier being added at each step. The selection for Group 2 and 3 variables (as shown in Table II) was made after the variables of Group 1 and Group 2 had been added, respectively. After adding the variables, a backward regression was performed. A *P*-value of 0.2 was used as threshold. Each variable was added together with its treatment interaction term, which was a multiplication of the variable and a variable representing the difference between the two types of gonadotrophin treatment. Treatment interaction terms could be omitted from the model (in the backward step), although their main effect was still present, but main effects could not be removed if any of the interaction effects were still present. If a variable was removed completely, the best fitting variable of the ones not yet present in the model was chosen without making any distinction between the three categories. The significance for the models and the main variables was set, respectively, at $P < 0.01$ and $P < 0.05$. Statistics were performed using R (www.R-project.org).

Graphs representing the relationships found in the statistical model

Significant correlations of gene expression with a variable or its treatment interaction term were represented in two-dimensional scatter plots. Linear regression lines calculated between the model's predicted values and the variable depicted in the horizontal axis were shown to suggest the direction of the correlation and to illustrate the significant differences (if any) between gonadotrophin treatments.

Results

All samples analyzed contained a sufficient amount of RNA to detect the endogenous control, ALU-J. All 10 genes were expressed in the CC of ICSI patients at the time of oocyte retrieval. On the basis of the melting curves, quantification was not reliable and was excluded for one sample in the ALCAM and SPROUTY4 analysis (this was the sample with the lowest ALU-J level) and four samples in the DUSP16 analysis.

Pregnancies (evaluated by positive serum hCG) were obtained in 9/31 (29%) and 5/26 (19%) of the HP-hMG and rFSH cycles with replacement of embryos, respectively ($P > 0.05$).

Biplot analysis of the 63 patients based on the 13 patient and treatment variables and the expression results for the 10 genes

To explore the correlations within the multivariable data set, taking into account all variables or expression values obtained for all 63 patients, two biplots were generated: one for the three categories of variables (patient characteristics, ovarian stimulation dependent variables and oocyte developmental competence related variables; Fig. 1A) and one for the gene expression values (Fig. 1B). The biplots shown here are the biplots according to the first and second principal component, which together represent 51% of the total variability. Points represent individual patients; the closer the distance between two points in Fig. 1B, the more similar their gene expression pattern; the closer the distance between arrows, the stronger the patient and treatment variables (Fig. 1A) or genes (Fig. 1B) are correlated. On the basis of the scatter and overlap of data points, there is no clear clustering of patients for either treatment. On the basis of Fig. 1 it can be concluded that serum progesterone and FSH concentrations as detected on the day of hCG injection are closely correlated. Oocyte quality related variables are closely correlated in a second group. Relative estradiol concentration on the day of hCG administration is closely correlated to fertilization (2PN) and degree of fragmentation (Low embryo Fragmentation). Ovarian Response and LH-levels at the time of hCG administration represent a third group. In the multivariable analysis (Biplot, Fig. 1), Age and Ovarian Response are on the same axis, but were inversely correlated, and serum LH, measured on day of hCG, was correlated to Ovarian Response.

On the basis of the arrow signs, each representing the expression values of one of the genes in Fig. 1B, the expression of HAS2 was closely related to the expression of PTGS2 and ALCAM. The fact that all expression related arrow signs were clustered and pointed in the same direction confirms the correlation between the different genes analyzed. The precise relationship between the ten factors is currently unknown, but a hypothetical scheme (Fig. 2) could be deduced from the literature (Richards *et al.*, 2005; Shimada *et al.*, 2006; Diaz *et al.*, 2007).

The statistical model describing the specific expression values

To determine which of the 13 variables most strongly influenced the expression level of each gene, and to investigate whether the expression differed depending on the type of gonadotrophin treatment, statistical models were generated using a multiple stepwise regression.

A significant model ($P < 0.01$, giving the best fit), containing three variables, led us to explain the differential expression for each gene except HAS2 ($P = 0.0292$). The equations describing the models are included in Table IV. For 5 of the 10 genes the equations were found to be gonadotrophin stimulation dependent. This was either revealed by an altered intercept and or an altered index (respectively, a or b, c, d in the formula $y = a + bx + cz + dt$; see also Table V for statistical significances).

Figure 3 contains explanatory graphs illustrating the three main relationships observed in this study (Fig. 3A–C). These graphs display the linear regression lines that show the direction of the correlation and illustrate the differences (if any) between the two gonadotrophin treatments. Two regression lines on the graphs indicate that the two gonadotrophin treatments significantly altered the expression of a given gene. Parallel regression lines indicate a similar correlation with the considered variable for the two gonadotrophin treatments (Fig. 3A). If a significant interaction term was retained in the model (Table V), the correlation with the biological variable was not identical in the two gonadotrophin groups and the regression lines crossed each other (Fig. 3B and E, F). Overlapping regression lines indicated that both patient populations had similar expression levels over the entire range (Fig. 3C).

Considering the equations obtained, the expression of all genes, except VCAN, was significantly correlated to patient Age or BMI ($P < 0.05$; Tables IV and V).

Patient Age or BMI and serum FSH concentration, as measured on the day of hCG administration, were identified as the three variables that most often significantly influenced gene expression (four of nine of the studied genes). PTGS1 and PTGS2 expression levels were found to be more correlated to patient characteristics and stimulation-dependent biological variables than to oocyte quality variables. VCAN expression was significantly correlated with oocyte nuclear maturity at retrieval, and high ALCAM and GREM1 expression levels were correlated with normal to fast embryo development: the latter three relationships are depicted in Fig. 3D–F and are also discussed below.

Because no significant model was obtained for HAS2 with three variables and because the PTGS models were the only models without an oocyte developmental competence related variable, further statistical analysis was performed to extend the three-variable models with one additional variables. This was the best fitting variable of the ones not yet present in the model and was chosen by the program without making any distinction between the three categories. This additional step did not improve the significances for the HAS2 or PTGS2 models, but showed that PTGS2 expression correlated with ≥7cells on Day 3 and that the PTGS2 correlation was different in the two gonadotrophin groups (Fig. 4). This embryo quality related variable was less significant ($P = 0.1085$) than the three initial variables in the PTGS2 model ($P < 0.005$).

