MHR
Basic science of reproductive medicine

ORIGINAL RESEARCH

Human antral follicles <6 mm: a comparison between *in vivo* maturation and *in vitro* maturation in non-hCG primed cycles using cumulus cell gene expression

L. Guzman[1],*, T. Adriaenssens[1], C. Ortega-Hrepich[2], F. K. Albuz[1], I. Mateizel[2], P. Devroey[2], M. De Vos[2], and J. Smitz[1]

[1]Laboratory of Follicular Biology, UZ Brussel, Laarbeeklaan 101, 1090 Brussels, Belgium [2]Centre for Reproductive Medicine, UZ Brussel, Laarbeeklaan 101, 1090 Brussels, Belgium

*Correspondence address. E-mail: lguzmanm@gmail.com

ABSTRACT: Within the context of an oocyte *in vitro* maturation (IVM) program for reproductive treatment, oocyte cumulus complexes (COCs) derived from follicles <6 mm in patients with PCOS were matured *in vitro*. Key transcripts related to meiotic maturation (*FSHR*, *LHCGR*, *EGFR*, *PGR*) and oocyte competence (*AREG*, *ADAMTS*, *HAS2*, *PTGS2*) were quantified in cumulus cells (CCs) before and after maturation. Control CC samples were collected from PCOS and normo-ovulatory patients who had undergone conventional gonadotrophin stimulation for IVF/ICSI. Additional control samples from a non-stimulated condition were obtained *ex vivo* from patients undergoing ovariectomy for fertility preservation. Expression data from CCs from follicles with a diameter of <6 mm before (IVM-CCs) and after *in vitro* maturation (IVM-CCs) were obtained after pooling CCs into four groups in relation to the percentage of matured (MII) oocytes obtained after 40 h of IVM (0; 40–60; 61–80; 100% MII) and values were compared with *in vivo* matured controls (IVO-CCs). Genes encoding key receptors mediating meiotic resumption are expressed in human antral follicles of <6 mm before and after IVM. The expression levels of *FSHR*, *EGFR* and *PGR* in CCs were significantly down-regulated in the IVO-CCs groups and in the 100% MII IVM group compared with the BM groups; all the receptors studied in the 100% MII IVM group reached an expression profile similar to that of IVO-CCs. However, after maturation in a conventional IVF/ICSI cycle, IVO-CCs from large follicles contained significantly increased levels of *ADAMTS1*, *AREG*, *HAS2* and *PTGS2* compared with IVM-CCs and IVM-CCs; the expression patterns for these genes in all IVM-CCs were unchanged compared with IVM-CCs. In conclusion, genes encoding receptors involved in oocyte meiotic resumption appeared to be expressed in CCs of small human antral follicles. Expression levels of genes-encoding factors reflecting oocyte competence were significantly altered in IVM-CCs compared with *in vivo* matured oocytes from large follicles. Observed differences might be explained by the different stimulation protocols, doses of gonadotrophin or by the intrinsic differences between *in vivo* and *in vitro* maturation.

Key words: *in vitro* maturation / cumulus cells / gene expression

Introduction

Oocytes develop within the protective and supportive environment of the follicle where communication between the oocyte and the surrounding somatic cells is crucial during follicle development and occurs until ovulation (Smitz *et al.*, 2010). Oocyte maturation and the acquisition of oocyte developmental competence is a tightly regulated process involving a high number of molecular pathways directed by gonadotrophin stimuli and gonadotrophin receptors expressed in granulosa cells (GCs) and cumulus cells (CCs).

Four key receptors have a cardinal role during the complex processes of oocyte meiotic resumption and were selected for the current study. Luteinizing hormone receptor (LHCGR) is a G-protein-coupled receptor (GPCR) that activates adenylate cyclase, resulting in an important intracellular surge of cAMP; this cAMP surge activates cAMP-dependent serine protein kinase A (PKA) to induce ovulation and luteinization of the follicles (Richards *et al.*, 1994). Furthermore,

LHCGR stimulates the production of epidermal growth-like factors in GCs and CCs (Park *et al.*, 2004; Freimann *et al.*, 2005; Adriaenssens *et al.*, 2011). Follicle-stimulating hormone receptor (FSHR) plays an important role in the development of pre-ovulatory follicles *in vivo* (Macklon and Fauser, 2001); FSHR is a GPCR closely related to LHCGR and activates adenylate cyclase, thus mediating cAMP synthesis and resulting in PKA-activated intracellular signaling (Zeleznik *et al.*, 2003). The epidermal growth factor receptor (EGFR) is involved in meiotic resumption within the cumulus—oocyte complex (COC) (Lorenzo *et al.*, 1994). EGF ligands activate the mitogen-activated protein kinase (MAPK1/3) signal transduction pathway, which is involved in the expression of extracellular matrix genes, CC expansion and ovulation (Lorenzo *et al.*, 2001). Progesterone receptor (PGR) is induced after the LH surge and is immediately activated by progesterone to induce CC expansion (Hedin *et al.*, 1987); PGR-knockout mice exhibit normal follicle growth and luteinization but no ovulation (Robker *et al.*, 2000).

It has been demonstrated that a number of genes expressed in CCs after *in vivo* maturation are linked to oocyte competence during conventional assisted reproduction technique (ART) (McKenzie *et al.*, 2004; Hamel *et al.*, 2008; Assou *et al.*, 2010; Wathlet *et al.*, 2011). Four markers of oocyte competence were selected: (i) a disintegrin and metalloproteinase with thrombospondin motifs-1 (ADAMTS1) known to be expressed and regulated in the ovary by LHCGR and PGR (Doyle *et al.*, 2004; Shimada *et al.*, 2004; Richards *et al.*, 2005); (ii) amphiregulin (AREG), an EGF-family member involved in the EGF signaling network and expressed in pre-ovulatory follicles (Freimann *et al.*, 2005; Zamah *et al.*, 2010); and (iii) prostaglandin-endoperoxide synthase 2 (*PTGS2*) and (iv) hyaluronan synthase 2 (*HAS2*), which are involved in signaling and extracellular matrix formation during the ovulatory response (McKenzie *et al.*, 2004; Russell and Robker, 2007; Adriaenssens *et al.*, 2011; Wathlet *et al.*, 2011).

While in conventional ART, gonadotrophins are used to stimulate the ovary to produce many large follicles with mature oocytes *in vivo*, IVM aims primarily to avoid the side effects of ovarian stimulation by retrieving immature oocytes from small follicles ($<$12 mm) from unstimulated or minimally stimulated ovaries. However, the intrinsic developmental competence of oocytes is reduced after IVM compared with conventional ART (Smitz *et al.*, 2011), although this might be compensated, at least partly, in PCOS patients due to the high number of antral follicles (Son and Tan, 2010). Nevertheless, it has recently been reported (Guzman *et al.*, 2012) that oocytes isolated from small antral follicles with a diameter of $<$6 mm can mature *in vitro*, support embryo development and can result in live birth. However, there is no information on the expression pattern of the genes related to meiotic resumption and maturation or linked to oocyte competence in cases where no hCG is given to prime the follicles before oocyte retrieval.

