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# The transcriptome of follicular cells: biological insights and clinical implications for the treatment of infertility

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**BACKGROUND:** Oocyte maturation is under strict regulatory control, not only from intrinsic cellular processes, but also extrinsic influences. While the oocyte is directly connected to the surrounding cumulus cells (CCs) via a network of gap junctions facilitating communication and exchange of molecules, it is also influenced by the greater follicular environment. In order to produce an oocyte capable of successfully transmitting the female genetic material and able to support the earliest stages of preimplantation development, cytoplasmic and nuclear maturation must be achieved. Granulosa and CCs play an essential role in the maturation and competence acquisition of the developing oocyte. The fact that these cells are closely associated with the oocyte, share the same microenvironment and can be easily collected during IVF procedures makes them attractive targets for basic research and the development of clinically relevant assays. Analysis of follicular cells is likely to reveal important information concerning the viability and genetic constitution of their associated oocyte, as well as increase our understanding of normal follicular processes and the impact of disorders or of medical interventions such as controlled ovarian stimulation (COS). This review summarizes results obtained during the investigation of granulosa and CCs, and considers the possibilities of using follicular cells as surrogate markers of stimulation response during IVF, oocyte/embryo competence and clinical outcome.

**METHODS:** In order to summarize the current knowledge obtained from the analysis of follicular cells, a thorough literature search was carried out. Relevant research articles published in English up to March 2013 were reviewed.

**RESULTS:** Multiple groups of genes expressed in follicular cells have been identified as possible indicators of ovulation, oocyte maturity, fertilization, chromosome status, ability to generate embryos capable of reaching the blastocyst stage of development, embryo morphology and the establishment of a pregnancy. However, there is a general lack of uniformity concerning groups of gene biomarkers among different studies.

**CONCLUSIONS:** Extensive investigation of genes and proteins of granulosa and CCs has provided a detailed insight into the follicular microenvironment surrounding oocytes. It was evident from the data reviewed that the gene expression of follicular cells influences and is influenced by the

oocyte, affecting factors such as maturity, chromosomal constitution, viability and competence. However, a general lack of overlap among genes identified as potentially useful biomarkers suggests that the transcriptome of follicular cells could be affected by multiple intrinsic factors, having to do with the patient and possibly the aetiology of infertility, as well as extrinsic factors, such as hormonal stimulation. Further work is required in order to establish a universally applicable, non-invasive test for the determination of oocyte competence based upon follicular cell assessment.

**Key words:** granulosa cells / cumulus cells / transcriptome analysis / oocyte competence / non-invasive biomarker

# Introduction

In female mammals, including humans, a reproductive cycle begins with the recruitment of a set of immature follicles and their enclosed oocytes, which start to grow and mature under the control of gonadotrophins (Edson *et al.*, 2009). The majority of these follicles and associated oocytes ultimately undergo atresia. Depending on the type of mammal (monoovulatory or polyovulatory), either a single oocyte or a small number of oocytes will reach maturity and subsequently be ovulated. A competent oocyte is not only capable of successful fertilization and transmission of the female genetic contribution, but also has responsibility for supporting and controlling the first few mitotic divisions, until the activation of the embryonic genome. Sustaining early embryo development depends upon stored mRNA transcripts and proteins, accumulated by the oocyte during its maturation period, prior to ovulation (Vasudevan *et al.*, 2006).

The follicles in which oocytes grow and mature consist of multiple layers of somatic cells, whose main functions are to protect and nurture the oocyte and, via hormonal influence, guide it through the series of events necessary for achieving cytoplasmic and nuclear competence. The follicular cells most closely associated with the oocyte are the mural granulosa cells (GCs) lining the wall of the follicle and the CCs, which are in direct contact with the oocyte and form what is known as the cumulus–oocyte complex (COC). Formation of gap junctions within the COC establishes a network, linking individual CCs to one another and to the oocyte they surround. These intimate connections allow bi-directional communication between the oocyte and the CCs and the exchange of different types of macromolecules, such as proteins and metabolites (reviewed in Feuerstein *et al.*, 2007). In this way CCs are able to fulfil their vital role in resourcing the growing oocyte.