Genes in CC reflect the type of gonadotrophin used

As shown in Table V, the expression levels of SDC4, VCAN, GREM1, SPROUTY4 and RPS6KA2 were found to be significantly different between the two gonadotrophin treatments. In rFSH stimulated patients, expression levels were consistently higher for SPROUTY4 and lower for SDC4 when compared with HP-hMG stimulated patients. This is reflected in different intercepts in the models obtained for these genes for the two treatments (Table IV). For the three remaining genes the relation between one or more of the variables and the expression was significantly different in the two gonadotrophin treatment groups as can be seen in the altered intercept and regression coefficients in the models for either treatment in Table IV. As such, VCAN expression was found to be negatively

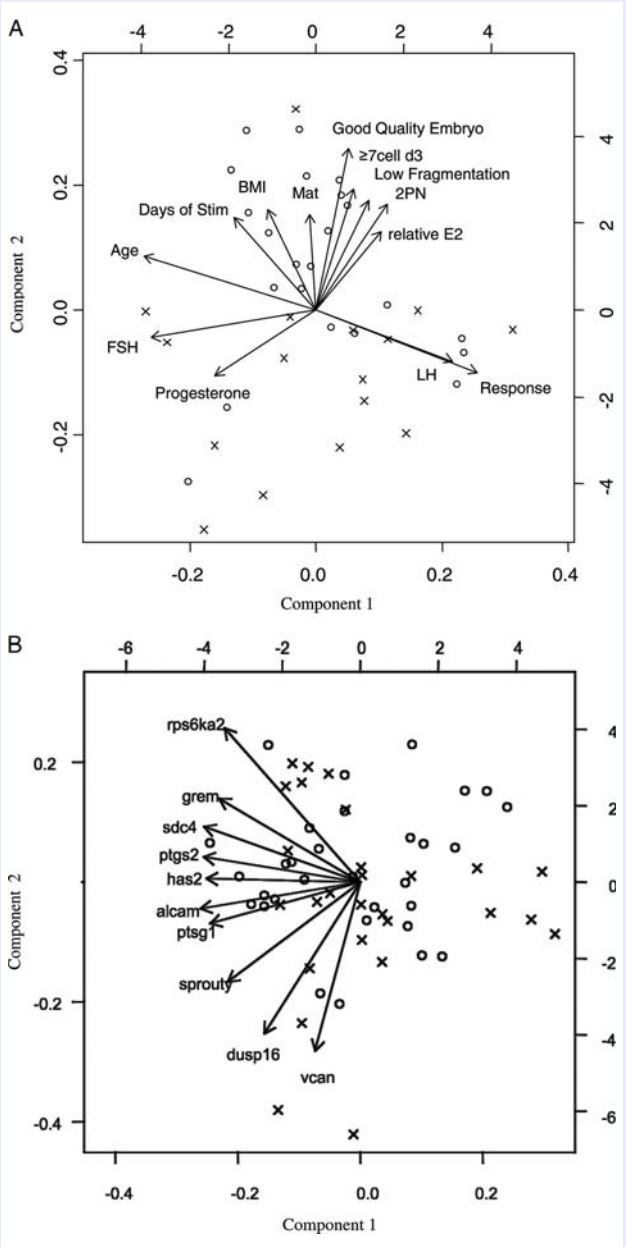

Figure 1 Biplot of the 63 patients based on the 13 patient and treatment variables (**A**) and the expression results for the 10 genes (**B**).

The multivariable dataset is represented by the two principal (descriptive) components for the highest variability with the first and second principal component, respectively in the lower and left axes for plotting the arrows and the upper and right axes for plotting the individual points. Circles and crosses representing, respectively, the 63 highly purified (HP)-hMG or rFSH stimulated patients. A biplot allows the display of the relationship between individual results (the points = the 63 patients) and correlations among the variables. The closer the points, the more similar the patient and treatment variables (A) or the gene expression pattern (B) for these patients according to the first two principal components. The closer the arrows to each other, the more the variables (A) or the gene expressions (B) are correlated. Gene abbreviations: HAS2, hyaluronan synthase 2; VCAN, versican; SDC4, syndecan 4; ALCAM, activated leukocyte cell adhesion molecule; PTGS1, prostaglandin-endoperoxide synthase 1; PTGS2, prostaglandin-endoperoxide synthase 2; DUSP16, dual specificity phosphatise 16; SPROUTY4, sprouty homolog 4; RPS6KA2, ribosomal protein S6 kinase; 90 kDa, polypeptide 2; GREM1, gremlin1.

Table IV The multiple regression models describing the expression values of the 10 genes after highly purified (HP)-hMG or recombinant FSH (rFSH) stimulation, using the three best fitting patient and treatment variables of the 13 available.

HP-hMG and rFSH:	Log(HAS2) = 2.6276 + 0.0127 × (Age) − 0.0163 × (Response) − 0.0030 × (2PN)
	Log(ALCAM) = 1.7412 + 0.0288 × (Age) − 0.1333 × (Progesterone) + 0.0045× (≥ 7 cell Day 3)
	Log(PTGS1) = 1.8688 − 0.0363 × (BMI) + 0.0209 × (FSH) − 0.0592 × (Response)
	Log(PTGS2) = 5.0784 + 0.0041 × (BMI) + 0.0022 × (FSH) − 0.0599 × (Response)
	Log(DUSP16) = 3.6097 − 0.0030 × (BMI) − 0.0186 × (LH) − 0.0021 × (Mat)
HP-hMG	Log(SDC4) = 4.1272 + 0.0260 × (Age) − 0.0001 × (Rel E2) + 0.0018 × (Low Fragmentation)
rFSH	Log(SDC4) = 3.8902 + 0.0260 × (Age) − 0.0001 × (Rel E2) + 0.0018 × (Low Fragmentation)
HP-hMG	Log(VCAN) = 5.5572 + 0.0014 × (E2) − 0.0016 × (FSH) − 0.0073 × (Mat)
rFSH	Log(VCAN) = 6.9966 + 0.0014 × (E2) − 0.0016 × (FSH) − 0.0285 × (Mat)
HP-hMG	Log(GREM1) = 1.6085 − 0.0009 × (Age) − 0.1101 × (Progesterone) − 0.0020 × (≥7 cell Day 3)
rFSH	Log(GREM1) = −0.7945 + 0.0713 × (Age) − 0.6993 × (Progesterone) + 0.0122 × (≥ 7 cell Day 3)
HP-hMG	Log(SROUTY4) = 1.9315 − 0.0341 × (BMI) + 0.0184 × (FSH) + 0.0037 × (≥ 7 cell Day 3)
rFSH	Log(SROUTY4) = 2.2870 − 0.0341 × (BMI) + 0.0184 × (FSH) + 0.0037× (≥ 7 cell Day 3)
HP-hMG	Log(RPS6KA2) = 2.4628 + 0.0035 × (Age) − 0.007 × (FSH) + 0.0031 × (Low Fragmentation)
rFSH	Log(RPS6KA2) = 0.4992 + 0.0637 × (Age) − 0.007 × (FSH) + 0.0031 × (Low Fragmentation)