All IVM-CC samples in this study were retrieved from small antral follicles with a diameter of $<$6 mm. The first aim of the study was to evaluate the expression patterns in CCs for selected genes involved in oocyte maturation and to relate these to oocyte maturation rates after IVM. Secondly, the sample sets were chosen to understand gene expression patterns between *in vitro* and *in vivo* matured oocytes, and in normo-ovulatory patients and patients with PCOS.

Materials and Methods

Patients

This study was approved by the Ethics Committee of UZ-VUB Brussel. CCs were obtained from normo-ovulatory patients (either cancer patients undergoing ovarian tissue cryopreservation or patients with fertility problems undergoing conventional ovarian stimulation with IVF/ICSI treatment) and PCOS patients who underwent either IVM or conventional IVF/ICSI were included. Overall, 39 patients undergoing 47 independent cycles were included in the study. The baseline patient characteristics are summarized in Table I.

IVM treatment protocol

The clinical and laboratory protocol for IVM treatment has previously been described (De Vos *et al.*, 2011). Briefly, patients received 150 IU of hMG (Menopur®, Ferring Pharmaceuticals A/S, Copenhagen, Denmark) daily for 3 consecutive days, starting on Day 3 after spontaneous menses or withdrawal bleeding. Oocyte retrieval was planned on cycle day 7 or 8 depending on the endometrial thickness ($>$5 mm), but before the largest follicle reached a diameter of 10 mm. No hCG trigger was administered. COCs were retrieved with a 17-gauge single lumen needle (Cook Medical, K-OPS-1230-VUB, Limerick, Ireland). During the ultrasound-guided procedure, two-dimensional diameter measurements were performed in all punctured follicles. Follicular aspirates were filtered (Falcon 1060; 70-μm mesh size, Franklin Lakes, NJ, USA). COCs were collected in a flushing medium (Origio, Måløv, Denmark). All the oocytes were at the germinal vesicle (GV) stage at the time of oocyte collection. COCs were subsequently matured for 40 h in MediCult IVM® System (Origio) supplemented with 75 mIU/ml FSH, 100 mIU/ml hCG and 10% human serum albumin (Vitrolife®, Göteborg, Sweden). After IVM, oocytes were inseminated by ICSI as described previously (Joris *et al.*, 1998). Oocytes and embryos were cultured individually in 25 μl medium droplets covered with mineral oil (Vitrolife®, Göteborg, Sweden). Embryos were cultured until Day 3, in sequential SAGE®-cleavage media (Cooper Surgical, Pasadena, USA).

Conventional IVF/ICSI

Controlled ovarian stimulation (COS) was performed following a conventional long agonist protocol using HP-hMG [Menopur, Ferring Pharmaceuticals (IVO-Normo group)] or rFSH [Puregon, Organon, The Netherlands (IVO-PCOS group)] in a GnRH-antagonist protocol [with ganirelix (Orgalutran)]. Daily gonadotrophin doses ranged from 150 to 225 IU/day for HP-hMG and from 100 to 225 IU/day for rFSH. Gonadotrophins were administered at a fixed dose for 7–13 days, starting on cycle day 2. GnRH-antagonist administration started on cycle day 7. The trigger to induce final follicular maturation (10 000 IU hCG) was administered as soon as 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 (Joris *et al.*, 1998).

Collection of samples

The different populations of CCs collected are depicted in Fig. 1. CCs from follicles $<$6 mm before oocyte *in vitro* maturation (BM-CC) were collected from five unstimulated normo-ovulatory patients (BM-Normo) during ovarian tissue handling after oophorectomy and before ovarian tissue cryopreservation. Briefly, follicles from the ovary were measured, punctured *ex vivo* with a needle and collected in flushing medium (Origio). COCs from PCOS patients (BM-PCOS) were retrieved after transvaginal ovarian puncture. BM-CCs were collected after mechanical

Table I Overview of study patients and baseline patient characteristics.

	Before maturation		After in vitro maturation				After in vivo maturation	
	BM normo-ovulatory (100% GV)	BM PCOS (100% GV)	IVM-CC1 (0% MII)	IVM-CC2 (40–60% MII)	IVM-CC3 (61–80% MII)	IVM-CC4 (100% MII)	IVO normo-ovulatory (100% MII)	IVO PCOS (100% MII)
Number of cycles (equal to number of samples)	5	6	6	6	6	6	6	6
Age ± SD	24.3 ± 3.1[a]	26.5 ± 3.3[a,b]	30.2 ± 3.1[a,b]	26.7 ± 3.4[a,b]	27.3 ± 3.7[a,b]	29.3 ± 3.1[a,b]	30.3 ± 3.6[a,b]	31.5 ± 3.9[b]
Cumulative FSH dose (IU ± SD)	0 ± 0	400 ± 155	475 ± 175	462 ± 146	437 ± 153	462 ± 230	2162 ± 409	1597 ± 465
Stimulation protocol	None	MS	MS	MS	MS	MS	COS	COS
In vivo ovulatory trigger (hCG)	–	–	–	–	–	–	+	+
Follicle diameter (mm)	<6	<6	<6	<6	<6	<6	>15	>15

Data represent the mean and standard deviation (SD) values. MII, metaphase II; PCOS, polycystic ovary syndrome; BM Normo-ovulatory, before oocyte maturation in normovulatory patients; BM PCOS, before oocyte maturation in PCOS patients; IVO PCOS, after in vivo maturation in PCOS patients; IVO Normo-ovulatory, after in vivo maturation in normo-ovulatory patients; MS, minimal stimulation; COS, controlled ovarian stimulation; (−) without in vivo hCG stimulation; (+) with in vivo hCG stimulation.
[a,b]ANOVA test showing a statistically significant difference between the groups ($P < 0.05$).

denudation with a pipette (Vitrolife, Göteborg, Sweden 190–210 μm). CCs were snap-frozen in liquid nitrogen.

For the collection of *in vitro* maturation CC (IVM-CC), COCs from PCOS patients were cultured in groups. CCs were collected after cumulase treatment (Origio). CCs were classified according to the oocyte maturation rate. The four categories were: all oocytes were arrested in the GV or germinal vesicle breakdown (GVBD) stage (IVM-CC 1); 40–60% of the oocytes were mature metaphase II (MII) (IVM-CC 2); 61–80% of the oocytes were MII (IVM-CC 3) and 100% of the oocytes were mature (IVM-CC 4).

As for *in vivo* maturation CC (IVO-CC), CCs from follicles >15 mm were aspirated in normo-ovulatory (IVO-Normo) and PCOS patients (IVO-PCOS) undergoing conventional IVF/ICSI treatment, 36 h post-hCG injection. CCs were detracted from the oocyte with ICSI cumulase (Origio) 4 h after oocyte retrieval; only CCs from MII oocytes were collected and pooled for each patient (Adriaenssens et al., 2010).

For each of the studied groups five or six samples were collected. Each sample was a pool of 3–6 COCs retrieved from one patient and one independent cycle.

RNA isolation and cDNA synthesis

The protocol was described previously by Adriaenssens et al. (2010). Briefly, total RNA was extracted from CCs using the total RNA extraction kit (RNeasy; Qiagen, Antwerp, Belgium) on the QiaCube robot (Qiagen) according to the manufacturer's instructions with minor modifications. A DNAse step was used to remove residual genomic DNA and extracted total RNA was subsequently eluted in 14 μl of RNAse-free water. Total RNA was reverse transcribed into cDNA with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Gent, Belgium) containing a blend of oligo (dT) and random primers, according to the manufacturer's instructions. Negative controls were generated by omitting the RNA or the reverse transcription enzyme. All resulting cDNA was frozen at $-80°C$.

