Differences in the transcriptional profiles of human cumulus cells isolated from MI and MII oocytes of patients with polycystic ovary syndrome

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Abstract

Polycystic ovary syndrome (PCOS) is a common endocrine and metabolic disorder in women. The abnormalities of endocrine and intra-ovarian paracrine interactions may change the microenvironment for oocyte development during the folliculogenesis process and reduce the developmental competence of oocytes in PCOS patients who are suffering from anovulatory infertility and pregnancy loss. In this microenvironment, the cross talk between an oocyte and the surrounding cumulus cells (CCs) is critical for achieving oocyte competence. The aim of our study was to investigate the gene expression profiles of CCs obtained from PCOS patients undergoing IVF cycles in terms of oocyte maturation by using human Genome U133 Plus 2.0 microarrays. A total of 59 genes were differentially expressed in two CC groups. Most of these genes were identified to be involved in one or more of the following pathways: receptor interactions, calcium signaling, metabolism and biosynthesis, focal adhesion, melanogenesis, leukocyte transendothelial migration, Wnt signaling, and type 2 diabetes mellitus. According to the different expression levels in the microarrays and their putative functions, six differentially expressed genes (*LHCGR*, *ANGPTL1*, *TNFIK*, *GRIN2A*, *SFRP4*, and *SOCS3*) were selected and analyzed by quantitative RT-PCR (qRT-PCR). The qRT-PCR results were consistent with the microarray data. Moreover, the molecular signatures (*LHCGR*, *TNFIK*, and *SOCS3*) were associated with developmental potential from embryo to blastocyst stage and were proposed as biomarkers of embryo viability in PCOS patients. Our results may be clinically important as they offer a new potential strategy for competent oocyte/embryo selection in PCOS patients.

Reproduction (2013) **145** 597–608

Introduction

Polycystic ovary syndrome (PCOS), which is characterized by increased circulating androgen levels, anovulatory infertility, and, frequently, insulin resistance and hyperinsulinemia, is a common endocrine and metabolic disorder (Franks 1995, Legro 2001, Ehrmann 2005). Although anovulation can be overcome via the use of pharmacological agents or lifestyle intervention, a number of women with PCOS are at an increased risk of pregnancy loss (Sagle *et al.* 1988, Carmina & Lobo 1999), which is possibly accounted for by prolonged folliculogenesis or a suboptimal intrauterine environment due to endocrinopathy or induction of ovulation itself (Glueck *et al.* 2002, Arredondo & Noble 2006). Previous microarray analyses have demonstrated that normal and PCOS oocytes exhibit different gene expression profiles, and annotation of the differentially expressed genes (DEGs) indicates that the reduced developmental competence of PCOS oocytes is associated with the defects in meiosis (Wood *et al.* 2007).

Oocyte maturation, which is promoted by the resumption of meiosis, can be divided into nuclear and cytoplasmic maturation. According to the different stages of meiosis, oocytes are at three different phases of nuclear maturation, which are as follows: germinal vesicle (GV), metaphase I (MI), and MII (Cha & Chian 1998, Martell *et al.* 2009). At the end of the nuclear maturation process, the oocyte that is arrested at MII following the extrusion of the first polar body (PB) is considered mature and able to be fertilized by the sperm. However, in PCOS patients, the main problems that hinder IVF-aided pregnancy are the abnormality of the folliculogenesis process and the incompetence of the oocytes with regard to embryonic development and implantation.

The cross talk between an oocyte and the surrounding cumulus cells (CCs) is critical for achieving oocyte competence, early embryonic development and CC expansion (Salustri *et al.* 1989, Cha & Chian 1998, Goud *et al.* 1998). Previous researches have proved that different gene expressions of CCs could indicate oocyte competence or predict the efficiency of embryo

development and pregnancy outcome (McKenzie *et al.* 2004, Zhang *et al.* 2005, Feuerstein *et al.* 2007, Assou *et al.* 2008, Hamel *et al.* 2008, van Montfoort *et al.* 2008, Kenigsberg *et al.* 2009, Adriaenssens *et al.* 2010, Assou *et al.* 2010). Nevertheless, among all these previous studies on the gene expression profiles of human CCs, only one study (Ouandaogo *et al.* 2011) has highlighted the distinct gene expression of human CCs isolated from oocytes at the GV, MI, and MII stages, while others have mainly focused on CCs surrounding the oocytes at MII stage. Furthermore, in studies on PCOS, differential gene expression patterns in CCs were analyzed by comparing CCs isolated from the oocytes of PCOS patients with those obtained from the oocytes of normal patients.

The aim of this study was to establish the gene expression profiles of human CCs isolated from oocytes at different maturation stages (MI and MII) in PCOS patients who were under controlled ovarian stimulation (COS) cycles by using a cDNA microarray technology. The results would help us to identify candidate genes involved in oocyte nuclear maturation in PCOS. By comparing our results with those of Ouandaogo's research on the gene expression profiles of human CCs at different nuclear maturation stages of non-PCOS patients, the understanding of different molecular mechanisms governing the process of oocyte nuclear maturation between PCOS and non-PCOS patients might be improved.

Results

Identification of the sets of genes differentially expressed in PCOS patient-derived CCs at different stages (MII or MI) of oocyte nuclear maturation

For the gene expression analysis, three CC_{MI} groups and three CC_{MI} groups (derived from nine PCOS patients) were analyzed using six microarrays. The raw microarray data have been deposited in NCBI's Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) and can be accessed through the GEO series accession number GSE40400. Of the 47 000 probe sets on the arrays, 24 360 had a present call. Most of these probe sets showed a similar expression between the CC_{MI} and CC_{MI} groups, except for 70 probe sets that were differentially expressed ($P < 0.05$). Annotations of the molecular functions of the DEGs by Gene Ontology are listed in Supplementary Table 2, see section on supplementary data given at the end of this article. For five of these probe sets, the corresponding gene is not yet known. Of the 65 remaining probe sets, which correspond to 59 different genes, 48 (74%) were upregulated and 17 (26%) were downregulated in the CC_{MI} groups compared with the CC_{MI} groups. Clustering analysis of the arrays based on the 59 DEGs perfectly clustered the CC_{MI} and CC_{MI} groups (Fig. 1).

Figure 1 Cluster of genes overexpressed in cumulus cells (CCs) obtained from PCOS patients. The supervised hierarchical clustering of genes overexpressed in CCs obtained from PCOS patients according to the oocyte nuclear maturation stages (MII vs MI) is shown. Distinct signatures were observed in the CC_{MI} and CC_{MI} groups. The value of each gene was adjusted by a median-centering algorithm in log scale, and the colors indicate the relative gene expression in the red–green heat map. 0 indicated by pure black represents no change from the median gene expression level in all samples. –3 indicated by pure green represents relatively lower expression. +3 indicated by pure red represents relatively higher expression. CC_{MI} and CC_{MI} CCs were isolated from oocytes at MII and MI stages respectively.

Of the 59 genes that were differentially expressed between the CC_{MII} and CC_{MII} groups ($P < 0.05$), 39 were categorized based on their involvement in one or more of the biological processes. Of these processes, inflammatory response, amino acid biosynthesis, synaptic transmission, signal transduction, epithelial-to-mesenchymal transition, anti-apoptosis, regulation of heart contraction, G-protein signaling, and regulation of protein amino acid phosphorylation were significantly overrepresented. In Table 1, the significant DEGs ($n=59$) are shown categorized based on their most prominent role.