Table V For each gene the three patient and treatment variables with the highest influence on the expression values as induced by the two gonadotrophin treatments are shown.

	HAS2	SDC4	ALCAM	VCAN	PTGS1	PTGS2	GREM1	DUSP16	SPROUTY4	RPS6KA2
Age	ns	*	*				*			***
BMI					*	***		***	***	
Days of Stimulation										
FSH				***	t	***			***	**
LH								*		
Relative E2		***		***						
Progesterone			*				***			
Ovarian Response	t				*	***				
Oocyte Maturity				***				ns		
2PN	t									
≥7 cell day 3			*				*ns*		ns	
Low Fragmentation		ns								*
Good Quality Embryo										
HP-hMG><rFSH	t	*	ns	***	ns	ns	*	t	***	**
Interaction	Age (t)		P (t)	Maturity (***)			Age (*) P (*) ≥7CELL D3 (*)	Maturity (t)	≥ 7CELL D3 (t)	Age (**)
p of the model	0.0292	0.0004	0.0030	0.0001	0.0001	0.0001	0.0005	0.0001	0.0001	0.0003

For all genes apart from HAS2, a significant model was found (*P*-values of the model <0.001). All variables marked with: ns, t, or * increased the significance of the specific model, though their direct relationship may not have been strong. The *, t and ns are the type III *P*-values obtained for this variable in the model and indicate the relationship of the individual variable with the gene expression. ns: not significant, t: $P<0.1$, *: $P<0.05$. **: $P<0.01$, ***: $P<0.005$. ns: the interaction with this term was significant, indicating a significant relation in at least 1 of the treatments or an inverse relation in both.

correlated with Oocyte Maturity in rFSH stimulated patients ($P < 0.0001$), but was not differentially expressed in HP-hMG patients with differences in Oocyte Maturity ($P = 0.1756$; Fig. 3E). RPS6KA2 expression had a significantly positive correlation with the increasing Age of the rFSH stimulated patients ($P < 0.001$), whereas there was no correlation between Age and RPS6KA2 expression in HP-hMG stimulated patients ($P = 0.7812$; Fig. 3B). GREM1 expression was dependent on Age, ≥7 cells on Day 3 (% of normal or fast dividing embryos, Fig. 3F) and serum progesterone on day hCG administration in rFSH stimulated patients, with a positive correlation for the first two variables and a negative correlation for the latter ($P < 0.01$, $P < 0.05$, $P = 0$). In HP-hMG patients, the GREM1 expression seems to be independent of these three variables (all $P > 0.05$).

The extend model for PTGS2 showed that PTGS2 expression was inversely correlated with the embryo development stage on Day 3 (≥7cells on Day 3) in the two gonadotrophin groups (Fig. 4).

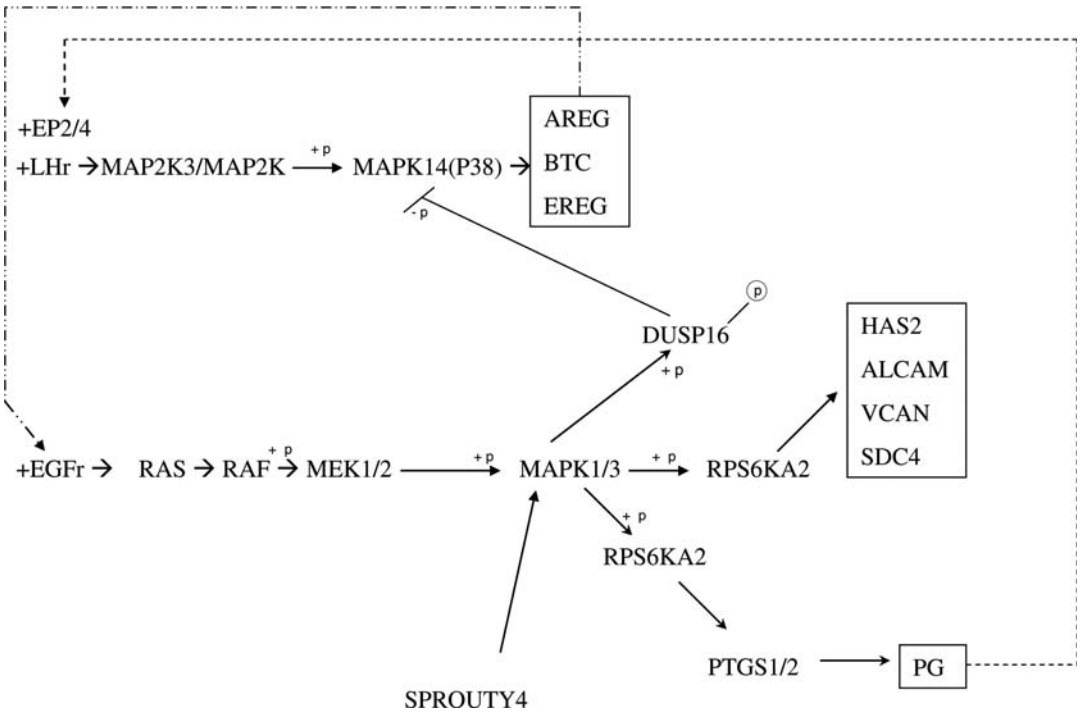

Figure 2 Hypothetical scheme of the interactions occurring in CC.