After oocyte retrieval during assisted reproductive technology (ART) procedures, the follicular cells surrounding the oocyte are generally considered to be superfluous and are discarded. In cases involving ICSI, CCs are usually removed before fertilization, while during regular IVF cycles, they become separate from the oocyte, once fertilization is complete. Since these follicular cells have experienced the same environment as the oocyte with which they are associated, they may be able to reveal important information concerning its status. For example, a suboptimal follicular environment that has a negative impact on the viability of the oocyte is also likely to affect the CCs, possibly producing characteristic alterations in gene expression. The fact that GCs and CCs are readily accessible during ART treatments and can be sampled without compromising the oocyte has made them attractive targets for the development of non-invasive assays of oocyte competence. This review summarizes the results obtained during the investigation of GCs and CCs, assesses their biological significance and considers the possibilities of using them as markers of stimulation response during fertility treatments and as indicators of oocyte/embryo competence and clinical outcome.

# Methods

In order to summarize the current knowledge obtained from the analysis of follicular cells, a thorough literature search was carried out. Relevant research articles published in English up to March 2013 were obtained and reviewed. Examples of key words included during this search were 'granulosa or cumulus cell gene expression', 'oocyte competence', 'non-invasive biomarker', 'non-invasive oocyte quality assessment' and 'non-invasive embryo viability assessment'.

# Oocyte and follicle development and maturation

Oogenesis involves two separate meiotic divisions, meiosis I (MI) and II (MII), and two different stages when the oocyte arrests. The process begins in female fetuses between 11 and 12 weeks of gestation (Gondos *et al.*, 1986) when immature oocytes, each with 46 chromosomes, enter prophase of MI. It is during this time that homologous chromosomes pair with each other and, via the formation of chiasmata, exchange genetic material. The oocytes then enter a protracted stage of arrest, known as dictyate, and remain there until meiosis is resumed. This takes place with the onset of puberty in females and the initiation of the menstrual cycle (reviewed in Huang *et al.*, 2012). Under the influence of gonadotrophins [follicle stimulating hormone (FSH) and luteinizing hormone (LH)], MI is resumed, the oocyte progresses to metaphase I, chiasmata are resolved and homologous chromosomes separate, one set ($n = 23$) remains in the oocyte, while the other enters the first polar body (PB) (reviewed in Sutton *et al.*, 2003; Fragouli *et al.*, 2011). The oocyte continues on, entering MII before arresting again at the second metaphase. MII is not completed until the oocyte is fertilized.

The proliferation and differentiation of the somatic follicular cells occur in synchrony with the maturing oocyte. The GCs represent the main cell type within the ovarian follicle and have the ability to alter their morphology and function so as to accommodate and support the developing oocyte. These structural and functional modifications are under both hormonal and oocyte signalling control. During their maturation, oocytes secrete several mitogenic factors which are involved in promoting follicular growth (Hutt and Albertini, 2007; Gilchrist *et al.*, 2008); examples include growth differentiation factor-9 and bone morphogenetic factor-15. Once the dividing GCs receive these factors, they also process and regulate them. The establishment of this regulatory loop between the oocyte and the surrounding follicular cells is necessary for oocyte competence acquisition. Completion of the oocyte growth phase coincides with the formation of the follicular antrum and the differentiation of GC into two anatomically different cell lines, the steroidogenic mural GCs, which line the follicular wall, and the CCs that encircle the oocyte (Chian *et al.*, 2004; Gilchrist *et al.*, 2008).

Oogenesis as well as folliculogenesis is heavily LH and FSH dependent. Both these hormones are generated by the pituitary in a cyclical manner (reviewed in Zuccotti et al., 2011). FSH is mostly involved in the regulation of follicular cell differentiation, proliferation and antrum formation. This takes place via FSH binding to its receptor, located on the cells of the follicle, and activating primarily the cAMP/protein kinase A and p38 signalling pathways, which result in transcriptional up-regulation of the aromatase gene (Richards, 2001). FSH is also responsible for extending the vital gap-junction connections between the CCs and the oocyte (Yeo et al., 2009). LH, on the other hand, is responsible for the triggering of meiotic resumption and the events leading to ovulation. Specifically, the so-called LH surge releases the oocyte from the inhibitory cAMP action exerted by the surrounding follicular cells (Mehlmann, 2005), via a maturation signal, which is transmitted from the extra-follicular environment through the CCs.