The thresholds of P value and false discovered rate (FDR) derived from the hypergeometric test were set < 0.05 to obtain the significantly represented pathway ($P < 0.05$) as indicated in Table 2. P value and FDR could reflect the significance of the correlation between the enriched DEGs and the respective pathways. As shown in Fig. 2, the pathway having the maximum enriched genes is 'neuroactive ligand–receptor interaction ($n=6$)', and in descending order follow the 'calcium signaling pathway ($n=4$)', cytokine–cytokine receptor interaction ($n=4$), focal adhesion ($n=3$), glycine, serine and threonine metabolism ($n=2$), prostate cancer ($n=2$), melanogenesis ($n=2$), leukocyte transendothelial migration ($n=2$), systemic lupus erythematosus ($n=2$), Wnt signaling pathway ($n=2$), vitamin B6 metabolism ($n=1$), cysteine, methionine, nitrogen, selenoamino acid metabolism ($n=1$), urea cycle and metabolism of amino groups ($n=1$), thyroid cancer ($n=1$), O-glycan biosynthesis ($n=1$), and type 2 diabetes mellitus ($n=1$).

Validation of the microarray results by quantitative RT-PCR

According to the relevant functional annotations and their high fold-change (FC) values, two genes involved in the neuroactive ligand–receptor interaction pathway (*LHCGR* and *GRIN2A*), two genes involved in the Wnt signaling pathway (*SFRP4* and *TNIF*), one gene involved in the type 2 diabetes mellitus pathway (suppressors of cytokine signaling (*SOCS*)), and one gene mediating angiogenesis (*ANGPTL1*) were chosen for quantitative RT-PCR (qRT-PCR) validation. For all the six genes, the qRT-PCR results were in accordance with the microarray data obtained using the original samples for the microarray analysis (Fig. 3).

In addition, the expression levels of the six selected genes were also tested in a cohort of CC samples isolated from the additional nine PCOS patients by qRT-PCR. On comparing the CC_{MII} groups with the CC_{MII} groups, it was observed that the mean transcript levels of three genes were significantly higher, with 4.55-, 3.76-, and 2.61-fold increases being observed for *LHCGR* (4.6 ± 0.13 vs 1.0 ± 0.03), *ANGPTL1* (3.6 ± 0.26 vs 0.95 ± 0.04), and *TNIF* (2.5 ± 0.67 vs 0.95 ± 0.16), respectively, while

the expression levels of the other three genes were significantly decreased in the CC_{MII} samples, with 2.06-, 3.69-, and 2.52-fold decreases being observed for *GRIN2A* (0.49 ± 0.05 vs 1.1 ± 0.02), *SFRP4* (0.29 ± 0.03 vs 1.1 ± 0.06), and *SOCS3* (0.62 ± 0.17 vs 1.56 ± 0.48) respectively (Fig. 4). The results of qRT-PCR for different samples further suggested the reasonable certainty of the fold change observed during the microarray analysis.

Differences in the transcript levels of target genes analyzed according to oocyte development outcome

The transcript levels of the target genes were evaluated as the follow-up of the oocytes with 2PN visualization after IVF (Fig. 5). CCs from MII oocytes were divided into two groups: CC_{B+} and CC_{B-}. The mean expression levels of *LHCGR* and *TNIF* were significantly higher in the CC_{B+} groups than in the CC_{B-} groups (*LHCGR* (2.19 ± 0.12 vs 1.02 ± 0.03) and *TNIF* (1.87 ± 0.08 vs 0.89 ± 0.04)). The expression level of *SOCS3* was significantly lower in the CC_{B+} groups than in the CC_{B-} groups (*SOCS3* (0.65 ± 0.08 vs 1.42 ± 0.03)). The levels of the other three genes (*ANGPTL1*, *SFRP4*, and *GRIN2A*) were not significantly different between the CC_{B+} and CC_{B-} groups.

Discussion

PCOS is the most common endocrine abnormality in women of reproductive age. Although sufficient oocytes are usually retrieved from PCOS patients who are under COS, high-quality mature oocytes are limited in number. Oocyte competence depends on the quality of the follicular microenvironment. The cross talk between CCs and oocytes plays a pivotal role in oocyte maturation and metabolism. At present, oocyte competence is evaluated on the basis of morphological features including the PB, zona pellucida, meiotic spindle, and cytoplasm. But a lot of evidence has proved that the morphological evaluation is not a reliable predictor of oocyte competence and embryo viability. Until now, the studies of gene expression profiles of CCs have reported a noninvasive method to predict oocyte and embryo competence (Assou *et al.* 2010). Together with morphological evaluation, CC genes may serve as biomarkers of oocyte and embryo selection during the IVF process or might result in an oocyte selection tool for those who are obliged to select a limited number of oocytes for fertilization in countries where embryo cryopreservation is legally restricted (Ludwig *et al.* 2000). Herein, for the first time, we have reported a significant alteration of gene expression in CCs isolated from mature oocytes and immature oocytes of PCOS patients under COS to identify molecular signatures in CCs that are associated with oocyte maturation or embryo development. This study introduces a new perspective that helps understanding oocyte nuclear maturation in PCOS

Table 1 Genes differentially expressed ($P < 0.05$) in the CC_{MII} groups vs the CC_{MI} groups categorized based on biological processes.

Gene ID	Gene description	Probe set	Fold ^a
Inflammatory response			
<i>CXCL3</i>	Chemokine (C-X-C motif) ligand 3	207850_at	0.3893
<i>CXCL1</i>	Chemokine (C-X-C motif) ligand 1	204470_at	0.3057
<i>CXCL2</i>	Chemokine (C-X-C motif) ligand 2	209774_x_at	0.2847
<i>ANXA1</i>	Annexin A1	201012_at	2.1445
<i>HRH1</i>	Histamine receptor H1	205579_at	2.0289
Amino acid biosynthesis			
<i>ALDH18A1</i>	Aldehyde dehydrogenase 18 family, member A1	217791_s_at	0.499
<i>CTH</i>	Cystathionase (cystathionine γ -lyase)	217127_at	2.1085
<i>PSAT1</i>	Phosphoserine aminotransferase 1	223062_s_at	3.9287
Synaptic transmission			
<i>CTNNA1</i>	Catenin (cadherin-associated protein), β 1	1554411_at	2.3465
<i>DLGAP1</i>	Discs, large (<i>Drosophila</i>) homolog-associated protein 1 /// hypothetical protein LOC284214	235527_at	3.188
<i>PDE7B</i>	Phosphodiesterase 7B	230109_at	2.2716
Signal transduction			
<i>NPY2R</i>	Neuropeptide Y receptor Y2	210729_at	2.1905
<i>ANGPTL1</i>	Angiopoietin-like 1	231773_at	2.1411
<i>LHCGR</i>	LH/choriogonadotropin receptor	207240_s_at	3.4612
<i>GABRA5</i>	γ -Aminobutyric acid (GABA) A receptor, α 5	206456_at	0.3149
<i>SFRP4</i>	Secreted frizzled-related protein 4	204052_s_at	0.2612
Epithelial-to-mesenchymal transition			
<i>HGF</i>	Hepatocyte growth factor (hepapoietin A; scatter factor)	210997_at	2.0589
Anti-apoptosis			
<i>SOCS3</i>	Suppressor of cytokine signaling 3	227697_at	0.3546
Regulation of heart contraction			
<i>CELF2 (CUGBP2)</i>	CUG triplet repeat, RNA binding protein 2	202158_s_at	2.0959
<i>HOPX</i>	HOP homeobox	211597_s_at	0.4748
G-protein signaling, coupled to IP3 second messenger (phospholipase C activating)			
<i>EDNRB</i>	Endothelin receptor type B	206701_x_at	0.4955
Regulation of protein amino acid phosphorylation			
<i>RAPGEF4</i>	Rap guanine nucleotide exchange factor (GEF) 4	205651_x_at	2.2647
Cell-matrix adhesion			
<i>ITGB5</i>	Integrin, β 5	201125_s_at	0.4905
Directional locomotion			
<i>GRIN2A</i>	Glutamate receptor, ionotropic, <i>N</i> -methyl <i>D</i> -aspartate 2A	242286_at	0.4388
Protein amino acid phosphorylation			
<i>STYK1</i>	Serine, threonine, and tyrosine kinase 1	220030_at	2.0848
<i>TNIK</i>	Traf2- and Nck-interacting kinase	213109_at	2.0698
<i>ROR1</i>	Receptor tyrosine kinase-like orphan receptor 1	232060_at	2.0609
Protein transport			
<i>RAB8B</i>	RAB8B, member RAS oncogene family	222846_at	2.0987
<i>TMED2</i>	Transmembrane emp24 domain trafficking protein 2	204426_at	2.3534
<i>RAB2A</i>	RAB2A, member RAS oncogene family	208733_at	2.2728
10-Formyltetrahydrofolate catabolism			
<i>ALDH1L2</i>	Aldehyde dehydrogenase 1 family, member L2	231202_at	2.1561
Cell cycle			
<i>11-Sep</i>	Septin 11	201308_s_at	2.2763
<i>STAG2</i>	Stromal antigen 2	209023_s_at	2.3415
Development			
<i>ENC1</i>	Ectodermal-neural cortex (with BTB-like domain)	201340_s_at	0.3252
<i>ADAMTS9</i>	ADAM metalloproteinase with thrombospondin type 1 motif, 9	1554697_at	2.3627
<i>EMP1</i>	Epithelial membrane protein 1	201324_at	2.1585
Proteolysis			
<i>C1S</i>	Complement component 1, s subcomponent	1555229_a_at	2.351
Actin cytoskeleton organization and biogenesis			
<i>TMSB15A (TMSL8)</i>	Thymosin β 15a	205347_s_at	0.4478
Regulation of small GTPase-mediated signal transduction			
<i>RASGEF1A</i>	RasGEF domain family, member 1A	230563_at	2.1574
Other or unknown functions			
<i>ITM2A</i>	Integral membrane protein 2A	202746_at	4.2735
<i>RUNX2</i>	Runt-related transcription factor 2	236859_at	2.6116
<i>SLC7A11</i>	Solute carrier family 7 (cationic amino acid transporter, y+ system) member 11	217678_at	2.5892
<i>LRRN3</i>	Leucine-rich repeat neuronal 3	209841_s_at	2.4714
<i>PTER</i>	Phosphotriesterase related	218967_s_at	2.4129
<i>SPDY1</i>	Speedy homolog E1 (<i>Xenopus laevis</i>)	232964_at	2.351
<i>CREB5</i>	cAMP-responsive element binding protein 5	229228_at	2.2827