Activation of the epidermal growth factor receptor (EGFr) will induce the phosphorylation of mitogen-activated protein kinase 1 (MAPK1) and 3. RPS6KA2 is a known intermediate in this pathway and its presence has been demonstrated in oocytes but its function in CC is currently unknown. The MAPK1/3 activation is required to obtain HAS2 expression in CC. The MAPK1/3 pathway will also allow PTGS2 induction, which via an autocrine regulation can activate the prostaglandin (PG) receptors on the CC. Prostaglandins, as well as LH, use the MAPK14 pathway in granulosa cells. This results in the production of EGF-like family members—an action which is also observed in the CC. The SPROUTY-family members are known inhibitors of growth factor pathways, such as the EGF pathway, but its action in CC has not yet been documented. DUSP16 is an inhibitor of the MAPK14, MAPK8 (not depicted here) and MAPK1/3 pathway, but with only a weak affinity for the latter. DUSP16 is an exceptional member of the DUSP family because it contains a MAPK1/3 phosphorylatable site, which will increase its half life (Katagiri *et al.*, 2005). As such, DUSP16 might be a key player in the balance between the prostaglandin/LH and the EGF-like family members stimulation. Phosphorylation is indicated by +p and dephosphorylation by −p. Abbreviations: MEK1/2 = MAP2K1/2, mitogen-activated protein kinase kinase 1 and 2; AREG, amphiregulin; BTC, betacellulin; EREG, epiregulin; EP2/4, prostaglandin E receptor 2, subtype EP2 and EP4.

Discussion

Novel genes in human CC related to the ovulatory response

The present investigation focused on 10 genes related to the ovulatory LH response found differentially expressed using micro array analysis as a function of oocyte competence (unpublished microarray data in mouse and human). The Q-PCR results confirmed the presence of mRNA of the ten genes in the CC of ICSI patients of both treatment groups 36 h after hCG injection. The biplot of the expression values confirmed the concerted expression of all selected genes and suggested that all are involved in the ovulatory response. The expression of SPROUTY4, DUSP16, RPS6KA2 and PTGS1 was previously undescribed in CC.

From a list of 13 predefined patient and treatment variables routinely recorded during ART, the three major variables co-determining the expression level were identified for each gene, using multiple stepwise regression analysis. To date the expression of PTGS1 in the human ovary has not been extensively documented. The expression of both PTGS molecules depends on the same variables, i.e. the amount of circulating FSH, the patient BMI and Ovarian Response. The degree of similarity for mRNA expression between the two isozymes suggests a common mechanism of regulation or synergy between PTGS1 and 2 in CC.

Three genes that potentially regulate epidermal growth factor (EGF) signaling in the CC, i.e. RPS6KA2, SPROUTY4 and DUSP16, positioned on the outside of the cluster in the biplot of the expression results (Fig. 1B) together with VCAN, which distinguishes them from the rest of the genes. The precise relationship between the 10 factors in the cumulus is currently unknown, but a hypothetical outline (Fig. 2) of the main interactions could be deduced from the literature (Richards *et al.*, 2005; Shimada *et al.*, 2006; Diaz *et al.*, 2007). Ribosomal Protein S6 kinase, 90 kDa, polypeptide 2 (RPS6KA2) is a member of the ribosomal S6 kinase (RSK) family whose action is downstream of mitogen-activated protein kinase (MAPK)1/3 (formerly ERK1/2). The involvement of RSK proteins such as RPS6KA1/3, formerly known as RSK1/2, in oocyte nuclear maturation, as triggered by the RAF-MEK1/2-MAPK1/3 cascade, has been recently documented (Pelech *et al.*, 2008). The presence of RPS6KA2 mRNA (also referred to as RSK3) has been reported in bovine oocytes (Dalbies-Tran and Mermillod, 2003), but for cumulus no data are available. It still needs