Among the various processes essential for oocyte maturation, those taking place just before ovulation within the COC are of crucial significance for the oocyte's future competence and acquisition of fertilization ability (Russell and Robker, 2007). Interestingly, experiments in a mouse model showed that correct spindle positioning, which is essential for accurate meiotic chromosome segregation, is regulated via the bi-directional communication established between the oocyte and the surrounding CCs (Barrett and Albertini, 2010).

## Follicular structure and cellular communication

The cytoplasmic projections that penetrate the zona pellucida bring the plasma membranes of the oocyte and the CCs into contact, facilitating the formation of gap junctions and the transfer of various molecules between the two cell types. The CCs provide pyruvate after they have metabolized it from glucose, which the oocyte utilizes for its own energy requirements (Sutton-McDowall et al., 2010). CCs also supply the oocyte with amino acids and nucleotides essential for the synthesis of macromolecules such as ribosomal and messenger RNAs (reviewed by Johnson, 2007). As well as being necessary for oocyte growth and maturation, the availability of such macromolecules is also vital for the embryo during the first few cellular divisions after fertilization (Eppig, 1991). The building blocks of gap junctions are connexins (Cx). Different types of Cxs are involved in communication between the somatic and oocyte compartments of the follicle. GCs are interconnected via Cx43 and Cx45, while connection to the oocyte via the surrounding CCs involves Cx37 (reviewed in Zuccotti et al., 2011). CCs also use Cx43 to establish the gap junctions used in their communication with the oocyte (Feuerstein et al., 2007). Studies have shown that different types of gap junctions have distinct permeability properties, suggesting that they are responsible for the transfer of specific signals and molecules (Gittens et al., 2005).

One of the main functions of mural GCs is to maintain the oocyte in meiotic arrest until the time of ovulation (Kawamura et al., 2011). This was concluded in a series of experiments during which COCs were removed from pre-ovulatory follicles, leading to a spontaneous resumption of meiosis (Tsafriri and Pomerantz, 1986). It was therefore evident that GCs secrete an oocyte maturation inhibitor (OMI) responsible for maintaining the oocyte in meiotic arrest. This OMI has recently been determined as the C-type natriuretic peptide (CNP) encoded by natriuretic peptide precursor type C (NPPC) (Zhang et al., 2010). In both mice and human ovaries, NPPC/CNP was found to be under strict gonadotrophin regulation and its action as a potent paracrine OMI was confirmed (Kawamura et al., 2011).

The external hormone signals responsible for stimulating the oocyte to resume MI are first received by the mural GCs. It has been shown that the LH surge leads to the production of epidermal growth factor (EGF)-like molecules, such as amphiregulin, epiregulin and betacellulin (Park et al., 2004). The growth factors have key roles in the activation of oocyte maturation, as well as the induction of expression of genes such as HAS2, PTGS2 and TNFAIP6, which are necessary for CC proliferation and expansion (Hsieh et al., 2009). After re-activation of meiosis by the LH surge, a signal is transmitted to the CCs, to produce neuregulin 1. This is thought to be a ligand for the ERBB3 receptor, which activates AREG-induced progesterone production in GCs (Noma et al., 2011, reviewed in Zuccotti et al., 2011). LH also activates the expression of the progesterone receptor (PR) in GCs surrounding oocytes that are destined to ovulate (Robker et al., 2009). PR is a nuclear receptor transcription factor which is actively involved in the regulation of ovulation, and its inactivation leads to a complete ovulation block (Robker et al., 2009). Luteinization of GCs follows the LH surge, and they convert to endocrine and auto/paracrine functions (Grøndahl et al., 2012). Another hormone which has received a lot of attention recently, as a possible indicator of oocyte competence, is AMH (anti-Mullerian hormone). This is solely produced by the GCs (Matzuk et al., 2002), and its amount is thought to decrease as follicular maturation progresses (Grøndahl et al., 2011).