Table 1 Continued.

Gene ID	Gene description	Probe set	Fold ^a
<i>TTMA</i>	Two transmembrane domain family member A	229523_at	2.2462
<i>TUBE1</i>	Tubulin, ϵ 1	226181_at	2.165
<i>OSBPL10</i>	Oxysterol binding protein-like 10	219073_s_at	2.1568
<i>C5ORF24</i>	Chromosome 5 open reading frame 24	224875_at	2.1151
<i>PBRM1</i>	Polybromo 1	223400_s_at	2.0873
<i>GCNT1</i>	Glucosaminyl (<i>N</i> -acetyl) transferase 1	239761_at	2.0839
<i>LOC401074</i>	Hypothetical LOC401074	1559827_at	2.0796
<i>GRAMD1C</i>	GRAM domain containing 1C	219313_at	2.0499
<i>MICU3 (EFHA2)</i>	EF-hand domain family, member A2	238458_at	2.0193
<i>CENPF</i>	Centromere protein F	207828_s_at	0.4872
<i>LOC129293</i>	Hypothetical protein LOC129293	227867_at	0.4871
<i>ANK2</i>	Ankyrin 2, neuronal	202920_at	0.4863
<i>SYNPO2</i>	Synaptopodin 2	227662_at	0.4124

^aCC_{MII}:CC_{MI} expression ratio.

patients and has an important clinical significance for increasing the oocyte competence of PCOS patients undergoing IVF.

It is worth mentioning that we have focused on a subtle biological question involving CCs that are fairly homogeneous and with almost no contamination from other cells. All the patients involved in our study suffered from PCOS and used the same GNRH agonist protocol in the IVF process. In contrast to other publications that have reported a large number of DEGs originating from the comparison of PCOS and non-PCOS patients (Wood *et al.* 2007, Kenigsberg *et al.* 2009), we report that there are only few genes ($n=59$) that are differentially expressed in CCs isolated from the mature or immature oocytes of PCOS patients. To the best of our knowledge, this is the first time that the gene expression profiles of CCs in PCOS have been studied according to oocyte nuclear maturation stages.

All the PCOS patients were administered exogenous hormones (GNRH agonist and recombinant FSH) to synchronize follicular development and ovarian hyperstimulation, respectively, and human chorionic gonadotrophin (hCG) was used to induce ovulation. The previous results from studies conducted on rats show that hormones used for estrus synchronization and ovarian hyperstimulation have minimal effects on gene expression and that the induction of ovulation causes major changes in the gene expression of cumulus–oocyte complexes (COCs; Agca *et al.* 2013). Compared with the different gene expression profiles of COCs in rats induced by hCG, only one gene, runt-related transcription factor (*Runx2*), was upregulated in the CC_{MII} groups of PCOS patients in our study. Moreover, all the PCOS patients involved in our study used the same ovarian stimulation and oocyte retrieval protocols. So, we thought that the 59 genes that were differentially expressed between the CC_{MII} and CC_{MI} groups of PCOS patients in our study were mainly involved in oocyte maturation and not induced by hCG.

Among the DEGs identified in our study, there were several previously described genes, including an

antagonist of the Wnt signaling pathway (*SFRP4*), a neurotransmitter receptor (*GABRA5*), a gene involved in cytoskeleton formation (*ANK2*) (Devjak *et al.* 2012), a growth factor (*HGF*; Haouzi *et al.* 2012), and a hormone receptor (*LHCGR*) (Kenigsberg *et al.* 2009). In addition, by comparing the list of DEGs ($n=59$) between the CC_{MII} and CC_{MI} groups of PCOS patients in our study with that of DEGs ($n=25$) of non-PCOS patients reported by Ouandaogo (Ouandaogo *et al.* 2011), we found that all genes were not exactly the same, while only two genes (*SLC7A11* in our study and *SLC38A2* in Ouandaogo's study) belonged to the same gene family. This may indicate that there are different molecular mechanisms governing the process of oocyte nuclear maturation in PCOS and non-PCOS patients.

The analysis of gene annotations in this study implied that the different expressed genes mainly play an important role in signal transduction and are involved in the Wnt signaling pathway, neuroactive ligand–receptor interaction, cytoskeleton and extracellular matrix formation, and angiogenesis process. These pathways or related genes have been previously proved to have a key role in folliculogenesis and oocyte maturation (Devjak *et al.* 2012).

Wnt signaling is involved in development, cell proliferation, cell migration, and angiogenesis (Richards *et al.* 2002). Three genes (*CTNNB1*, *SFRP4*, and *TNFK*) associated with the Wnt signaling pathway were identified in our study. It has recently been reported that the WNT/ β -catenin signaling pathway plays a complicated role in ovulation, and as a Wnt antagonist, *SFRP4* is an important marker of ovulation and luteinization (Fan *et al.* 2010). Moreover, *SFRP4* is expressed in human GCs and its expression, which is inhibited by LH/HCG, declines during late antral follicular growth (Maman *et al.* 2011). Traf2- and Nck-interacting kinase (*TNFK*) is another gene that is a novel activator of Wnt signaling. It is an activating kinase for T-cell factor-4 (TCF4) and is essential for β -catenin–TCF4 transactivation (Satow *et al.* 2010). Because the aberrant activation of Wnt signaling is also

Table 2 Genes differentially expressed ($P < 0.05$) in the CC_{MII} groups vs the CC_{MI} groups per significantly overrepresented ($P < 0.05$) pathway.