Quantitative PCR

cDNA quantification by real-time PCR was performed on a LightCycler 480 apparatus (Roche Applied Science, Mannheim, Germany). PCR primer design for qPCR was performed using the universal probe library software (Roche Diagnostics) (Table II). Primers were selected to be specific and complementary to the human sequences of *FSHR*, *EGFR*, *PGR*, *ADAMTS1* and *AREG*. Primer sequences for *LHCGR*, *PTGS2* and *HAS2* have been published previously (Adriaenssens et al., 2010; Sanchez et al., 2010). The expression of the genes was normalized using beta 2 microglobulin (β2M) and ubiquitin (UBC). The total volume for the amplification reaction was 10 μl, containing 2 μl cDNA, 5 μl SYBR Green PCR Master Mix 2× (Roche Applied Science), 1 μl of 0.6 μM primer mix and 2 μl of nuclease-free water per sample. The amplification conditions were as follows: 10 min at 95°C, followed by 50 cycles at 95°C for 10 s and 60°C for 30 s, followed by a melting curve. A log6 dilution series of a synthetic oligonucleotide corresponding to the amplicon sequence was included in each experiment. The number of copies for each gene within each sample was normalized with the endogenous controls. Each sample was tested in triplicate and all negative controls were as expected. All results mentioned hereafter are the normalized values.

Statistical analysis

Q-PCR results were compared using two-tailed *t*-test or ANOVA followed by the Tukey post-hoc test. Values were determined to be significant when $P < 0.05$. Two subanalyses were performed: (i) to explore the differences in gene expression between subgroups of patients classified according to their endocrine background (normo-ovulatory patients and patients with PCOS) before and after IVM and (ii) to analyze the influence of repetitive patients included in different groups with independent cycles.
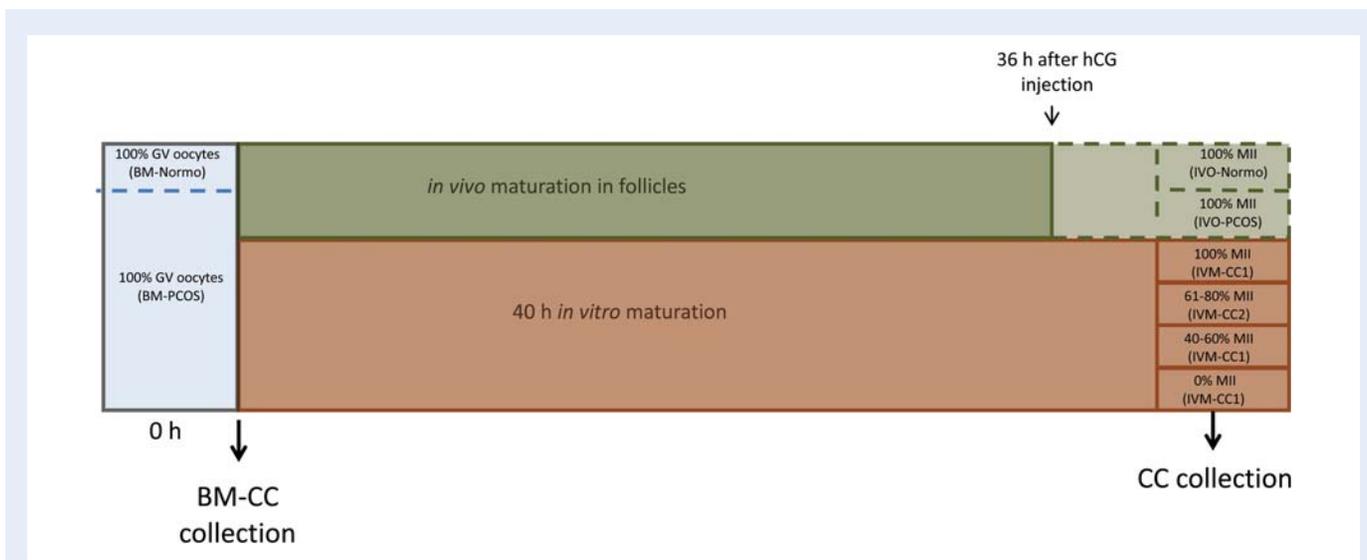
Figure 1 The scheme shows the CC groups included in the study, indicating the collection time points. h, hours; GV, germinal vesicle; BM, before maturation, IVM, *in vitro* maturation; IVO, *in vivo* matured; Normo, normo-ovulatory; PCOS, polycystic ovary syndrome.

Table II Q-PCR primer sequences.

Gene	Accession no.	Forward primer sequences	Reverse primer sequences	Product size (pb)
FSHR	NM_000145.3	GGTGCATTTTCAGGATTTGG	TCACCTCCAAGACATCATTCTG	67
EGFR	NM_005228.3	CATGTCGATGGACTTCCAGA	GGGACAGCTTGGATCACACT	61
PGR	NM_000926.4	GTCATAGACCCCCGTTGCTA	GCTAAGCCAGCAAGAAATGG	124
ADAMTS1	NM_006988.3	GGCGTCAATGCTTTCCAA	AGGCTTGTCCATCAAACATTC	112
AREG	NM_001657.2	TGATCCTCACAGCTGTTGCT	CCATTCTCTTGTCGAAGTTTCTTT	106

FSHR, follicle-stimulating hormone receptor; *EGFR*, epidermal growth factor receptor; *PGR*, progesterone receptor; *ADAMTS1*, A disintegrin and metalloproteinase with thrombospondin motifs-1; *AREG*, amphiregulin.

All analyses and graphs were generated using Graphpad Prim V4.0 (Graphpad software, CA, USA).

Results

Table I summarizes the basal patient characteristics. The mean age of patients in the BM-normo group was statistically lower than that of patients in the IVO-PCOS group. Additionally, patients in the BM-normo group did not receive any ovarian stimulation, whereas patients in the BM-PCOS and IVM-CC groups received minimal ovarian stimulation with a cumulative dose of 450 IU HP-HMG per cycle. Patients from IVO-CC groups received statistically higher doses of gonadotrophins in GnRH-analog treated cycles compared with the other groups.

All biological material of the study was collected in a single center and all molecular analyses were performed in one single experiment. Each experimental group contained six samples obtained from independent cycles (five samples from different patients were included in BM-Normo group). In total, CCs from 224 COCs from 39 patients in 47 independent cycles were analyzed and classified according to their maturation status. All oocytes in COCs obtained before oocyte IVM (BM-groups) were at the GV stage and the CCs were still compact. After 40 h of IVM, COCs were expanded and the maturation status was assessed; mature oocytes were microinjected with partner sperm and CCs were collected.