## Transcriptomic analysis of follicular cells: methodological strategies for global and specific gene expression assessment

The intimate connection of the somatic and oocyte compartments of a follicle means that any change in the microenvironment is likely to have a pronounced effect on both. Virtually all cellular processes experience some degree of regulation at the gene expression level. Adjusting the activity of specific genes leads to alterations in the quantities of mRNA transcripts that they produce, ultimately changing the levels of their corresponding proteins and thereby influencing the cellular pathways that they regulate. Given the importance of gene expression, analysis of the transcriptome of a cell should shed light on the processes active at the moment it was collected. Moreover, the gene expression profile could provide an insight into cellular health and viability.

There are various approaches for the investigation of gene activity. The most common strategy involves a combination of a technique capable of simultaneously evaluating large numbers of genes, such as a microarray, followed by validation of the findings using quantitative real-time PCR. While microarrays provide vast amounts of transcriptomic data in a single experiment, highlighting genes of potential importance, real-time PCR gives a much more reliable quantification of mRNA transcript levels and is essential for confirming the validity of microarray findings. Further assessment at the protein and/or functional level is also valuable, proving whether or not a change in the quantity of mRNA level has a

downstream effect. All these methods have been applied for the examination of GCs and CCs.

An example of global gene expression analysis is a study carried out by Kõks *et al*. (2010). During this investigation, a microarray approach was employed to assess the transcriptome of floating GCs (FGCs), obtained from the follicular fluid, and to compare it with that of CCs coming from the same follicle. Samples from both cell types were obtained from stimulated follicles of 19 women (average age, 32 years) undergoing ICSI. As was expected, marked differences were observed between the transcriptome of the two cell populations, with 222 genes being relatively over-expressed in FGCs and 267 being relatively overxpressed in CCs. In comparison with CCs, FGCs displayed elevated activity of genes involved in steroidogenic processes. Genes, such as the cytoplasmic cholesterol transporter SCP2 (sterol-carrier protein 2) and the cholesterol side-chain cleavage enzymes, CYP11A1 (Cytochrome P450, family 11, subfamily A, polypeptide 1) and CYP17A1 (Cytochrome P450, family 17, subfamily A, polypeptide 1), responsible for converting cholesterol to pregnenolone and androstenedione respectively, were more active in FGCs. Intra- and inter-cellular signalling and interaction pathways were seen for both cell types (Kõks *et al.*, 2010). This study provided some interesting findings concerning pathways prominent in FGCs and CCs and the functional diversity of different classes of follicular cells. However, no further confirmatory validation of the identified genes via real-time PCR in a larger sample size took place.

Another example is the comprehensive analysis of the GC and CC transcriptomes described in a more recent investigation carried out by Grøndahl *et al*. (2012). As with the study carried out by Kõks *et al.* (2010), follicular cells were obtained during stimulated cycles (from 21 women undergoing assisted reproductive procedures). The average female age of this patient group was 33 years. Unlike, Kõks *et al.* (2010) however, the microarray results, were further validated with the use of real-time PCR. A total of 18 119 transcripts were present for the vast majority (80%) of the GCs, corresponding to 11 739 unique genes. Among the findings, the steroidogenic function of GCs during ovulation was highlighted by the up-regulation of CYP11A1, while other processes such as cell–cell communication and extracellular matrix modelling, key factors in the organization and regulation of the follicle, were also observed to be active in this cell type. A total of 16 544 transcripts were detected in the CCs examined, representing 11 090 unique genes. Of these, 64 transcripts (61 genes) were exclusively expressed in CCs compared with GCs. Genes such as LHR (LH receptor), involved in detecting and responding to the LH surge, AR (androgen receptor), important for steroid hormone signalling, Cx43, Cx40 and Cx45 (Cxs 43, 40 and 45, respectively), required for the formation of gap junctions, IL6R and IL7R (receptors of interleukins 6 and 7), involved in inflammatory response, and PTX3 (pentraxin-related gene 3), VCAN (vesicant), HAS2 (hyaluronan synthase 2) and fibronectin, that function in extracellular matrix formation and remodelling, were all actively expressed in CCs of pre-ovulatory follicles.