Gene ID	Gene description	Probe set	Fold ^a
Neuroactive ligand–receptor interaction ($n=6$)*			
<i>NPY2R</i>	Neuropeptide Y receptor Y2	210729_at	2.1905
<i>GRIN2A</i>	Glutamate receptor, ionotropic, <i>N</i> -methyl D-aspartate 2A	242286_at	0.4388
<i>LHCGR</i>	LH/choriogonadotropin receptor	207240_s_at	3.4612
<i>GABRA5</i>	γ -Aminobutyric acid (GABA)	206456_at	0.3149
<i>HRH1</i>	Histamine receptor H1	205579_at	2.0289
<i>EDNRB</i>	Endothelin receptor type B	206701_x_at	0.4955
Calcium signaling pathway ($n=4$)*			
<i>GRIN2A</i>	Glutamate receptor, ionotropic, <i>N</i> -methyl D-aspartate 2A	242286_at	0.4388
<i>LHCGR</i>	LH/choriogonadotropin receptor	207240_s_at	3.4612
<i>HRH1</i>	Histamine receptor H1	205579_at	2.0289
<i>EDNRB</i>	Endothelin receptor type B	206701_x_at	0.4955
Cytokine–cytokine receptor interaction ($n=4$)*			
<i>CXCL3</i>	Chemokine (C-X-C motif) ligand 3	207850_at	0.3893
<i>CXCL1</i>	Chemokine (C-X-C motif) ligand 1	204470_at	0.3057
<i>HGF</i>	Hepatocyte growth factor (hepapoietin A; scatter factor)	210997_at	2.0589
<i>CXCL2</i>	Chemokine (C-X-C motif) ligand 2	209774_x_at	0.2847
Focal adhesion ($n=3$)*			
<i>CTNNB1</i>	Catenin (cadherin-associated protein), β 1	1554411_at	2.3465
<i>ITGB5</i>	Integrin, β 5	201125_s_at	0.4905
<i>HGF</i>	Hepatocyte growth factor (hepapoietin A; scatter factor)	210997_at	2.5519
Glycine, serine, and threonine metabolism ($n=2$)*			
<i>CTH</i>	Cystathionase (cystathionine γ -lyase)	206085_s_at	2.0034
<i>CTH</i>	Cystathionase (cystathionine γ -lyase)	217127_at	2.1085
<i>PSAT1</i>	Phosphoserine aminotransferase 1	223062_s_at	3.9287
Prostate cancer ($n=2$)*			
<i>CTNNB1</i>	Catenin (cadherin-associated protein), β 1	1554411_at	2.3465
<i>CREB5</i>	cAMP-responsive element binding protein 5	229228_at	2.2827
Vitamin B6 metabolism ($n=1$)*			
<i>PSAT1</i>	Phosphoserine aminotransferase 1	223062_s_at	3.9287
Melanogenesis ($n=2$)*			
<i>CTNNB1</i>	Catenin (cadherin-associated protein), β 1	1554411_at	2.3465
<i>EDNRB</i>	Endothelin receptor type B	206701_x_at	0.4955
Leukocyte transendothelial migration ($n=2$)*			
<i>CTNNB1</i>	Catenin (cadherin-associated protein), β 1	1554411_at	2.3465
<i>RAPGEF4</i>	Rap guanine nucleotide exchange factor (GEF) 4	205651_x_at	2.2647
Systemic lupus erythematosus ($n=2$)*			
<i>GRIN2A</i>	Glutamate receptor, ionotropic, <i>N</i> -methyl D-aspartate 2A	242286_at	0.4388
<i>C1S</i>	Complement component 1, s subcomponent	1555229_a_at	2.3510
Wnt signaling pathway ($n=2$) [†]			
<i>CTNNB1</i>	Catenin (cadherin-associated protein), β 1	1554411_at	2.3465
<i>SFRP4</i>	Secreted frizzled-related protein 4	204052_s_at	0.2612
Cysteine, methionine, nitrogen, and selenoamino acid metabolism ($n=1$) [†]			
<i>CTH</i>	Cystathionase (cystathionine γ -lyase)	206085_s_at	2.0034
<i>CTH</i>	Cystathionase (cystathionine γ -lyase)	217127_at	2.1085
Urea cycle and metabolism of amino groups ($n=1$) [†]			
<i>ALDH18A1</i>	Aldehyde dehydrogenase 18 family, member A1	217791_s_at	0.4990
Thyroid cancer ($n=1$) [†]			
<i>CTNNB1</i>	Catenin (cadherin-associated protein), β 1	1554411_at	2.3465
O-glycan biosynthesis ($n=1$) [†]			
<i>GCNT1</i>	Glucosaminyl (<i>N</i> -acetyl) transferase 1, core 2	239761_at	2.0839
Type 2 diabetes mellitus ($n=1$) [†]			
<i>SOCS3</i>	Suppressor of cytokine signaling 3	227697_at	0.3546

* $P < 0.01$; [†] $P < 0.05$.^aCC_{MII}:CC_{MI} expression ratio.

associated with several types of cancers, most reports on *TNIK* concern its role in cancer growth (Shitashige *et al.* 2010). In our study, the upregulated expression of *TNIK* (2.61-fold) and the downregulated expression of *SFRP4* (3.69-fold) in the CC_{MII} groups compared with the CC_{MI} groups may further verify that the Wnt signaling pathway is involved in oocyte maturation in PCOS patients. Moreover, as the expression level of *TNIK* is higher (2.1-fold) in the CC_{B+} groups than in the

CC_{B-} groups, we propose that the activation of *TNIK* on Wnt signaling is important for embryo development and *TNIK* might serve as a biomarker of embryo viability in the CCs of PCOS patients.

Besides the signal transduction genes in Wnt signaling, there are several genes that are involved in neuroactive ligand–receptor interaction (*GABRA5*, *GRIN2A*, and *LHCGR*) or mediate angiogenesis (*ANGPTL1*). It has been supposed that neurotransmitters

Figure 2 Number of genes enriched in the significantly represented pathway ($P < 0.05$). The thresholds of P value and FDR derived from the hypergeometric test were set at < 0.05 to find the significantly represented pathway. The significantly represented pathway is shown on the y-axis and the number of genes is shown on the x-axis.

play an important role in folliculogenesis and oocyte maturation and the gene (*GABRA5*) responsive to γ -aminobutyric acid, GABA, might serve as a biomarker of oocyte maturation in CCs (Devjak *et al.* 2012). In our study, the expression levels of two genes (*GABRA5* and *GRIN2A*) that encoded receptors for two neurotransmitters, GABA and glutamate, respectively, were down-regulated in the CC_{MII} groups compared with the CC_{MI} groups. So far, little is known about the function of *GRIN2A* in folliculogenesis and oocyte maturation. In view of the fact that ovarian steroids increased *GRIN2A* expression in serotonin neurons of macaques (Betha & Reddy 2012), we guessed that the downregulated expression of *GRIN2A* in the CC_{MII} groups perhaps was related to steroid levels in the microenvironment for oocyte development. In agreement with Devjak's opinion, we also supposed that *GABRA* and *GRIN2A* might be used as biomarkers to predict oocyte maturation in CCs.