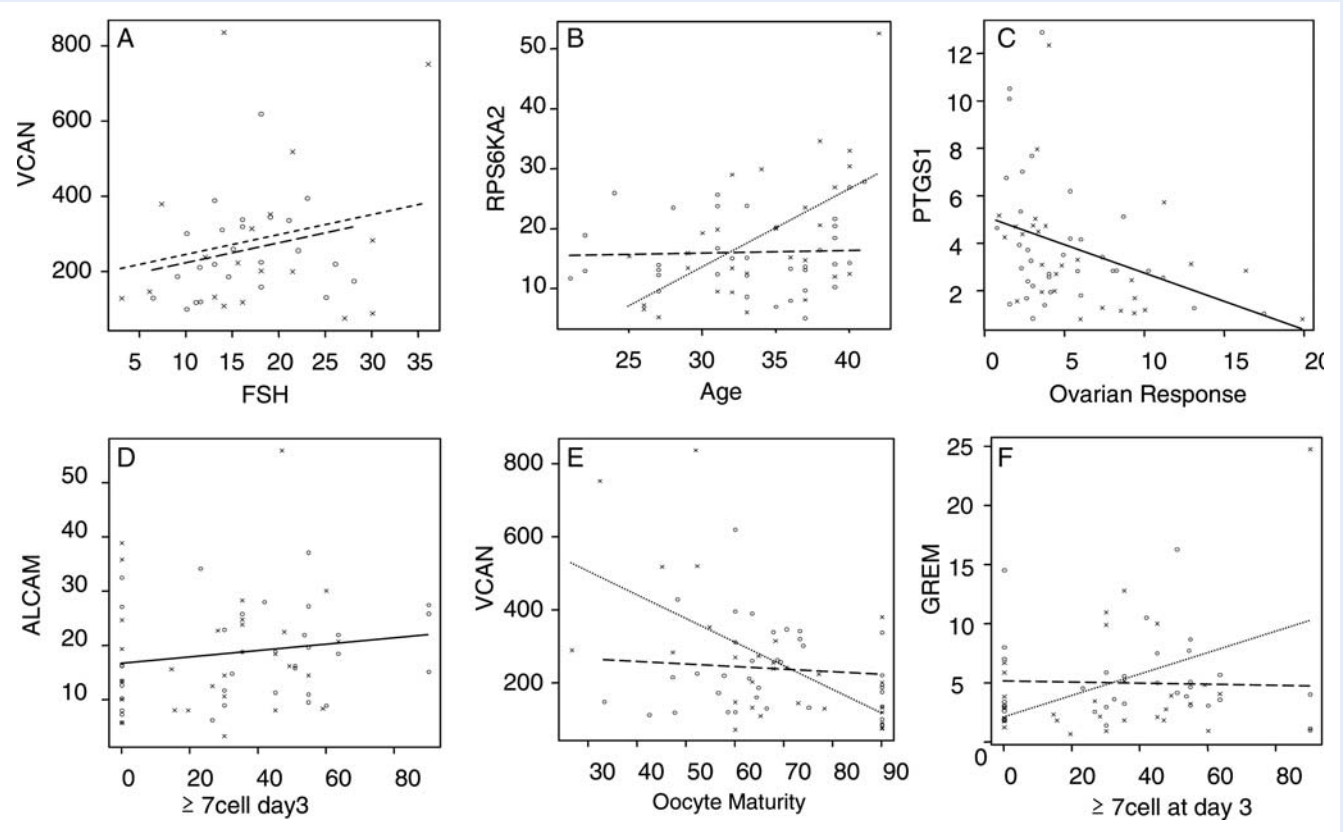

Figure 3 Graphical representation of the three types of interactions observed between the two gonadotrophin treatments.

Crosses and circles represent rFSH and HP-hMG stimulated patients respectively. Regression lines are dotted and dashed for rFSH and HP-hMG stimulated patients, respectively, and full lines are used if the relationship was equal in both gonadotrophin treatment groups. The results as mentioned here in brief are deduced from Table V. To facilitate the interpretation, only two variables were taken into consideration, and significance of the correlations is higher in the multivariable analysis (Table V). (**A**) VCAN expression is significantly correlated to FSH and the HP-hMG>⁄<rFSH variable, but the interaction of HP-hMG>⁄<rFSH with FSH was not retained in the model. Graph A indicates that VCAN is positively ($P < 0.005$) correlated with FSH in both treatment groups and is systematically higher in rFSH stimulated patients. (**B**) RPS6KA2 expression was significantly influenced by Age and HP-hMG>⁄<rFSH and the interaction of HP-hMG>⁄<rFSH with Age. The two-dimensional graph demonstrates that RPS6KA2 expression is positively correlated to Age in rFSH ($P < 0.001$) and independent of Age in HP-hMG stimulated patients ($P > 0.05$). (**C**) PTGS1 expression is significantly correlated to Ovarian Response and was not different between the two treatments. The expression of PTGS1 is therefore lower in patients with a more responsive ovary, independently of the gonadotrophin preparation ($P < 0.05$). (**D**) ALCAM expression is significantly correlated to the developmental stage of the embryo (≥7 cell Day 3) and there was no difference between the two treatments. This graph indicates that ALCAM expression is positively correlated with the developmental potential of the embryo independently of the gonadotrophin preparation. (**E**) VCAN expression was significantly correlated to the interaction of HP-hMG>⁄<rFSH with the % of oocyte mature at oocyte retrieval and the % of oocyte mature at retrieval and HP-hMG>⁄<rFSH. VCAN expression is negatively correlated with oocyte maturity in the rFSH group ($P < 0.0001$) and is rather independent of oocyte maturity in the HP-hMG group ($P = 0.1756$). (**F**) GREM1 expression was significantly influenced by the interaction of HP-hMG>⁄<rFSH with the ≥7 cell Day 3 and HP-hMG>⁄<rFSH. GREM1 expression is positively correlated with the embryonic development in the rFSH group, whereas this correlation is not apparent in HP-hMG stimulated patients ($P = 0.02$ in rFSH and $P = 0.6$ in HP-hMG).

to be determined whether RPS6KA2 is involved in both cascades following MAPK1/3 expression.

The specific action of SPROUTY4 has not yet been documented in the ovary. SPROUTY molecules have mainly been associated with growth factor inhibition [fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF)], but also with amplifying effects on the EGF signaling pathway (Cabrita and Christofori, 2008). Increased SPROUTY2 expression in bovine GC has been associated with competent oocytes (Robert *et al*., 2001) and has been described in human luteal cells where its expression was induced by EGF and FGF (Haimov-Kochman *et al*., 2005). SPROUTY heterodimers are described to be very potent, and recent data from mice confirmed a key role for SPROUTY2 in the oocyte CC communication, regulating the balance between FGF and EGF signaling in the CC and resulting in an amplification of the EGF signal after ovulation induction (Cabrita and Christofori, 2008; Sugiura *et al*. 2009).

DUSP16 is a unique member of the DUSP family in as far as it can be phosphorylated by activated ERK, which results in a longer lifespan. Since DUSP16 preferentially inactivates the mitogen-activated protein kinase 8 (MAPK8 earlier referred to as c-Jun N-terminal kinase or JNK) or mitogen-activated protein kinase 14 (MAPK14 earlier referred to as p38; Masuda *et al*., 2001), there might be a switching-off of the p38 pathway used by LH, thereby generating a preference for EGF signaling in the CC. Further study is required to better understand the exact interactions and functions of the genes involved.

Age, BMI and ovarian response to stimulation, the main variables influencing gene expression in CC

Inherent patient characteristics, such as Age and BMI, influenced the CC gene expression since they were significantly correlated with the expression of 9 in 10 genes. Age and BMI are well-known factors influencing ovarian responsiveness to gonadotrophins and ART outcome. HAS2, SDC4, ALCAM and RPS6KA2 showed a positive correlation to Age. The well-known inverse relationship between Age and Ovarian Response was also confirmed in the current population (Fig. 1A). Hence, in patients where the ovaries were less sensitive to gonadotrophin action (because of increased age), the expression of PTGS1 and 2 was also higher.