All CC samples contained enough total RNA to detect the endogenous controls and selected genes. Figure 2 shows the relative gene expression of the receptors under study. In CCs of MII oocytes after IVM (IVM-CC 4), the levels of *FSHR*, *EGFR* and *PGR* transcripts were significantly down-regulated, respectively, 7-fold ($P < 0.001$), 3-fold ($P < 0.001$) and 2.5-fold ($P = 0.01$) compared with BM-PCOS-CC. In contrast, IVM-CC 4 *LHCGR* expression was 3.5-fold up-regulated compared with BM-PCOS, although this was not significant ($P = 0.188$). Similar expression patterns were observed in CCs from *in vivo* matured oocytes, although the increased *LHCGR* expression level after *in vivo* oocyte maturation was statistically significant compared with the those before maturation ($P < 0.001$) (Fig. 2).

Gene expression patterns of markers of oocyte competence in CCs are shown in Fig. 3. The gene expression levels of *ADAMTS1*, *AREG*, *HAS2* and *PTGS2* after 40 h of IVM culture were not statistically different from those before IVM culture in any of the IVM subgroups. However, the expression levels of *ADAMTS1*, *AREG* and *PTGS2* were
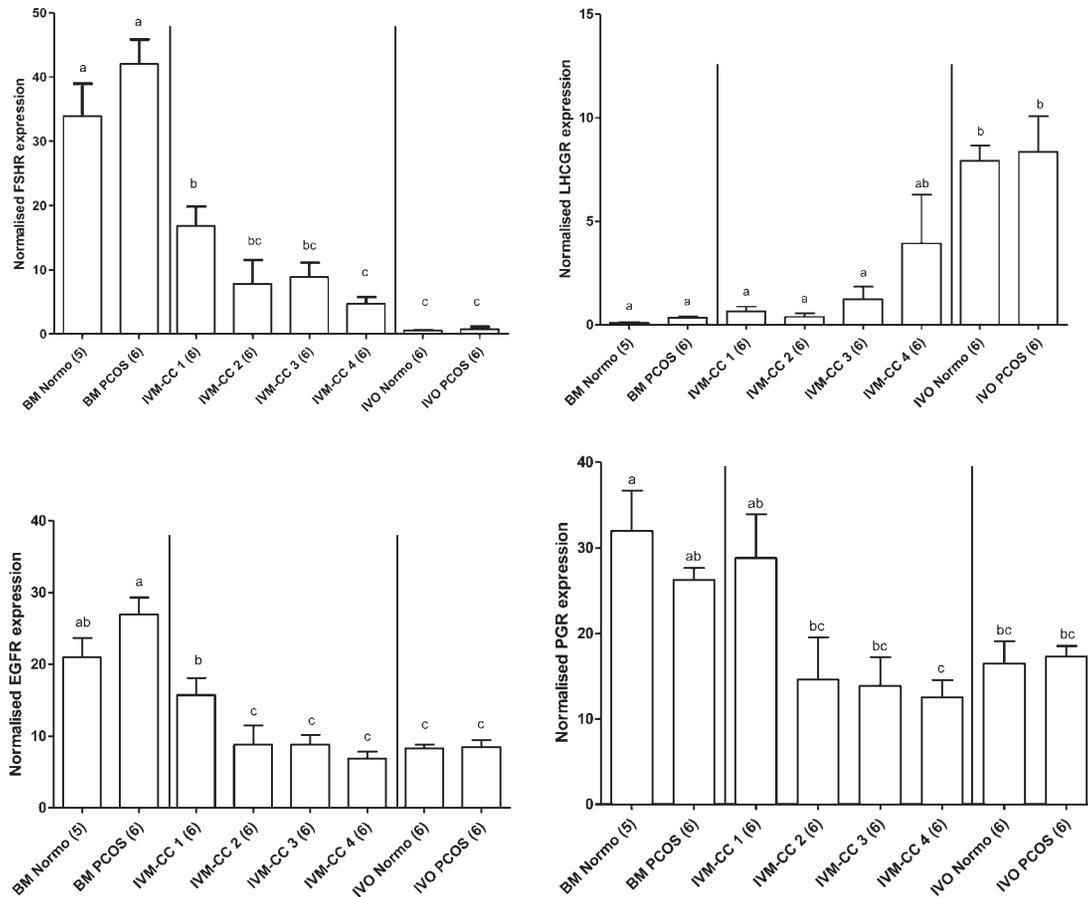
Figure 2 Gene expression patterns for the receptors. BM, before maturation, IVM, *in vitro* maturation; IVO, *in vivo* matured; Normo, normo-ovulatory; PCOS, polycystic ovary syndrome. *FSHR*, follicle-stimulating hormone receptor; *LHCGR*, luteinizing hormone receptor; *EGFR*, epidermal growth factor receptor; *PGR*, progesterone receptor. IVM-CC1, cumulus cells after 40 h of IVM culture of a group where all oocytes were arrested in the GV or germinal vesicle breakdown (GVBD) stage; IVM-CC2, 40–60% of the oocytes were mature MII; IVM-CC3, 61–80% of the oocytes were MII; IVM-CC4, 100% of the oocytes were MII. The expression of the genes was normalized using β2M and UBC. a, b, c, ANOVA test showing a statistically significant difference between the groups (*P* < 0.05).

significantly up-regulated after *in vivo* maturation in normo-ovulatory and PCOS patients (all *P*-values <0.005). Interestingly however, the *HAS2* expression was not statistically different between the IVO-PCOS and any of the IVM-CC groups, but the *HAS2* expression in the IVO-Normo reached statistically higher values than those in the IVM-CC groups.

COCs were cultured in groups and were classified following their divergent maturation rates (IVM-CC 1–4). Gene expression levels of *FSHR*, *EGFR* and *PGR* were significantly higher in CCs associated with oocytes that did not mature *in vitro* (IVM-CC 1) than that in CC in the IVM-CC 4 group, where all oocytes reached MII stage (*P* = 0.003, 0.006 and 0.015 respectively). In contrast, *LHCGR* expression showed a trend to increase in IVM-CC4 compared with that in IVM-CC1 (*P* = 0.196). However, *ADAMTS1*, *AREG*, *HAS2* and *PTGS2* expression levels did not differ significantly between IVM groups in relation to different oocyte maturation rates.

Finally, a subanalysis using a *t*-test was performed to explore the differences in CC gene expression between normo-ovulatory and PCOS patients before IVM and after *in vivo* oocyte maturation: this analysis did not reveal any statistical differences between patient types. To exclude bias that may occur as a result of the analysis of CCs from multiple treatment cycles in a single patient, we performed a subanalysis excluding patients who underwent multiple cycles from each group. This resulted in a minimal number of four patients in each group, but did not alter the statistically significant differences that had been obtained with the initial analysis, in all genes and groups studied (data not shown).