# Gene expression, aneuploidy and advancing female age

A recent investigation of GC gene expression attempted to shed some light on the follicular environment of reproductively older women or women with a reduced ovarian reserve (Pacella *et al.*, 2012). The declining quality of oocytes derived from older women is well known, but the extent to which this is due to intrinsic (oocyte) factors as opposed to extrinsic (follicular) factors remains unclear. Pacella *et al.* (2012) examined the expression of two enzymes involved in glycolysis, namely phosphofructokinase platelet (PFKP) and lactate dehydrogenase A (LDHA), in GCs obtained from follicles of young women with normal ovarian reserve (control group), young women with reduced ovarian reserve (test group 1) and reproductively older women (test group 2). Metabolic analysis as well as measurement of concentrations of carbohydrates, hormones and selected ions in the follicular fluid also took place. The results obtained showed a higher level of expression for both glycolytic enzymes in GCs from the two test groups, coupled with lower production of progesterone (P). Additionally, the authors observed that GCs of women with reduced ovarian reserve or of advanced reproductive age have a decreased capacity to differentiate into CCs. They attributed this to the declining quality of the enclosed oocyte, which may not be able to generate adequate levels of paracrine factors to drive GC differentiation (Pacella *et al.*, 2012).

Another study examining the effect of female age on gene and protein expression in CCs utilized a combination of liquid chromatography–tandem mass spectrometry (LC-MS/MS) and real-time PCR (McReynolds *et al.*, 2012). The examined CCs were obtained from two groups of patients: infertile women of advanced reproductive age (40–45 years, test group) and young fertile donors (20–33 years, control group). LC-MS/MS analysis revealed a total of 110 proteins being differentially expressed between the two groups. In their majority, the proteins and their associated genes were down-regulated in CCs surrounding oocytes from older women. Examples include CYP11A1, a catalyst of the initial and rate-limiting step in steroid hormone synthesis, VCAN, involved in CC extracellular matrix formation, ATP5I (ATP synthase subunit e), NDUFA1 [NADH dehydrogenase (ubiquinone) I alpha subcomplex subunit 5], ATP6V1A and COX10, regulating oxidative phosphorylation and mitochondrial function, and SFPQ, KHSRP, DDX46 and SNRPF, involved in post-transcriptional splicing. The only process to be up-regulated in CCs of older women compared with the control group was amino acid metabolism (ACAT2, HSD17B4). These findings combined with the results of the GC analysis carried out by Pacella *et al.* (2012) show a significant impact of advancing female age on the processes occurring in follicular cells. It is likely that disturbances of key pathways (e.g. steroid hormone synthesis) would lead to alterations in the follicular microenvironment in which the oocytes mature, and could in part explain the decline in their general quality.

One of the most important aspects of oocyte competence and corresponding embryo viability is the chromosome constitution of the female gamete. Chromosome abnormalities are extremely common in human oocytes and are almost always lethal to the developing embryo or fetus. Aneuploidy increases rapidly with advancing female age, affecting more than half of all oocytes from women over 40 years (Fragouli *et al.*, 2011). In order to gain an insight into the follicular environment of aneuploid oocytes, our group compared the gene expression of CCs associated with chromosomally normal and abnormal oocytes (Fragouli *et al.*, 2012). Similar to previous investigations, a combination of gene expression microarray and real-time PCR was employed. The examined CCs were obtained by women of an average age of 32.8 years. Interestingly, CCs enclosing aneuploid oocytes were found to have lower levels of mRNA in general, compared with those surrounding chromosomally normal oocytes, indicative of reduced transcriptional

activity. The microarray data obtained showed that various biological processes were affected in CCs of aneuploid oocytes, including metabolism, signalling pathways, apoptosis and transport.

Further real-time PCR validation confirmed the statistically significant difference in expression ($P < 0.05$) of two genes, SPSB2 [splA/ryanodine receptor domain and suppressor of cytokine signalling (SOCS) box containing 2] and TP53I3 (tumour protein p53 inducible protein 3) between the groups of CCs. Both these were under-expressed in CCs of aneuploid oocytes. These two genes were involved in the regulation of ubiquitination (Kuang et al., 2010) and DNA damage response (Lee et al., 2010), respectively. Reduced levels of expression for both these genes suggest that aneuploid oocytes tend to be surrounded by CCs which are dysfunctional or damaged. It is possible that the atypical expression pattern seen in CCs associated with chromosomally abnormal oocytes is directly responsible for an increase in the risk of aneuploidy, a consequence of inappropriate signalling or insufficient transfer of resources to the oocyte. Alternatively, an abnormal follicular environment might cause a predisposition to aneuploidy in the oocyte, while simultaneously effecting gene expression in the CCs. A third possibility is that an aneuploid oocyte might induce changes in the surrounding CCs. Determining which of these possibilities, if any, is correct will require further investigation.