Half the genes studied were also significantly influenced by the FSH concentration on the day of hCG administration. In long GnRH-agonist protocols, serum FSH levels correlated well with the units of gonadotrophins administered. This emphasizes the importance of gonadotrophin treatment doses on the CC gene expression. It has been demonstrated that treatment with high doses of FSH increases progesterone production in normo-ovulatory patients (Ubaldi *et al.*, 1996). A similar correlation is also observed in our patients (Fig. 1A) and coincided with lower VCAN and RPS6KA2 expression and higher PTGS1 and PTGS2 and SPROUTY4 expression in both treatment protocols.

E_2 concentrations, oocyte quality and CC gene expression

The only stimulation-dependent variable that featured a close relationship with the oocyte quality related parameters (Fig. 1A) was relative serum E_2. An increased level of E_2 per COCs retrieved, which shows that follicles are well advanced in maturation, correlated with an increased VCAN and reduced SDC4 mRNA production in the CC (Table IV). SDC4 protein has already been described in the follicular fluid of IVF patients at 34 h post-hCG and its abundance was inversely correlated with the E_2 levels (Sakata *et al.*, 2000): the inverse correlation with E_2 seems to confirm the inverse relationship on the CC mRNA level observed in the current study. Higher VCAN and lower SDC4 might as such be potential markers for good oocyte quality.

Oocyte development variables related to CC gene expression

Although the current study population was quite heterogenous, some oocyte development related variables already correlated significantly with gene expression: maturity of the oocyte (VCAN), percentage of fragmentation in embryos (RPS6KA2) and embryo cleaving speed (GREM1 and ALCAM).

Patients with a higher percentage of mature (polar body) oocytes had less VCAN expression. This correlation with maturity was most prominent in rFSH stimulated patients (Fig. 3E).

Expression of ALCAM mRNA and protein during the ovulatory response in mouse COCs was more pronounced in CC than in mural GC, reaching maximal levels in the mouse CC immediately prior to ovulation (Hernandez-Gonzalez *et al.*, 2006). ALCAM protein is localized at the surface of all CC, but its function, originally related to immune cell function, remains to be elucidated in the COC. A positive correlation of ALCAM expression with normal to fast embryo cleavage has not been reported before and makes it a promising new marker.

In rFSH stimulated patients, GREM1 expression was positively correlated to good embryo development. In this series of HP-hMG patients, GREM1 expression appeared to be independent of this variable ($P > 0.05$), although McKenzie *et al.* (2004) described that a high GREM1 expression in eight patients stimulated with menotropin was positively correlated with embryo development. In the same study, increased HAS2 and PTGS2 expression was also proposed to predict embryo development. In the current study, no significant relation with gamete quality-related variables was retained for HAS2. In contrast to the McKenzie study, the current study was not specifically designed to detect quality related expression differences. Other reasons could be related to differences in the gonadotrophin used or to the patient series effect. In another recently published study, which included only GnRH agonist/rFSH stimulated patients, PTGS2 expression was found to be inversely correlated with embryo development (Feuerstein *et al.*, 2007). The extend PTGS2 model obtained on our data, showed a positive correlation with PTGS2 expression in GnRH agonist/HP-hMG and a negative correlation for GnRH agonist/rFSH stimulated patients with the embryo development stage on Day 3 ($\geq$7cells on Day 3; Fig. 4) and thus confirms that the apparent contradiction found in the McKenzie and Feuerstein studies might result from the difference in the stimulation protocol.

Implication of the gonadotrophin preparation used

It has been shown that gonadotrophin preparations that differ in LH bioactivity are associated with different endocrine and paracrine profiles (Smitz *et al.*, 2007). The use of rFSH and HP-hMG significantly modified the expression in the COCs of half the genes selected here (Table III). Patients who had been treated with rFSH presented a constitutive 1.4 times increased SPROUTY4 expression level and a 1.3 times lower SDC4 expression level when compared with patients who had been stimulated with HP-hMG. The expression of VCAN, GREM1 and RPS6KA2 also diverged significantly in the two treatment groups, but this depended on the clinical and biological parameters.

SDC4 expression in GC has been shown to be regulated during follicular growth and is drastically increased in mural GC during follicular atresia. In CC, SDC4 expression increases during oocyte maturation and might be associated with the final differentiation of the CC (Ishiguro *et al.*, 1999; Sakata *et al.*, 2000). The 1.3-fold increased expression of SDC4 in HP-hMG CC possibly suggests a more advanced ovulatory response due to the pre-exposure to hCG in the HP-hMG during the growth period of the antral follicle, although the implications of such an increase are as yet unknown. SDC4 modulates signaling induced by growth factors, such as transforming growth factor B, basic FGF and VEGF, by acting as a (co-)receptor for these growth factors (Beauvais and Rapraeger, 2004) and might regulate the ovulation and the associated immune response in the CC.

SPROUTY4 might, as SPROUTY2, be involved in the regulation of the EGF response in the CC. Since in this study SPROUTY4 expression was significantly associated with higher serum FSH levels, which were associated with increased progesterone levels, increased

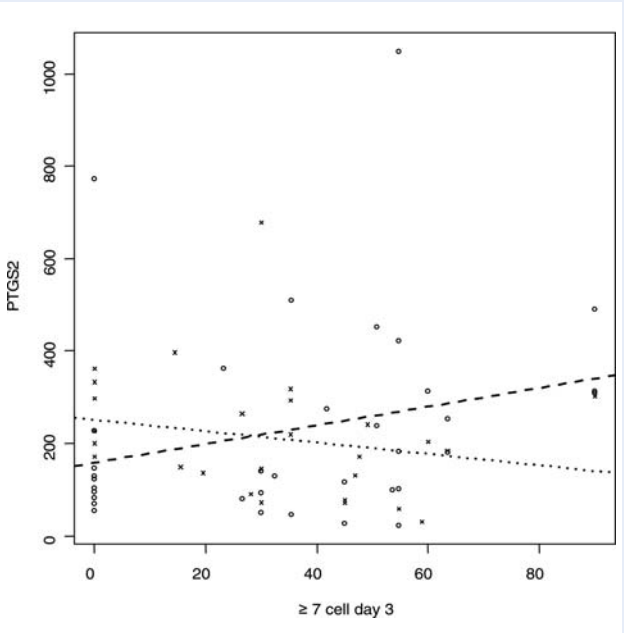

Figure 4 Illustration of the correlation observed between PTGS2 expression and embryo development.