Discussion

After hCG triggering, human oocytes are able to resume meiosis, CCs start to expand and the processes leading to extrusion of the first polar body are initiated. These morphological changes are observed at 15, 22 and 35 h after hCG triggering, respectively (Bomsel-Helmreich *et al*., 1987). The cellular modifications are associated with gene expression modifications at different time points, as reported in mice and pigs (Kawashima *et al*., 2008; Adriaenssens *et al*., 2011). *In vivo*, in a fully grown follicle, the LH stimulus induces
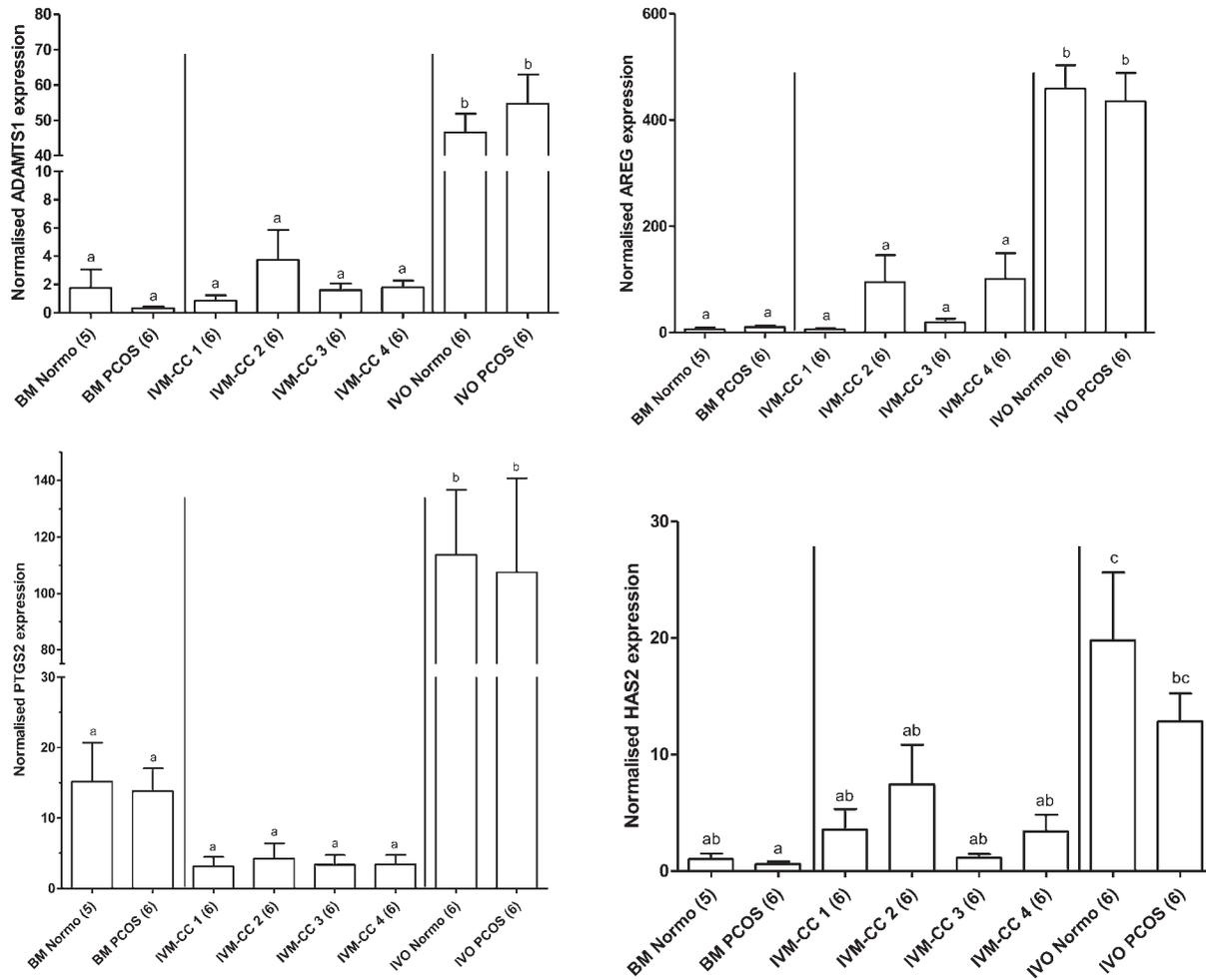
Figure 3 Gene expression patterns for the cellular markers. BM, before maturation, IVM, *in vitro* maturation; IVO, *in vivo* matured; Normo, normo-ovulatory; PCOS, polycystic ovary syndrome. *ADAMTS1*, a disintegrin and metalloproteinase with thrombospondin motifs-1; *AREG*, amphiregulin; *PTGS2*, prostaglandin-endoperoxide synthase 2; *HAS2*, hyaluronan synthase 2. IVM-CC1, cumulus cells after 40 h of IVM culture of a group where all oocytes were arrested in the GV or GVBD stage; IVM-CC2, 40–60% of the oocytes were mature MII; IVM-CC3, 61–80% of the oocytes were MII; IVM-CC4, 100% of the oocytes were MII. The expression of the genes was normalized using β2M and UBC. a, b, c, ANOVA test showing a statistically significant difference between the groups ($P < 0.05$).

the maturation cascade in GCs and transduces this stimulus to the CCs and the oocyte. Genuine IVM treatment is without hCG triggering, because oocytes are retrieved at the GV stage with compacted CCs, and the processes of meiotic resumption, CC expansion and oocyte maturation have to be completed *in vitro*. Therefore, COCs cultured *in vitro* have to be able to mature and produce paracrine signals to allow the oocyte to resume meiosis without having been exposed to the normal physiological meiosis inducing factors *in vivo* (e.g. LH stimuli to mural GC, EGF-like factors and prostaglandins).

Receptors involved in oocyte maturation

An antral follicle contains two types of GCs: mural GCs and CCs; *LHCGR* is highly expressed in mural GCs but its expression is very low in CCs (Peng *et al.*, 1991; Yang *et al.*, 2005). Recently, *LHCGR* was studied in mural GCs and CCs in follicles from 3 to 20 mm; they report the *LHCGR* expression in the mural GCs for the complete

follicle diameter range; additionally, *LHCGR* was also expressed in IVM-CC expanded and compact after *in vivo* hCG priming but before IVM culture (Jeppesen *et al.*, 2012). Our results, obtained in a non-hCG-triggered IVM system, confirm the presence of *LHCGR* in human CCs derived from follicles of <6 mm before and after IVM. Moreover, the *LHCGR* expression in CCs was up-regulated after *in vivo* maturation and a similar trend was observed in the IVM group with 100% oocyte maturation but it did not reach statistical significance. *LHCGR* transcript levels in CCs were not significantly different between normo-ovulatory and PCOS patients. Others have found that *LHCGR* gene expression in large follicle GCs and theca cells from PCOS follicles is significantly higher in PCOS patients (Jakimiuk *et al.*, 2001), the contradictory findings might be explained by the fact that the CCs are not the main site expression/action for *LHCGR* or by the limited number of patients in the current and referenced manuscript.

In mice, CCs do not expand in response to the LH stimulus *in vitro*, but they do expand in response to FSH (Eppig, 1979); similar results have been obtained in rats where FSH was able to induce mucification and progesterone production in CCs (Hillensjo *et al.*, 1981). Additionally, FSH increases intracellular cAMP, promotes maturation of EGFs and activates the tyrosine kinase cascade in CCs (Dekel and Kraicer, 1978; Prochazka *et al.*, 2003). Following the activation of the EGF signaling, there is phosphorylation of MAPK1/3 promoting CC expansion and meiotic activation (Park *et al.*, 2004; Diaz *et al.*, 2006). Furthermore, it has been proposed that the supplementation of IVM medium with hCG is not necessary to complete oocyte maturation (Ge *et al.*, 2008). Our findings confirm that *FSHR* is expressed at higher levels compared with *LHCGR* in CCs before oocyte IVM. Therefore, the *FSHR* pathway *in vitro* may be the main signal transducer to achieve oocyte meiotic resumption. Regarding the kinetics during the ovulatory process, our results in IVM without hCG triggering showed that *FSHR* is significantly down-regulated after *in vitro* and *in vivo* maturation. Remarkably, these results are in concordance with those of an IVM system with hCG triggering (Jeppesen *et al.*, 2012).