# Transcriptomic analysis of follicular cells associated with oocytes at various maturational stages

The transcriptome of CCs surrounding oocytes at different stages of maturation [i.e. at the germinal vesicle (GV), meiosis I (MI) and meiosis II (MII)] was assessed during investigations carried out by two different research groups (Ouandaogo et al., 2011; Feuerstein et al., 2012). Both studies examined CCs removed from oocytes generated by younger women (<36 years). Ouandaogo et al. (2011), via the use of a microarray, observed that the gene expression profiles of CCs surrounding oocytes at the three stages of maturity were very similar to each other, with only 25 genes displaying differential expression. Of these, the number of genes over-expressed was 10 in CCs from GV oocytes, 4 in CCs from MI oocytes and 11 in CCs from MII oocytes. The changes in gene activity among the three groups were confirmed for 15 genes in real-time PCR experiments. An interesting finding was that the transcriptomic signature of the surrounding CCs was not necessarily related to the maturity of the corresponding oocyte. Hence, mature MII oocytes can sometimes be enclosed in CCs whose gene expression resembles that of earlier stages (GV or MII) (Ouandaogo et al., 2011). It is conceivable that a lack of synchrony between the oocyte and the CCs could have significant consequences for oocyte competence, although this remains to be conclusively demonstrated.

Using a similar approach to Ouandaogo et al., but a different microarray, Feuerstein et al. (2012) identified a total of 724 genes differentially expressed between CCs surrounding GV and MII oocytes. Of these, 634 were up-regulated and 90 were down-regulated in CCs from MII oocytes, compared with those from GVs. The genes displaying relative over-expression in the CCs of MII oocytes were involved in the regulation of a total of 16 processes, including the mitogen-activated protein kinase pathway, lipid biosynsthesis and apoptosis. Those which were under-expressed regulated a total of 37 functions, including tRNA processing and metabolism and induction of programmed cell death. A total of three of the genes identified during the microarray comparisons were further validated as potential biomarkers of oocyte competence via real-time PCR. These were ANG (angiogenin), PLIN2 (perilipin 2) and RGS2 (regulator of G-protein signalling 2). These three genes were selected as possibly useful biomarkers, as they were differentially expressed in CCs in relation to the oocyte's maturity as well as the oocyte's ability to develop into a blastocyst embryo. Interestingly, the expression of RGS2, a GTPase-activating protein that hydrolyses GTP to GDP on the α-subunit of an activated G-protein (Dohlman and Thorner, 1997), was seen to be correlated not only with oocyte quality, but also with the establishment of a clinical pregnancy (Feuerstein et al., 2012). Feurstein's findings on the usefulness of RGS2 were confirmatory of two other investigations of follicular cell gene expression carried out by Hamel et al. (2008, 2010). In agreement with these authors, Feuerstein suggested that the expression patterns of RGS2 could be indicative of follicular competence.

# Disturbances of cumulus cell gene expression induced by controlled ovarian stimulation

Oocytes acquire competence through a series of events occurring internally as well as events within the surrounding follicular cells (Eppig, 2001; Barrett and Albertini, 2010). It has been speculated that controlled ovarian stimulation (COS), employed during assisted reproductive treatments, could interfere with these events, impacting the quality of the ovulated oocyte. This was assessed in a study which compared the gene expression of CCs surrounding oocytes produced in unstimulated cycles to that seen in CCs from oocytes generated using COS (de los Santos et al., 2012). Oocytes and CCs were obtained from donors with very similar characteristics. An initial microarray analysis demonstrated that a total of 18 genes were differentially expressed between the two groups of CCs. The expression of three of these genes, MYH11 (myosin heavy chain 11), SOX4 (SRY box 4) and PRB2 (proline-rich protein BstNI subfamily 2) was further assessed via real-time PCR. Of these MYH11 and SOX4 were up-regulated in natural cycles, whereas PRB2 was up-regulated in COS cycles. It was apparent that COS had a significant effect on pathways involved in leukocyte differentiation, T cell activation and regulation (SOX4), and angiogenesis (MYH11). The obtained results suggested that COS not only activates numerous immune cell-like functions, which could be involved in innate immune responses during ovulation (Hernandez-Gonzalez et al., 2006; Shimada et al., 2006a, b), but also could affect the oocyte maturation process (de los Santos et al., 2012).