Regression lines are dotted and dashed for rFSH (crosses) and HP-hMG (circles) stimulated patients. The three most robust variables determining PTGS2 expression did not contain oocyte quality-related variables. An additional regression step led to the addition of the variable ≥7 cell day 3 together with its interaction factor. This embryo quality variable was less strong ($P = 0.1085$) than the three initial variables. The two-dimensional representation of this correlation illustrates the positive correlation with PTGS2 expression in HP-hMG and the negative correlation for rFSH stimulated patients.

SPROUTY4 expression might indicate a poor prognosis. The exact implication of the 1.4-fold increased expression observed in COCs of the rFSH group is currently unknown.

The current study indicates that the correlation between certain variables and gene expression in cumulus is gonadotrophin preparation dependent.

As an example: Age (RPS6KA2, GREM1), Oocyte Maturity (VCAN), progesterone (GREM1) and embryo development on Day 3 (GREM1) were differentially correlated in the gonadotrophin treatments. A clear correlation was often observed in the rFSH stimulated patients, whereas expression levels in the HP-hMG stimulated patients seemed independent of these variables. We speculate that exposure to LH bioactivity in HP-hMG preparations during the entire follicular phase might lead to a damping effect on LH responsive genes after 36 h. Given the complexity of the interactions observed (dependency of multiple patient characteristics) and the currently limited knowledge about the function of the CC genes, it is still difficult to fully explain the biological/causal correlations.

Conclusions

For the first time, the presence of PTGS1, SPROUTY4, DUSP16 and RPS6KA2 mRNA in CC is demonstrated. CC gene expression analyzed at oocyte retrieval proved to be correlated to multiple patient and treatment related variables, such as serum FSH concentration at the end of stimulation, Age and BMI. Increased ALCAM, GREM1 and RPS6KA2 expression, and possibly also increased VCAN and lower SDC4 expression, are correlated to better embryo development. For the other genes, a relationship with oocyte quality might still exist, but is of less predictive value than the three patient or stimulation related variables in the model. We hypothesize that a prospective study on gene expression in individual cumulus complexes might reveal a higher discriminative power of the selected cumulus genes, if they are normalized for the confounding variables identified in this study. Cumulus complexes from HP-hMG or rFSH stimulations respond differentially during a critical period of oocyte maturation. Patients who had been treated with rFSH presented a constitutive increased SPROUTY4 and lower SDC4 expression when compared with patients who had been stimulated with HP-hMG. The expression of VCAN, GREM1 and RPS6KA2 also diverged significantly in the two treatment groups, but this depended on the clinical and biological parameters. CC gene expression analysis might prove to be a valuable tool for further optimization of stimulation protocols.

Acknowledgements

The authors thank J.-C. Arce, Clinical Research & Development, Ferring Pharmaceuticals for his support to the study and M. Whitburn, ITO, Vrije Universiteit Brussel for his editorial assistance.

Funding

This research has been supported by funds from Fonds voor Wetenschappelijk onderzoek (FWO) (No. FWOAL389), Ferring Pharmaceuticals and Vrije Universiteit Brussel Onderzoeksraad (VUB OZR).

References

Albertini DF, Combelles CMH, Benecchi E, Carabatsos MJ. Cellular basis for paracrine regulation of ovarian follicle development. *Reproduction* 2001;**121**:647–653.

Beauvais DM, Rapraeger AC. Syndecans in tumor cell adhesion and signaling. *Reprod Biol Endocrinol* 2004;**2**:3.

Cabrita MA, Christofori G. Sprouty proteins, masterminds of receptor tyrosine kinase signaling. *Angiogenesis* 2008;**11**:53–62.

Dalbies-Tran R, Mermillod P. Use of heterologous complementary DNA array screening to analyze bovine oocyte transcriptome and its evolution during in vitro maturation. *Biol Reprod* 2003;**68**:252–261.

Davis BJ, Lennard DE, Lee CA, Tiano HF, Morham SG, Wetsel WC, Langenbach R. Anovulation in cyclooxygenase-2-deficient mice is restored by prostaglandin E2 and interleukin-1beta. *Endocrinology* 1999;**140**:2685–2695.

Diaz FJ, Wigglesworth K, Eppig JJ. Oocytes determine cumulus cell lineage in mouse ovarian follicles. *J Cell Sci* 2007;**120**:1330–1340.

Espey LL, Richards JS. Temporal and spatial patterns of ovarian gene transcription following an ovulatory dose of gonadotrophin in the rat. *Biol Reprod* 2002;**67**:1662–1670.

Feuerstein P, Cadoret V, Dalbies-Tran R, Guerif F, Bidault R, Royere D. Gene expression in human cumulus cells: one approach to oocyte competence. *Hum Reprod* 2007;**22**:3069–3077.

Foster R, Segers I, Smart D, Adriaenssens T, Smitz J, Arce JC, Princivalle M. A differential cytokine expression profile is induced by highly purified human menopausal gonadotropin and recombinant follicle-stimulating hormone in a pre- and postovulatory mouse follicle culture model. *Fertil Steril* 2009 (in press).

Fujiwara H, Tatsumi K, Kosaka K, Sato Y, Higuchi T, Yoshioka S, Maeda M, Ueda M, Fujii S. Human blastocysts and endometrial epithelial cells express activated leukocyte cell adhesion molecule (ALCAM/CD166). *J Clin Endocrinol Metab* 2003;**88**:3437–3443.

Haimov-Kochman R, Ravhon A, Prus D, Greenfield C, Finci-Yeheskel Z, S Goldman-Wohl D, Natanson-Yaron S, Reich R, Yagel S, Hurwitz A. Expression and regulation of Sprouty-2 in the granulosa-lutein cells of the corpus luteum. *Mol Hum Reprod* 2005;**11**:537–542.

Hamel M, Dufort I, Robert C, Gravel C, Leveille MC, Leader A, Sirard MA. Identification of differentially expressed markers in human follicular cells associated with competent oocytes. *Hum Reprod* 2008;**23**: 1118–1127.