In addition, another gene that is involved in the neuroactive ligand–receptor interaction pathway is *LHCGR*, which encodes a G-protein-coupled receptor for LH and HCG. In the ovary, the induction of *LHCGR* during granulosa cell differentiation allows the pre-ovulatory follicle to respond to the mid-cycle surge of LH, leading to the ovulation and release of the mature oocytes. In women, inactivating mutations of *LHCGR* are associated with increased LH levels, enlarged ovaries, oligomenorrhea, resistance to LH or hCG, and infertility (Toledo *et al.* 1996, Latronico *et al.* 1998). In our study, the expression level of *LHCGR* in the CC_{MII} samples was 4.55-fold higher than that in the CC_{MI} samples. This indicates that *LHCGR* expression in CCs may contribute to oocyte maturation in PCOS patients. It should be kept in mind that the regulation of *LHCGR* expression in CCs has previously been reported to depend on oocyte-secreted factors and also on the FSH dose (Kawashima *et al.* 2008, Romero *et al.* 2011). Moreover, a genome-wide association study aimed at identifying susceptibility loci for PCOS indicated that *LHCGR* may influence the expression of FSH receptor

(*FSHR*) even though the genes are located in different LD blocks (Chen *et al.* 2011). *FSHR* has been identified as a biomarker in bovine CCs that helps to predict oocyte competence and select higher embryo quality (Assidi *et al.* 2008). We found that the mRNA level of *LHCGR* was significantly higher (2.15-fold) in the CC_{B+} groups than in the CC_{B-} groups. Maybe *LHCGR* could also serve as an early marker of embryo viability in PCOS patients.

Another gene involved in signal transduction is *ANGPTL1*, which is a member of the angiopoietin-related protein family and known to mediate angiogenesis. Previous studies have suggested that *ANGPTL1* and *ANGPTL2* interact with unidentified receptors on endothelial cells to modulate angiogenesis in a context-dependent manner (Dhanabal *et al.* 2002). Both *ANGPTL1* and *ANGPTL2* exert anti-apoptotic effects via the phosphatidylinositol 3-kinase/Akt pathway, and their cooperative activity is likely required for vascular development during zebrafish embryogenesis (Kubota

Figure 3 Differentially expressed genes (*LHCGR*, *ANGPTL1*, *TNK1*, *GRIN2A*, *SFRP4*, and *SOCS3*) studied through microarray experiments and validated by qRT-PCR. The qRT-PCR results were in line with the microarray data set. The gray bars show the relative gene expression measured using qRT-PCR. The white bars show the relative gene expression measured using the Affymetrix microarrays. The same cumulus cells from PCOS COCs at MII and MI stages were used for qRT-PCR and microarray analysis. The y-axis represents the fold change CC_{MII}/CC_{MI} and the selected genes are shown on the x-axis.

Figure 4 mRNA expression of candidate genes (*LHCGR*, *ANGPTL1*, *TNF*, *GRIN2A*, *SFRP4*, and *SOCS3*) in human CCs, organized according to the oocyte nuclear maturation stage (MI vs MII stages). The signal intensity for each gene is shown on the y-axis in arbitrary units determined by qRT-PCR with *GAPDH* as an endogenous reference. Asterisk (*) indicates a significant difference in gene expression between CC categories (** $P < 0.01$ and * $P < 0.05$). The results are presented as the means $\pm$ S.E.M. CC_{MI}, cumulus cells from oocytes at MI stage; CC_{MII}, CCs from oocytes at MII stage.

et al. 2005a, 2005b). Recently, it has been suggested that the change in *ANGPT1* and *ANGPT2* levels may be associated with follicular growth and angiogenesis during the preovulatory period (Nishigaki *et al.* 2011). We observed a higher expression level of *ANGPTL1* in the CC_{MII} groups in our study and considered that the results further testified the relationship between angiogenesis and folliculogenesis.

It has been proved that the extracellular matrix of CCs plays a major role in folliculogenesis and is crucial for ovulation, oviduct passage, and fertilization (Irving-Rodgers & Rodgers 2005). We found that *ADAMTS9*, a gene involved in extracellular matrix binding, was upregulated in the CC_{MII} groups compared with the CC_{MI} groups. Moreover, it has been reported that *Adamts9* is widely expressed during mouse embryo development (Jungers *et al.* 2005). *ANK2*, another gene involved in cytoskeleton formation, was downregulated in the CC_{MII} samples. It has been suggested that *ANK2* contains SOWAHC (ANKRD57) protein, which has already been recognized to be influenced by oocyte maturation in CCs (Ouandaogo *et al.* 2011). Changes in the expression levels of *ADAMTS9* and *ANK2* during oocyte maturation further confirmed that cytoskeleton and extracellular

matrix formation was important for folliculogenesis and oocyte maturation in PCOS.

One interesting gene that we identified was *SOCS3*, a member of the SOCS family, which is involved in the type 2 diabetes mellitus pathway. SOCS family members are regulatory proteins that are rapidly induced in response to intracellular JAK–STAT signaling, a cascade controlling biological functions including cytokine-induced immunological responses and reproductive processes. It has been reported that *SOCS3* plays a vital role in reproduction by regulating trophoblast differentiation (Fitzgerald *et al.* 2009). In PCOS, *SOCS3* affects adipogenesis and insulin resistance (Chazenbalk *et al.* 2012). The results of our study showed decreased *SOCS3* expression (2.52-fold) in the CC_{MII} groups compared with the CC_{MI} groups and a downregulated (2.18-fold) expression level in the CC_{B+} groups compared with the CC_{B-} groups. We propose that the expression of *SOCS3* is not only associated with the increased prevalence of obesity in PCOS patients but also related to oocyte or embryo competence and might serve as a biomarker of oocyte maturation or embryo viability in CCs.

In conclusion, the comparison of the gene expression profiles of CCs isolated from oocytes at different nuclear

Figure 5 mRNA expression of candidate genes (*LHCGR*, *ANGPTL1*, *TNIK*, *GRIN2A*, *SFRP4*, and *SOCS3*) in human CCs, organized according to oocyte development capability after normal fertilization. The signal intensity for each gene is shown on the y-axis in arbitrary units determined by qRT-PCR with *GAPDH* as an endogenous reference. *Significant difference in gene expression between CC categories (** $P < 0.01$ and * $P < 0.05$). The results are presented as the means $\pm$ S.E.M. CC_{B+}, cumulus cells from oocytes yielding blastocysts after 5–6 days of *in vitro* culture; CC_{B-}, CCs from oocytes that had not developed into blastocysts on days 5–6.

maturation stages (MII and MI) of PCOS patients suggested that the gene expression of CCs was altered according to the oocyte maturation process. Specifically, major signaling pathways (particularly the Wnt signaling pathway, neuroactive ligand–receptor interaction, and cytoskeleton and extracellular matrix formation) play a key role in the oocyte nuclear maturation of PCOS patients. Moreover, several genes (*LHCGR*, *TNIK*, and *SOCS3*) were suggested to serve as biomarkers of oocyte or embryo competence in CCs of PCOS patients.

Materials and Methods

Processing of CCs

PCOS patients referred to our center for IVF were included in this study after obtaining written informed consent. This study was approved by the Institutional Ethical Review Board of Yuhuangding Hospital of Yantai. All the PCOS patients were diagnosed by the presence of two or more of the following features: chronic oligo-ovulation or anovulation, androgen excess, and polycystic ovaries. We excluded patients with Cushing's syndrome, congenital adrenal hyperplasia, and androgen-secreting tumors. The inclusion criteria of the

recruited PCOS patients in this study were as follows: age < 36 years, BMI ranging between 20 and 26 kg/m², number of obtained oocytes ranging between 12 and 28 per cycle, number of obtained blastocysts ranging between 4 and 12 per cycle, basal serum LH/FSH more than 2.0, serum androgen more than 0.5 ng/ml, and normal spermogram of the partner according to the WHO criteria.