The findings of all of the above-mentioned studies are summarized in Table I. It is evident from the data obtained that appropriate functioning of the transcriptome of follicular cells is essential for oocyte maturation and competence acquisition. It is also clear that factors such as COS and advancing female age can have an adverse effect on the follicular microenvironment with consequences for oocyte and embryo quality.

**Table I** Genes and pathways in follicular cells.

| Study | Cell type | Methods used | Selection measure | Genes | Function | Expression pattern |
|---|---|---|---|---|---|---|
| Kõks et al. (2010) | FGCs | Microarray | Global gene expression analysis | SCP2<br>CYP11A1<br>CYP17A1 | Steroidogenesis | Up-regulated<br>Up-regulated<br>Up-regulated |
| Grøndahl et al. (2012) | Granulosa and CCs | Microarray and real-time PCR | Global gene expression analysis before ovulation | CYP11A1 | Steroidogenesis | Up-regulated in GCs |
|  |  |  |  | GHR | Proliferation and metabolism | Up-regulated in GCs |
|  |  |  |  | GAP43 | Proliferation and metabolism | Up-regulated in CCs |
|  |  |  |  | RYR2 | Cell−cell communication | Up-regulated in CCs |
|  |  |  |  | CACNA1C | Cell−cell communication | Down-regulated in GCs and up-regulated in CCs |
|  |  |  |  | TNC | Extracellular matrix | Down-regulated in GCs and up-regulated in CCs |
|  |  |  |  | ESR1 | Gonadotrophin, steroid hormone, steroidogenesis and nuclear receptors | Up-regulated in GCs and CCs |
| Pacella et al. (2012) | GCs | Real-time PCR | Female age and ovarian reserve | PFKP<br>LDHA | Glycolysis | Up-regulated in relation to advancing female age or reduced ovarian reserve |
| McReynolds et al. (2012) | CCs | LC-MS/MS and real-time PCR | Female age | CYP11A1 | Steroidogenesis | Down-regulated with advancing female age |
|  |  |  |  | VCAN | Extracellular matrix | Down-regulated with advancing female age |
|  |  |  |  | ATP5I, NDUFA1, ATP6V1A and COX10 | Oxidative phosphorylation and mitochondrial function | Down-regulated with advancing female age |
|  |  |  |  | SFPQ, KHSRP, DDX46 and SNRPF | Post-transcriptional slicing | Down-regulated with advancing female age |
|  |  |  |  | ACAT2, HSD17B4 | Amino acid metabolism | Up-regulated with advancing female age |
| Fragouli et al. (2012) | CCs | Microarray and real-time PCR | Oocyte chromosome status | SPSB2 | Ubiquitination | Down-regulated in CCs of aneuploid oocytes |
|  |  |  |  | TP53I3 | DNA damage response | Down-regulated in CCs of aneuploid oocytes |
| de los Santos et al. (2012) | CCs | Microarray and real-time PCR | Natural versus COS cycles | MYH11 | Angiogenesis | Up-regulated in natural cycles |
|  |  |  |  | SOX4 | Leukocyte differentiation and T-cell activation | Up-regulated in natural cycles |
|  |  |  |  | PRB2 | Not specified | Up-regulated in COS cycles |

CC, cumulus cells; COS, controlled ovarian stimulation; FGC, floating granulosa cell; GC, granulosa cell.