The EGFR is located on the cell surface and is activated by its binding to specific ligands (EGF-like factors) and *PGR* expression can be induced either by LH or FSH through a cAMP-mediated pathway. It has been described that both receptors are expressed in CCs (el-Danasouri *et al.*, 1993; Zhao *et al.*, 2005) and our results show high expression levels for both receptors before and after oocyte IVM. Furthermore, our study demonstrates that expression levels of *EGFR*, *FSHR* and *PGR* in the IVM-CC 1 group (absence of oocyte maturation after 40 h of IVM culture) are not different from those before IVM (BM-groups) and are significantly higher than those in the IVM-CC 4 group (full oocyte maturation to MII stage) and in *in vivo* matured COCs. This relative high expression level of *EGFR*, *FSHR* and *PGR* may reflect the incapacity of the oocyte to mature *in vitro* and indicate the insensitivity of the CCs to the factors driving the initiation of meiotic progression.

Markers of oocyte competence in IVM

Overall, the efficiency of IVM is limited and oocyte developmental competence in IVM is still lower than conventional ART (Smitz *et al.*, 2011). Nevertheless, micro-array gene expression data in a non-human primate model has shown a limited number of transcripts differentially expressed between *in vitro* and *in vivo* mature oocytes (Lee *et al.*, 2008). Despite this, a large number of genes were shown to be differentially expressed in CCs between *in vitro* and *in vivo* maturation, and the main mis-regulated pathways were DNA replication, cell cycle, cell death and lipid metabolism, suggesting that the interaction between CCs and oocytes could be affected in IVM (Lee *et al.*, 2011). The current study in human CCs also suggests some differences between the CCs of *in vivo* and *in vitro* matured COCs.

Female *ADAMTS-1* mouse knockouts are subfertile due to impaired ovulation, with mature oocytes remaining confined in the follicles (Mittaz *et al.*, 2004; Shozu *et al.*, 2005). *ADAMTS-1* is induced by the LH surge in ovulating follicles and this induction depends on the expression of the *PGR* gene (Doyle *et al.*, 2004; Shimada *et al.*, 2004; Richards *et al.*, 2005). *PGR* mouse knockouts fail to ovulate and have reduced expression levels of mRNA and proteins of ADAMTS-1 (Robker *et al.*, 2000; Russell *et al.*, 2003). Our results

show that *PGR* is expressed by all CC groups analyzed. Nevertheless, the expression of *ADAMTS-1* in CCs appeared not to be regulated after IVM culture (IVM-CC), not even when oocytes were able to mature *in vitro*. Interestingly, the results of Yung *et al.* (2010) are in line with our observations, with a lower expression level of *ADAMTS-1* in the mural GCs isolated from small antral follicles (<10 mm), a higher expression in larger follicles (>10 mm), but still with a lower expression level in both compared with the mural GCs of conventional IVF/ICSI patients.

EGF-like growth factors (AREG, BTC and EREG) are known to activate EGFRs in COCs, and trigger oocyte maturation and CC expansion (Park *et al.*, 2004). AREG plays a major role in oocyte maturation in humans (Zamah *et al.*, 2010). We observed low levels of *AREG* expression after 40 h of IVM culture, compared with *AREG* expression levels after *in vivo* oocyte maturation. Several mechanisms may underlie this observation, for example, the doses of hCG in *in vitro* culture are low compared with the dose of hCG administered during oocyte *in vivo* maturation. Furthermore, *AREG* has been shown to be predominantly expressed during the first stage of *in vitro* oocyte maturation, and its expression decreases to basal levels at the end of the maturation process (Kawashima *et al.*, 2008).

It has been demonstrated that *PTGS2* and *HAS2* are needed for CC expansion (Ochsner *et al.*, 2003) and they are related to embryo quality (McKenzie *et al.*, 2004). In mouse, *Ptgs2* and *Has2* are up-regulated in CCs at the GBVD and GV stages, respectively, and the expression of these genes drops to basal levels at the time of ovulation (Adriaenssens *et al.*, 2011). However, in human IVM-CC, the expression of *PTGS2* and *HAS2* may display a similar profile. In order to explain the similar gene expression before and after IVM, studies evaluating the developmental transitional stages throughout the maturation period rather than extreme time points should be considered. A reduced expression of *PTGS2* and *HAS2* in CCs after IVM compared with CCs obtained after *in vivo* maturation was observed, in spite of CC expansion and mucification after IVM culture. It should be stated that the CC expansion observed in an IVM system is morphologically different from that after *in vivo* CC expansion (Smitz *et al.*, 2011). These observations might be due to alternative pathways regulated in IVM-CC compared with IVO-CC, coinciding with the differential gene expression for oocyte quality markers (Lee *et al.*, 2011).

All CC markers of oocyte competence analyzed in this study are involved in oocyte maturation and have previously been implicated in oocyte/embryo competence in conventional ART (McKenzie *et al.*, 2004; Feuerstein *et al.*, 2007; Yung *et al.*, 2010; Zamah *et al.*, 2010). The results of our study reveal that these markers are expressed at significantly lower levels in CCs after IVM compared with that after *in vivo* oocyte maturation. Nevertheless, *ADAMTS1*, *AREG* and *HAS2* expression levels increase, although not significantly, after IVM in IVM-CC4 compared with BM-PCOS, while the expression of *PTGS2* decreases ($P > 0.05$). The absence of prostaglandin E2 (PGE2) production *in vitro* might compromise the maturation in IVM because PGE2 is a known up-regulator of the EGF-like family member response in human GCs (Ben-Ami *et al.*, 2006). Reduced expression of CC genes after IVM has also been described in the monkey model using a similar experimental approach (Lee *et al.*, 2011). These results could either be explained by the inability of certain receptors to respond to *in vitro* stimuli or by the different expression profiles of these markers during IVM. The markedly different gonadotrophin

stimulation protocols between *in vitro* and *in vivo* oocyte maturation treatments may also contribute to the divergent gene expression patterns (Adriaenssens et al., 2010; Sanchez et al., 2010; Wathlet et al., 2011). In the IVM-CC group, low doses of gonadotrophins are used in all patients and low doses of FSH and hCG were added to the maturation medium. Additionally, IVO-CC are exposed to high doses of gonadotrophins and exacerbated cellular responses to gonadotrophin treatment might illustrate similar gene expression kinetics as described in animal models (Kawashima et al., 2008; Adriaenssens et al., 2011). Further studies would be valuable to demonstrate the transitional kinetics of gene expression during oocyte maturation.