Ovarian stimulation and oocyte retrieval protocols were carried out as described previously (Wood *et al.* 2007, Kenigsberg *et al.* 2009). All the selected PCOS patients were administered GnRH agonist triptorelin acetate (Diphereline, Ipsen Pharma Biotech, Paris, France) from the mid-luteal phase at a daily dose of 0.05 mg subcutaneously. Once adequate pituitary downregulation was confirmed (serum LH levels < 3.0 ng/ml and serum estradiol (E₂) levels < 30 pg/ml), the patients were s.c. administered 150–187.5 IU recombinant FSH (Gonal-f, Follitropin Alfa, Serono) for COS. When two or more follicles were at least 18 mm in diameter and the serum E₂ levels were at least 300 pg/ml per dominant follicle, all the patients were administered 250 μ g hCG (Profasi, Serono). COCs were recovered under ultrasound guidance 36 h after hCG administration. After COC retrieval, a proportion of the CCs surrounding a single oocyte were removed using a sharp needle, lysed in 80 μ l RLT buffer (RNeasy Mini Kit, Qiagen),

snap-frozen in liquid nitrogen, and stored at -80°C (CCs from one oocyte per vial). A total of 162 CC samples obtained from 30 patients were used in this study.

Oocytes were further inseminated and cultured individually in 20 μl droplets covered by mineral oil. Oocytes were denuded to assess the maturation stage 3 h after insemination. Immature MI oocytes had no polar bodies or GV, while mature MII oocytes extruded a clearly visible PB. Fertilization status was evaluated 16–18 h after insemination by observing the appearance of two pronuclei. Only fertilized oocytes (2PN) were further cultured to the blastocyst stage for 5 or 6 days, and unfertilized oocytes were discarded. On day 5/6, one or two embryos at the blastocyst or morula stage were transplanted and the remaining blastocysts were cryopreserved. CCs obtained from MI and MII oocytes were considered for a transcriptome analysis.

Experimental design

As the total RNA extracted from a single CC is too limited, CCs separated from COCs at the same stage were pooled together for this study. Because very few GV-stage COCs were retrieved from the PCOS patients under controlled ovarian hyperstimulation, we focused on COCs at MII and MI stages. For microarray analysis, 54 individual CC samples obtained from nine patients were isolated from COCs at MI and MII stages and divided into six groups (CC_{MI}-1, CC_{MI}-2, CC_{MI}-3, CC_{MI}-1, CC_{MI}-2, and CC_{MI}-3). That is, each group had nine CC samples isolated from oocytes at MI or MII stage. The original samples were also analyzed using qRT-PCR to validate the microarray results.

To further confirm the differences in the expression of candidate genes in CCs isolated from oocytes at different nuclear maturation stages, 54 CC samples obtained from an additional nine PCOS patients were used. All the CCs were classified into two groups (CC_{MI} and CC_{MI}) and tested using qRT-PCR.

Furthermore, to evaluate whether the specific MII CC molecular signatures observed correlate with embryo viability, 54 CC samples from the other 12 PCOS patients were also tested using qRT-PCR. The 54 CC samples derived from the normally fertilized (2PN) oocytes were classified into the following two groups as described previously (Gardner & Schoolcraft 1999, Guerif *et al.* 2007): the 'blastocyst' group (CC_{B+}), defined as CCs from COCs that yielded top-quality embryos on day 2 and developed into blastocysts on day 5/6, and the 'unable to develop into blastocyst' group (CC_{B-}), defined as CCs from COCs that formed weak- or low-quality embryos on day 2 and failed to develop into blastocysts on day 5/6.

Each group (CC_{MI}, CC_{MI}, CC_{B+}, or CC_{B-}) had three biological replicates.

Complementary RNA preparation and microarray hybridization

RNA extraction and microarray hybridization were performed at CapitalBio Corporation (Beijing, China). Total RNA isolation was performed using the Qiagen RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. This RNA isolation kit significantly reduces contamination from both genomic DNA and proteins. Only the extracted RNA that had a

rRNA 28S:18S intensity ratio of 1.0–1.5:1 was used in the microarray and qRT-PCR assays.

Aliquots (2 μg) of total RNA from three CC_{MI} (CC_{MI}-1, CC_{MI}-2, and CC_{MI}-3) and three CC_{MI} (CC_{MI}-1, CC_{MI}-2, and CC_{MI}-3) groups were used to synthesize a double-stranded cDNA, which was subsequently transcribed into a biotin-tagged cDNA using the MessageAmp II aRNA Amplification Kit (Ambion, Austin, TX, USA). The cDNA was then fragmented to produce strands that were 35–200 bases in length in accordance with the published protocols (Affymetrix, Santa Clara, CA, USA). The fragmented cDNA was hybridized to the Affymetrix GeneChip Human Genome U133 Plus 2.0 Array, which contains 47 000 transcripts. Microarray hybridization was performed at 45°C with rotation for 16 h using an Affymetrix GeneChip Hybridization Oven 640. The arrays were washed and stained (streptavidin–phycoerythrin) at an Affymetrix GeneChip Fluidics Station 450 and later scanned on an Affymetrix GeneChip Scanner 3000 to analyze the hybridization data. Overall, six microarray chips were analyzed in this study.

Data processing and microarray data analysis

The scanned images that were obtained were first assessed by visual inspection and then analyzed using the Affymetrix GeneChip Operating Software (GCOS 1.4). The detection algorithm uses probe pair intensities to generate a detection *P* value and assign a present, a marginal or an absent call, which represents whether the measured transcript is detected (present) or not detected (absent). *P* value was derived from one-sided Wilcoxon's signed rank test with the default value 0.015 defined by the Affymetrix Corporation. Any *P* value that falls below 0.04 is assigned a present call and above 0.06 is assigned an absent call. Marginal calls are given to probe sets that have *P* values between 0.04 and 0.06. The lesser the *P* value correlates, the more likely the given transcript is truly present in the sample. The raw data were filtered to mask genes with signal intensities < 100, which is at the background threshold, and to retain only genes that were called present by the GCOS in at least two replicates. To normalize the different arrays, dChip Software was used in a global scaling procedure.

In a comparison analysis, a two-class, unpaired method from the Significance Analysis of Microarrays (SAM version 3.02, Stanford University, Stanford, CA, USA) software was used to compare significantly DEGs in the CC_{MI} and CC_{MI} groups. The algorithm used to sort the statistically significant DEGs was a modified *t*-test, and the criteria for DEGs were FDR < 0.05 and fold change > 2.0 or < 0.5. FDR was a corrected *P* value by *post hoc* test. The SAM-M results were used to perform a supervised hierarchical clustering based on the expression level of the probe sets (multi-class gene set), and the cluster was visualized using the Tree View Software (Stanford University, Stanford, CA, USA) (Eisen *et al.* 1998). All the DEGs were analyzed using a free web-based Molecular Annotation System 3.0 (MAS 3.0, www.capitalbio.com), which integrates three different open-source pathway resources: KEGG, BioCarta, and GenMAPP. The significantly represented pathway was chosen by the threshold of *P* value and FDR (corrected *P* value) < 0.05 derived from the hypergenomic test.

Quantitative RT-PCR

qRT-PCR was conducted on candidate genes that were found to be differentially expressed in the microarrays and whose functions were found, upon a biological function analysis, to be closely related to oocyte maturation and/or embryo development. The first-strand complementary synthesis reaction was performed using a PrimeScript RT reagent kit (Perfect Real Time; TaKaRa Biotechnology (Dalian) Co. Ltd., Dalian, China). Amplification reactions were conducted using SYBR Premix Ex Taq (Perfect Real Time; TaKaRa Biotechnology (Dalian) Co. Ltd.) and an ABI PRISM 7300 system. Gene-specific qRT-PCR primers that were used are listed in [Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article. *GAPDH* served as an internal control to normalize the loading of the template cDNA. Each set of qRT-PCR reactions was repeated three times, and the fold change in the expression of each gene of interest was analyzed using the $\Delta\Delta C_t$ method (Livak & Schmittgen 2001). Student's *t*-test of independent data was used for statistical analysis. The differences among groups were considered significant when the *P* value was <0.05.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-13-0005>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This study was supported by the National Basic Research Program (grant 81170622), Science and Technology Development Project of Shangdong Population and Family Planning Commission (grant 2011-15), and Science and Technology Development Project of Yantai (grant 2010-148-26).