Hernandez-Gonzalez I, Gonzalez-Robayna I, Shimada M, Wayne CM, Ochsner SA, White L, Richards JS. Gene expression profiles of cumulus cell oocyte complexes during ovulation reveal cumulus cells express neuronal and immune-related genes: does this expand their role in the ovulation process? *Mol Endocrinol* 2006;**20**:1300–1321.

Hizaki H, Segi E, Sugimoto Y, Hirose M, Saji T, Ushikubi F, Matsuoka T, Noda Y, Tanaka T, Yoshida N *et al*. Abortive expansion of the cumulus and impaired fertility in mice lacking the prostaglandin E receptor subtype EP(2). *Proc Natl Acad Sci USA* 1999;**96**: 10501–10506.

Hsu DR, Economides AN, Wang X, Eimon PM, Harland RM. The Xenopus dorsalizing factor Gremlin identifies a novel family of secreted proteins that antagonize BMP activities. *Mol Cell* 1998;**1**:673–683.

Ishiguro K, Kojima T, Taguchi O, Saito H, Muramatsu T, Kadomatsu K. Syndecan-4 expression is associated with follicular atresia in mouse ovary. *Histochem Cell Biol* 1999;**112**:25–33.

Katagiri C, Masuda K, Urano T, Yamashita K, Araki Y, Kikuchi K, Shima H. Phosphorylation of Ser-446 determines stability of MKP-7. *J Biol Chem* 2005;**280**:14716–14722.

Masuda K, Shima H, Watanabe M, Kikuchi K. MKP-7, a novel mitogen-activated protein kinase phosphatase, functions as a shuttle protein. *J Biol Chem* 2001;**276**:39002–39011.

McKenzie LJ, Pangas SA, Carson SA, Kovanci E, Cisneros P, Buster JE, Amato P, Matzuk MM. Human cumulus granulosa cell gene expression: a predictor of fertilization and embryo selection in women undergoing IVF. *Hum Reprod* 2004;**19**:2869–2874.

Pangas SA, Jorgez CJ, Matzuk MM. Growth differentiation factor 9 regulates expression of the bone morphogenetic protein antagonist gremlin. *J Biol Chem* 2004;**279**:32281–32286.

Pelech S, Jelinkova L, Susor A, Zhang H, Shi X, Pavlok A, Kubelka M, Kovarova H. Antibody microarray analyses of signal transduction protein expression and phosphorylation during porcine oocyte maturation. *J Proteome Res* 2008;**7**:2860–2871.

Princivalle M, Hasan S, Hosseini G, de Agostini AI. Anticoagulant heparan sulfate proteoglycans expression in the rat ovary peaks in preovulatory granulosa cells. *Glycobiology* 2001;**11**:183–194.

Richards JS, Hernandez-Gonzalez I, Gonzalez-Robayna I, Teuling E, Lo Y, Boerboom D, Falender AE, Doyle KH, LeBaron RG, Thompson V *et al*. Regulated expression of ADAMTS family members in follicles and cumulus oocyte complexes: evidence for specific and redundant patterns during ovulation. *Biol Reprod* 2005;**72**:1241–1255.

Robert C, Gagne D, Bousquet D, Barnes FL, Sirard MA. Differential display and suppressive subtractive hybridization used to identify granulosa cell messenger rna associated with bovine oocyte developmental competence. *Biol Reprod* 2001;**64**:1812–1820.

Russell DL, Robker RL. Molecular mechanisms of ovulation: co-ordination through the cumulus complex. *Hum Reprod Update* 2007;**13**:289–312.

Russell DL, Ochsner SA, Hsieh M, Mulders S, Richards JS. Hormone-regulated expression and localization of versican in the rodent ovary. *Endocrinology* 2003a;**144**:1020–1031.

Russell DL, Doyle KM, Ochsner SA, Sandy JD, Richards JS. Processing and localization of ADAMTS-1 and proteolytic cleavage of versican during cumulus matrix expansion and ovulation. *J Biol Chem* 2003b; **278**:42330–42339.

Sakata M, Kobayashi H, Sun GW, Mochizuki O, Takagi A, Kojima T. Ryudocan expression by luteinized granulosa cells is associated with the process of follicle atresia. *Fertil Steril* 2000;**74**:1208–1214.

Shimada M, Hernandez-Gonzalez I, Gonzalez-Robayna I, Richards JS. Paracrine and autocrine regulation of epidermal growth factor-like factors in cumulus oocyte complexes and granulosa cells: key roles for prostaglandin synthase 2 and progesterone receptor. *Mol Endocrinol* 2006;**20**:1352–1365.

Smitz J, Andersen AN, Devroey P, Arce JC. Endocrine profile in serum and follicular fluid differs after ovarian stimulation with HP-hMG or recombinant FSH in IVF patients. *Hum Reprod* 2007;**22**:676–687.

Sugiura K, Su YQ, Li Q, Wigglesworth K, Matzuk MM, Eppig JJ. Fibroblast Growth Factors and Epidermal Growth Factor Cooperate with Oocyte-Derived Members of the TGFbeta Superfamily to Regulate Spry2 mRNA Levels in Mouse Cumulus Cells. *Biol Reprod* 2009; **81**:833–841.

Ubaldi F, Camus M, Smitz J, Bennink HC, Van Steirteghem A, Devroey P. Premature luteinization in in vitro fertilization cycles using gonadotropin-releasing hormone agonist (GnRH-a) and recombinant follicle-stimulating hormone (FSH) and GnRH-a and urinary FSH. *Fertil Steril* 1996;**66**:275–280.

Van Landuyt L, De Vos A, Joris H, Verheyen G, Devroey P, Van Steirteghem A. Blastocyst formation in in vitro fertilization versus intracytoplasmic sperm injection cycles: influence of the fertilization procedure. *Fertil Steril* 2005;**83**:1397–1403.

van Montfoort AP, Geraedts JP, Dumoulin JC, Stassen AP, Evers JL, Ayoubi TA. Differential gene expression in cumulus cells as a prognostic indicator of embryo viability: a microarray analysis. *Mol Hum Reprod* 2008;**14**:157–168.