With regard to the patient's endocrine profile, several previously published studies have reported similar gene expression profiles. However, in patients with PCOS, a number of studies have reported altered gene expression patterns in the CCs of these patients, such as dysregulated expression of genes involved in the Wnt signaling pathway, immunological factors and extracellular matrix genes. *ADAMTS-1* dysregulation has also been associated with PCOS (Jansen et al., 2004). However, with regard to the expression patterns of the genes studied here, no statistically significant differences were observed between CCs from normo-ovulatory patients and patients with PCOS, neither before oocyte IVM nor after *in vivo* maturation. This observation led us to suggest that there are no gene expression differences in this specific cohort of CCs retrieved from small antral follicles, at least for the genes under study. However, further experiments should confirm this by increasing the number of genes and patients studied.

Conclusion

In the current study, we describe for the first time the expression of a number of key genes in human CCs isolated from follicles with a diameter not exceeding 6 mm in patients undergoing IVM without hCG triggering. Genes encoding receptors involved in oocyte meiotic resumption appear to be expressed in these small human antral follicles, and the relative expression levels and profiles of these genes in CCs of oocytes capable of reaching MII after IVM are similar to those observed after *in vivo* oocyte maturation. However, the expression profiles of *ADAMTS1*, *AREG*, *PTGS2* and *HAS2*, genes that are induced by an ovulation trigger and reflect oocyte competence, are significantly altered after oocyte IVM, in comparison with follicles that reached full growth *in vivo*. Notwithstanding this observation, nearly 50% of oocytes derived from follicles <6 mm can mature *in vitro* and a subset of these oocytes have been shown to result in life births (Guzman et al., 2012). Hence, the 'classical' CC biomarkers of oocyte competence identified in conventional IVF/ICSI treatment are probably not predictive of nuclear competence in follicles that are <6 mm of diameter in the absence of hCG triggering before oocyte retrieval.

Authors' roles

L.G. drafted the manuscript, performed data analysis and executed the IVM culture procedure. T.A. corrected the manuscript, performed data analysis and provided interpretation. C.O.H. undertook patient recruitment and management. F.K.A. executed the IVM culture procedure and did sample collection. IM did sample and data collection.

P.D. supervised the clinical activities. M.D.V. reviewed and edited the manuscript and performed patient recruitment and management. J.S. reviewed and edited the manuscript, and is a principle investigator of the oocyte maturation project.

Funding

Conflict of interest

None declared.

References

Adriaenssens T, Segers I, Wathlet S, Smitz J. The cumulus cell gene expression profile of oocytes with different nuclear maturity and potential for blastocyst formation. *J Assist Reprod Genet* 2011;**28**:31–40.

Adriaenssens T, Wathlet S, Segers I, Verheyen G, De Vos A, Van der Elst J, Coucke W, Devroey P, Smitz J. Cumulus cell gene expression is associated with oocyte developmental quality and influenced by patient and treatment characteristics. *Hum Reprod* 2010;**25**:1259–1270.

Assou S, Haouzi D, De Vos J, Hamamah S. Human cumulus cells as biomarkers for embryo and pregnancy outcomes. *Mol Hum Reprod* 2010;**16**:531–538.

Ben-Ami I, Freimann S, Armon L, Dantes A, Strassburger D, Friedler S, Raziel A, Seger R, Ron-El R, Amsterdam A. PGE2 up-regulates EGF-like growth factor biosynthesis in human granulosa cells: new insights into the coordination between PGE2 and LH in ovulation. *Mol Hum Reprod* 2006;**12**:593–599.

Bomsel-Helmreich O, Huyen LV, Durand-Gasselin I, Salat-Baroux J, Antoine JM. Timing of nuclear maturation and cumulus dissociation in human oocytes stimulated with clomiphene citrate, human menopausal gonadotropin, and human chorionic gonadotropin. *Fertil Steril* 1987;**48**:586–595.

Dekel N, Kraicer PF. Induction in vitro of mucification of rat cumulus oophorus by gonadotrophins and adenosine 3′,5′-monophosphate. *Endocrinology* 1978;**102**:1797–1802.

De Vos M, Ortega-Hrepich C, Albuz FK, Guzman L, Polyzos NP, Smitz J, Devroey P. Clinical outcome of non-hCG-primed oocyte in vitro maturation treatment in patients with polycystic ovaries and polycystic ovary syndrome. *Fertil Steril* 2011;**96**:860–864.

Diaz FJ, O'Brien MJ, Wigglesworth K, Eppig JJ. The preantral granulosa cell to cumulus cell transition in the mouse ovary: development of competence to undergo expansion. *Dev Biol* 2006;**299**:91–104.

Doyle KM, Russell DL, Sriraman V, Richards JS. Coordinate transcription of the ADAMTS-1 gene by luteinizing hormone and progesterone receptor. *Mol Endocrinol* 2004;**18**:2463–2478.

el-Danasouri I, Frances A, Westphal LM. Immunocytochemical localization of transforming growth factor-alpha and epidermal growth factor receptor in human fallopian tubes and cumulus cells. *Am J Reprod Immunol* 1993;**30**:82–87.

Eppig JJ. FSH stimulates hyaluronic acid synthesis by oocyte-cumulus cell complexes from mouse preovulatory follicles. *Nature* 1979;**281**:483–484.

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.

Freimann S, Ben-Ami I, Dantes A, Armon L, Ben Ya'cov-Klein A, Ron-El R, Amsterdam A. Differential expression of genes coding for EGF-like factors and ADAMTS1 following gonadotropin stimulation in normal and transformed human granulosa cells. *Biochem Biophys Res Commun* 2005;**333**:935–943.

Ge HS, Huang XF, Zhang W, Zhao JZ, Lin JJ, Zhou W. Exposure to human chorionic gonadotropin during in vitro maturation does not improve the maturation rate and developmental potential of immature oocytes from patients with polycystic ovary syndrome. *Fertil Steril* 2008;**89**:98–103.

Guzman L, Ortega-Hrepich C, Albuz FK, Verheyen G, Devroey P, Smitz J, De Vos M. Developmental capacity of in vitro-matured human oocytes retrieved from polycystic ovary syndrome ovaries containing no follicles larger than 6 mm. *Fertil Steril* 2012;**98**:503–507; e502.

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.

Hedin L, McKnight GS, Lifka J, Durica JM, Richards JS. Tissue distribution and hormonal regulation of messenger ribonucleic acid for regulatory and catalytic subunits of adenosine 3′,5′-monophosphate-dependent protein kinases during ovarian follicular development and luteinization in the rat. *Endocrinology* 1987;**120**:1928–1935.

Hillensjo T, Magnusson C, Svensson U, Thelander H. Effect of luteinizing hormone and follicle-stimulating hormone on progesterone synthesis by cultured rat cumulus cells. *Endocrinology* 1981;**108**:1920–1924.

Jakimiuk AJ, Weitsman SR, Navab A, Magoffin DA. Luteinizing hormone receptor, steroidogenesis acute regulatory protein, and steroidogenic enzyme messenger ribonucleic acids are overexpressed in thecal and granulosa cells from polycystic ovaries. *J Clin Endocrinol Metab* 2001;**86**:1318–1323.

Jansen E, Laven JS, Dommerholt HB, Polman J, van Rijt C, van den Hurk C, Westland J, Mosselman S, Fauser BC. Abnormal gene expression profiles in human ovaries from polycystic ovary syndrome patients. *Mol Endocrinol* 2004;**18**:3050–3063.