Acknowledgements

We gratefully acknowledge the CapitalBio Corporation for conducting the RNA extractions and microarray analysis.

References

- Adriaenssens T, Wathlet S, Segers I, Verheyen G, De Vos A, Van der Elst J, Coucke W, Devroey P & Smits J 2010 Cumulus cell gene expression is associated with oocyte developmental quality and influenced by patient and treatment characteristics. *Human Reproduction* **25** 1259–1270. (doi:10.1093/humrep/deq049)
- Agca C, Yakan A & Agca Y 2013 Estrus synchronization and ovarian hyperstimulation treatments have negligible effects on cumulus–oocyte complex gene expression whereas induction of ovulation causes major expression changes. *Molecular Reproduction and Development* **80** 102–117. (doi:10.1002/mrd.22141)
- Arredondo F & Noble LS 2006 Endocrinology of recurrent pregnancy loss. *Seminars in Reproductive Medicine* **24** 33–39. (doi:10.1055/s-2006-931799)
- Assidi M, Dufort I, Ali A, Hamel M, Algriany O, Dielemann S & Sirard MA 2008 Identification of potential markers of oocyte competence expressed in bovine cumulus cells matured with follicle-stimulating hormone and/or phorbol myristate acetate *in vitro*. *Biology of Reproduction* **79** 209–222. (doi:10.1095/biolreprod.108.067686)
- Assou S, Haouzi D, Mahmoud K, Aouacheria A, Guillemin Y, Pantescio V, Reme T, Dechaud H, De Vos J & Hamamah S 2008 A non-invasive test for assessing embryo potential by gene expression profiles of human cumulus cells: a proof of concept study. *Molecular Human Reproduction* **14** 711–719. (doi:10.1093/molehr/gan067)
- Assou S, Haouzi D, De Vos J & Hamamah S 2010 Human cumulus cells as biomarkers for embryo and pregnancy outcomes. *Molecular Human Reproduction* **16** 531–538. (doi:10.1093/molehr/gaq032)
- Bethea CL & Reddy AP 2012 Ovarian steroids increase glutamatergic related gene expression in serotonin neurons of macaques. *Molecular and Cellular Neurosciences* **49** 251–262. (doi:10.1016/j.mcn.2011.11.005)
- Carmina E & Lobo RA 1999 Polycystic ovary syndrome (PCOS): arguably the most common endocrinopathy is associated with significant morbidity in women. *Journal of Clinical Endocrinology and Metabolism* **84** 1897–1899. (doi:10.1210/jc.84.6.1897)
- Cha KY & Chian RC 1998 Maturation *in vitro* of immature human oocytes for clinical use. *Human Reproduction Update* **4** 103–120. (doi:10.1093/humupd/4.2.103)
- Chazenbalk G, Chen YH, Heneidi S, Lee JM, Pall M, Chen YD & Azziz R 2012 Abnormal expression of genes involved in inflammation, lipid metabolism, and Wnt signaling in the adipose tissue of polycystic ovary syndrome. *Journal of Clinical Endocrinology and Metabolism* **97** E765–E770. (doi:10.1210/jc.2011-2377)
- Chen ZJ, Zhao H, He L, Shi Y, Qin Y, Li Z, You L, Zhao J, Liu J, Liang X *et al.* 2011 Genome-wide association study identifies susceptibility loci for polycystic ovary syndrome on chromosome 2p16.3, 2p21 and 9q33.3. *Nature Genetics* **43** 55–59. (doi:10.1038/ng.732)
- Devjak R, Fon Tacer K, Juvan P, Virant Klun I, Rozman D & Vrtacnik Bokal E 2012 Cumulus cells gene expression profiling in terms of oocyte maturity in controlled ovarian hyperstimulation using GnRH agonist or GnRH antagonist. *PLoS ONE* **7** e47106. (doi:10.1371/journal.pone.0047106)
- Dhanabal M, LaRochelle WJ, Jeffers M, Herrmann J, Rastelli L, McDonald WF, Chillakuru RA, Yang M, Boldog FL, Padigar M *et al.* 2002 Angioarrestin: an antiangiogenic protein with tumor-inhibiting properties. *Cancer Research* **62** 3834–3841.
- Ehrmann DA 2005 Polycystic ovary syndrome. *New England Journal of Medicine* **352** 1223–1236. (doi:10.1056/NEJMra041536)
- Eisen MB, Spellman PT, Brown PO & Botstein D 1998 Cluster analysis and display of genome-wide expression patterns. *PNAS* **95** 14863–14868. (doi:10.1073/pnas.95.25.14863)
- Fan HY, O'Connor A, Shitanaka M, Shimada M, Liu Z & Richards JS 2010 β -Catenin (CTNNB1) promotes preovulatory follicular development but represses LH-mediated ovulation and luteinization. *Molecular Endocrinology* **24** 1529–1542. (doi:10.1210/me.2010-0141)
- Feuerstein P, Cadoret V, Dalbès-Tran R, Guerif F, Bidault R & Royere D 2007 Gene expression in human cumulus cells: one approach to oocyte competence. *Human Reproduction* **22** 3069–3077. (doi:10.1093/humrep/dem336)
- Fitzgerald JS, Toth B, Jeschke U, Schleussner E & Markert UR 2009 Knocking off the suppressors of cytokine signaling (SOCS): their roles in mammalian pregnancy. *Journal of Reproductive Immunology* **83** 117–123. (doi:10.1016/j.jri.2009.07.010)
- Franks S 1995 Polycystic ovary syndrome. *New England Journal of Medicine* **333** 853–861. (doi:10.1056/NEJM199509283331307)
- Gardner DK & Schoolcraft WB 1999 Culture and transfer of human blastocysts. *Current Opinion in Obstetrics & Gynecology* **11** 307–311. (doi:10.1097/00001703-199906000-00013)
- Glueck CJ, Wang P, Goldenberg N & Sieve-Smith L 2002 Pregnancy outcomes among women with polycystic ovary syndrome treated with metformin. *Human Reproduction* **17** 2858–2864. (doi:10.1093/humrep/17.11.2858)
- Goud PT, Goud AP, Qian C, Laverge H, Van der Elst J, De Sutter P & Dhont M 1998 *In-vitro* maturation of human germinal vesicle stage

- oocytes: role of cumulus cells and epidermal growth factor in the culture medium. *Human Reproduction* **13** 1638–1644. (doi:10.1093/humrep/13.6.1638)
- Guerif F, Le Gouge A, Giraudeau B, Poindron J, Bidault R, Gasnier O & Royere D 2007 Limited value of morphological assessment at days 1 and 2 to predict blastocyst development potential: a prospective study based on 4042 embryos. *Human Reproduction* **22** 1973–1981. (doi:10.1093/humrep/dem100)
- Hamel M, Dufort I, Robert C, Gravel C, Leveille MC, Leader A & Sirard MA 2008 Identification of differentially expressed markers in human follicular cells associated with competent oocytes. *Human Reproduction* **23** 1118–1127. (doi:10.1093/humrep/den048)
- Haouzi D, Assou S, Monzo C, Vincens C, Dechaud H & Hamamah S 2012 Altered gene expression profile in cumulus cells of mature MII oocytes from patients with polycystic ovary syndrome. *Human Reproduction* **27** 3523–3530. (doi:10.1093/humrep/des325)
- Irving-Rodgers HF & Rodgers RJ 2005 Extracellular matrix in ovarian follicular development and disease. *Cell and Tissue Research* **322** 89–98. (doi:10.1007/s00441-005-0042-y)
- Jungers KA, Le Goff C, Somerville RP & Apte SS 2005 Adamts9 is widely expressed during mouse embryo development. *Gene Expression Patterns* **5** 609–617. (doi:10.1016/j.modgep.2005.03.004)
- Kawashima I, Okazaki T, Noma N, Nishibori M, Yamashita Y & Shimada M 2008 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* **136** 9–21. (doi:10.1530/REP-08-0074)
- Kenigsberg S, Bentov Y, Chalifa-Caspi V, Potashnik G, Ofir R & Birk OS 2009 Gene expression microarray profiles of cumulus cells in lean and overweight-obese polycystic ovary syndrome patients. *Molecular Human Reproduction* **15** 89–103. (doi:10.1093/molehr/gan082)
- Kubota Y, Oike Y, Satoh S, Tabata Y, Niikura Y, Morisada T, Akao M, Urano T, Ito Y, Miyamoto T *et al.* 2005a Cooperative interaction of Angiopoietin-like proteins 1 and 2 in zebrafish vascular development. *PNAS* **102** 13502–13507. (doi:10.1073/pnas.0501902102)
- Kubota Y, Oike Y, Satoh S, Tabata Y, Niikura Y, Morisada T, Akao M, Urano T, Ito Y & Miyamoto T 2005b Isolation and expression patterns of genes for three angiopoietin-like proteins, Angptl1, 2 and 6 in zebrafish. *Gene Expression Patterns* **5** 679–685. (doi:10.1016/j.modgep.2005.02.006)
- Latronico AC, Chai Y, Arnhold IJ, Liu X, Mendonca BB & Segaloff DL 1998 A homozygous microdeletion in helix 7 of the luteinizing hormone receptor associated with familial testicular and ovarian resistance is due to both decreased cell surface expression and impaired effector activation by the cell surface receptor. *Molecular Endocrinology* **12** 442–450. (doi:10.1210/me.12.3.442)
- Legro RS 2001 Polycystic ovary syndrome: the new millennium. *Molecular and Cellular Endocrinology* **184** 87–93. (doi:10.1016/S0303-7207(01)00640-2)
- Livak KJ & Schmittgen TD 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2^{−(ΔΔC_T)} method. *Methods* **25** 402–408. (doi:10.1006/meth.2001.1262)
- Ludwig M, Schopper B, Katalinic A, Sturm R, Al-Hasani S & Diedrich K 2000 Experience with the elective transfer of two embryos under the conditions of the German embryo protection law: results of a retrospective data analysis of 2573 transfer cycles. *Human Reproduction* **15** 319–324. (doi:10.1093/humrep/15.2.319)
- Maman E, Yung Y, Cohen B, Konopnicki S, Dal Canto M, Fadini R, Kanety H, Kedem A, Dor J & Hourvitz A 2011 Expression and regulation of sFRP family members in human granulosa cells. *Molecular Human Reproduction* **17** 399–404. (doi:10.1093/molehr/gar010)
- Marteil G, Richard-Parpaillon I & Kubiak JZ 2009 Role of oocyte quality in meiotic maturation and embryonic development. *Reproductive Biology* **9** 203–224. (doi:10.1016/S1642-431X(12)60027-8)
- McKenzie LJ, Pangas SA, Carson SA, Kovanci E, Cisneros P, Buster JE, Amato P & Matzuk MM 2004 Human cumulus granulosa cell gene expression: a predictor of fertilization and embryo selection in women undergoing IVF. *Human Reproduction* **19** 2869–2874. (doi:10.1093/humrep/deh535)
- van Montfoort AP, Geraedts JP, Dumoulin JC, Stassen AP, Evers JL & Ayoubi TA 2008 Differential gene expression in cumulus cells as a prognostic indicator of embryo viability: a microarray analysis. *Molecular Human Reproduction* **14** 157–168. (doi:10.1093/molehr/gam088)
- Nishigaki A, Okada H, Tsuzuki T, Cho H, Yasuda K & Kanzaki H 2011 Angiopoietin 1 and angiopoietin 2 in follicular fluid of women undergoing a long protocol. *Fertility and Sterility* **96** 1378–1383. (doi:10.1016/j.fertnstert.2011.09.031)
- Ouandaogo ZG, Haouzi D, Assou S, Dechaud H, Kadoch IJ, De Vos J & Hamamah S 2011 Human cumulus cells molecular signature in relation to oocyte nuclear maturity stage. *PLoS ONE* **6** e27179. (doi:10.1371/journal.pone.0027179)
- Richards JS, Russell DL, Ochsner S, Hsieh M, Doyle KH, Falender AE, Lo YK & Sharma SC 2002 Novel signaling pathways that control ovarian follicular development, ovulation, and luteinization. *Recent Progress in Hormone Research* **57** 195–220. (doi:10.1210/rp.57.1.195)
- Romero S, Sanchez F, Adriaenssens T & Smits J 2011 Mouse cumulus–oocyte complexes from *in vitro*-cultured preantral follicles suggest an anti-luteinizing role for the EGF cascade in the cumulus cells. *Biology of Reproduction* **84** 1164–1170. (doi:10.1095/biolreprod.110.087551)
- Sagle M, Bishop K, Ridley N, Alexander FM, Michel M, Bonney RC, Beard RW & Franks S 1988 Recurrent early miscarriage and polycystic ovaries. *BMJ* **297** 1027–1028. (doi:10.1136/bmj.297.6655.1027)
- Salustri A, Yanagishita M & Hascall VC 1989 Synthesis and accumulation of hyaluronic acid and proteoglycans in the mouse cumulus cell–oocyte complex during follicle-stimulating hormone-induced mucification. *Journal of Biological Chemistry* **264** 13840–13847.
- Satow R, Shitashige M, Jigami T, Honda K, Ono M, Hirohashi S & Yamada T 2010 Traf2- and Nck-interacting kinase is essential for canonical Wnt signaling in *Xenopus* axis formation. *Journal of Biological Chemistry* **285** 26289–26294. (doi:10.1074/jbc.M109.090597)
- Shitashige M, Satow R, Jigami T, Aoki K, Honda K, Shibata T, Ono M, Hirohashi S & Yamada T 2010 Traf2- and Nck-interacting kinase is essential for Wnt signaling and colorectal cancer growth. *Cancer Research* **70** 5024–5033. (doi:10.1158/0008-5472.CAN-10-0306)
- Toledo SP, Brunner HG, Kraaij R, Post M, Dahia PL, Hayashida CY & Kremer HTAP 1996 An inactivating mutation of the luteinizing hormone receptor causes amenorrhea in a 46,XX female. *Journal of Clinical Endocrinology and Metabolism* **81** 3850–3854. (doi:10.1210/jc.81.11.3850)
- Wood JR, Dumesic DA, Abbott DH & Strauss JF III 2007 Molecular abnormalities in oocytes from women with polycystic ovary syndrome revealed by microarray analysis. *Journal of Clinical Endocrinology and Metabolism* **92** 705–713. (doi:10.1210/jc.2006-2123)
- Zhang X, Jafari N, Barnes RB, Confino E, Milad M & Kazer RR 2005 Studies of gene expression in human cumulus cells indicate pentraxin 3 as a possible marker for oocyte quality. *Fertility and Sterility* **83** (Suppl 1) 1169–1179. (doi:10.1016/j.fertnstert.2004.11.030)

Received 8 January 2013

First decision 11 March 2013

Accepted 16 April 2013