Jeppesen JV, Kristensen SG, Nielsen ME, Humaidan P, Dal Canto M, Fadini R, Schmidt KT, Ernst E, Yding Andersen C. LH-receptor gene expression in human granulosa and cumulus cells from antral and preovulatory follicles. *J Clin Endocrinol Metab* 2012;**97**:E1524–E1531.

Joris H, Nagy Z, Van de Velde H, De Vos A, Van Steirteghem A. Intracytoplasmic sperm injection: laboratory set-up and injection procedure. *Hum Reprod* 1998;**13**(Suppl. 1):76–86.

Kawashima I, Okazaki T, Noma N, Nishibori M, Yamashita Y, Shimada M. Sequential exposure of porcine cumulus cells to FSH and/or LH is critical for appropriate expression of steroidogenic and ovulation-related genes that impact oocyte maturation in vivo and in vitro. *Reproduction* 2008;**136**:9–21.

Lee YS, Latham KE, Vandevoort CA. Effects of in vitro maturation on gene expression in rhesus monkey oocytes. *Physiol Genomics* 2008;**35**:145–158.

Lee YS, VandeVoort CA, Gaughan JP, Midic U, Obradovic Z, Latham KE. Extensive effects of in vitro oocyte maturation on rhesus monkey cumulus cell transcriptome. *Am J Physiol Endocrinol Metab* 2011;**301**:E196–E209.

Lorenzo PL, Illera MJ, Illera JC, Illera M. Enhancement of cumulus expansion and nuclear maturation during bovine oocyte maturation in vitro by the addition of epidermal growth factor and insulin-like growth factor I. *J Reprod Fertil* 1994;**101**:697–701.

Lorenzo PL, Liu IK, Illera JC, Picazo RA, Carneiro GF, Illera MJ, Conley AJ, Enders AC, Illera M. Influence of epidermal growth factor on mammalian oocyte maturation via tyrosine-kinase pathway. *J Physiol Biochem* 2001;**57**:15–22.

Macklon NS, Fauser BC. Follicle-stimulating hormone and advanced follicle development in the human. *Arch Med Res* 2001;**32**:595–600.

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.

Mittaz L, Russell DL, Wilson T, Brasted M, Tkalcevic J, Salamonsen LA, Hertzog PJ, Pritchard MA. Adamts-1 is essential for the development and function of the urogenital system. *Biol Reprod* 2004;**70**:1096–1105.

Ochsner SA, Day AJ, Rugg MS, Breyer RM, Gomer RH, Richards JS. Disrupted function of tumor necrosis factor-alpha-stimulated gene 6 blocks cumulus cell-oocyte complex expansion. *Endocrinology* 2003;**144**:4376–4384.

Park JY, Su YQ, Ariga M, Law E, Jin SL, Conti M. EGF-like growth factors as mediators of LH action in the ovulatory follicle. *Science* 2004;**303**:682–684.

Peng XR, Hsueh AJ, LaPolt PS, Bjersing L, Ny T. Localization of luteinizing hormone receptor messenger ribonucleic acid expression in ovarian cell types during follicle development and ovulation. *Endocrinology* 1991;**129**:3200–3207.

Prochazka R, Kalab P, Nagyova E. Epidermal growth factor-receptor tyrosine kinase activity regulates expansion of porcine oocyte-cumulus cell complexes in vitro. *Biol Reprod* 2003;**68**:797–803.

Richards RG, Gadsby JE, Almond GW. Differential effects of LH and PGE2 on progesterone secretion by small and large porcine luteal cells. *J Reprod Fertil* 1994;**102**:27–34.

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.

Robker RL, Russell DL, Espey LL, Lydon JP, O'Malley BW, Richards JS. Progesterone-regulated genes in the ovulation process: ADAMTS-1 and cathepsin L proteases. *Proc Natl Acad Sci USA* 2000;**97**:4689–4694.

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

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* 2003;**278**:42330–42339.

Sanchez F, Adriaenssens T, Romero S, Smitz J. Different follicle-stimulating hormone exposure regimens during antral follicle growth alter gene expression in the cumulus-oocyte complex in mice. *Biol Reprod* 2010;**83**:514–524.

Shimada M, Nishibori M, Yamashita Y, Ito J, Mori T, Richards JS. Down-regulated expression of A disintegrin and metalloproteinase with thrombospondin-like repeats-1 by progesterone receptor antagonist is associated with impaired expansion of porcine cumulus-oocyte complexes. *Endocrinology* 2004;**145**:4603–4614.

Shozu M, Minami N, Yokoyama H, Inoue M, Kurihara H, Matsushima K, Kuno K. ADAMTS-1 is involved in normal follicular development, ovulatory process and organization of the medullary vascular network in the ovary. *J Mol Endocrinol* 2005;**35**:343–355.

Smitz J, Dolmans MM, Donnez J, Fortune JE, Hovatta O, Jewgenow K, Picton HM, Plancha C, Shea LD, Stouffer RL et al. Current achievements and future research directions in ovarian tissue culture, in vitro follicle development and transplantation: implications for fertility preservation. *Hum Reprod Update* 2010;**16**:395–414.

Smitz JE, Thompson JG, Gilchrist RB. The promise of in vitro maturation in assisted reproduction and fertility preservation. *Semin Reprod Med* 2011;**29**:24–37.

Son WY, Tan SL. Laboratory and embryological aspects of hCG-primed in vitro maturation cycles for patients with polycystic ovaries. *Hum Reprod Update* 2010;**16**:675–689.

Wathlet S, Adriaenssens T, Segers I, Verheyen G, Van de Velde H, Coucke W, Ron El R, Devroey P, Smitz J. Cumulus cell gene expression predicts better cleavage-stage embryo or blastocyst development and pregnancy for ICSI patients. *Hum Reprod* 2011;**26**:1035–1051.

Yang SH, Son WY, Yoon SH, Ko Y, Lim JH. Correlation between in vitro maturation and expression of LH receptor in cumulus cells of the oocytes collected from PCOS patients in HCG-primed IVM cycles. *Hum Reprod* 2005;**20**:2097–2103.

Yung Y, Maman E, Konopnicki S, Cohen B, Brengauz M, Lojkin I, Dal Canto M, Fadini R, Dor J, Hourvitz A. ADAMTS-1: a new human ovulatory gene and a cumulus marker for fertilization capacity. *Mol Cell Endocrinol* 2010;**328**:104–108.

Zamah AM, Hsieh M, Chen J, Vigne JL, Rosen MP, Cedars MI, Conti M. Human oocyte maturation is dependent on LH-stimulated accumulation of the epidermal growth factor-like growth factor, amphiregulin. *Hum Reprod* 2010;**25**:2569–2578.

Zeleznik AJ, Saxena D, Little-Ihrig L. Protein kinase B is obligatory for follicle-stimulating hormone-induced granulosa cell differentiation. *Endocrinology* 2003;**144**:3985–3994.

Zhao Z, Garbett D, Hill JL, Gross DJ. Epidermal growth factor receptor downregulation in cultured bovine cumulus cells: reconstitution of calcium signaling and stimulated membrane permeabilization. *Reproduction* 2005;**130**:517–528.