# Transcriptomic analysis of follicular cells: clinical implications

The concept of a non-invasive method capable of identifying the most competent oocyte(s) or embryo(s) is highly desirable, potentially allowing the number of embryos transferred to the uterus to be reduced, ideally to just one per transfer, without harming ART success rates. Currently, the only non-invasive tools available for the selection of oocytes and embryos in the IVF laboratory are based on the assessment of various morphological parameters. These include the presence of the first PB as an indication of oocyte nuclear maturity, the number of blastomeres and the presence of cellular fragmentation or multinucleation at the cleavage stage, and the extent of blastocoel expansion and the appearance of the inner cell mass and trophectoderm at the blastocyst stage. Unfortunately, these criteria are subjective and fail to provide a conclusive assessment of developmental potential. Morphological examination is unable to detect genetic abnormalities, such as aneuploidy or deficiencies in critical cellular functions. Currently, the only method yielding a definitive evaluation of oocytes/embryos is aneuploidy diagnosis, which provides a simple black or white assessment of the chromosomal constitution (i.e. normal or abnormal). However, the various methods used for aneuploidy detection require breach of the zona pellucida and biopsy of polar bodies or cells from the oocyte/embryo. These procedures are time consuming, require a highly trained embryologist, and may carry a small risk of damage to the embryo (Cohen et al., 2007). The limitations of morphological analysis and the risks of embryo biopsy have added impetus for research into novel non-invasive methods of viability assessment.

Both GCs and CCs are readily available during ART procedures, and can be removed from the oocyte without compromising it. Theoretically, transcriptomic information obtained from these cells might shed light on the viability of the associated oocyte, thereby providing a non-invasive method of oocyte/embryo selection. It is also possible that the data obtained might indicate whether follicular processes are taking place normally, potentially allowing individualization of COS protocols in order to maximize the production of competent oocytes.

Our understanding of follicle biology is currently incomplete and consequently the possibility of tailored ovarian stimulation procedures, based upon transcriptomic analysis of follicular cells, might seem like a distant prospect. However, a study carried out by Gerasimova et al. (2010) demonstrates that optimization of COS, guided by the results of genetic analysis might not be far off. The authors examined the genetic constitution of the FSH receptor (FSHR) in relation to female infertility and response to COS. The investigated CCs were obtained from oocytes generated by women undergoing ART procedures who were at the extremes of the normal distribution of ovarian response to FSH according to their age. Interestingly, 37% (13 of 35) of these women carried one of four genetic FSHR splicing variants. Specific variants were associated with either low or high response to COS with the use of FSH. The presence of these variants is indicative of a possible genetic basis for certain forms of female infertility. They also provide a potential biological explanation for some cases of ovarian hyperstimultion syndrome and other cases in which COS fails to elicit an adequate response (Gerasimova et al., 2010).

Another study examined the expression of 10 CC genes in relation to 13 different variables, including the type of COS protocol used (Adriaenssens et al., 2010). These genes were selected from findings obtained from preliminary microarray experiments and were HAS2, VCAN, SDC4 (syndecan 4), ALCAM (activated leukocyte cell adhesion molecule), GREM1 (Gremlin 1), PTGS1 and PTGS2 (prostaglandin-endoperoxide synthase 1 and 2), DUSP16 (dual specificity phosphatise 16), SPROUTY4 (Sprouty homologue 4) and RPS6KA2 (ribosomal protein S6 kinase, 90 kDa, polypeptide 2). Real-time PCR was used for the analysis of their expression patterns. CCs were obtained from 63 women being treated for infertility via two different stimulation protocols involving highly purified (HP) hMG or recombinant FSH (rFSH). The type of COS protocol employed influenced the expression of five of these genes, namely VCAN (component of CC extracellular matrix), RPS6KA2 (intermediate in EGF signalling), SDC4 (transforming growth factor beta signalling), GREM1 (transforming growth factor beta signalling) and SPROUTY4 (EGF signalling). Of these, SPROUTY4 was consistently over-expressed in CCs obtained from rFSH, compared with HP-hMG, while SDC4 was under-expressed (Adriaenssens et al., 2010). Interestingly, both these genes are involved in the regulation of different signalling pathways in CCs. It can therefore be speculated that different types of stimulation regimes may have a significant effect on signalling within the COC and possibly on oocyte quality. Other variables which were frequently associated with differential expression of the examined genes included body mass index, serum FSH level and female age, all factors known to influence the likelihood of successful IVF treatment (Adriaenssens et al., 2010).

The influence of polycystic ovarian syndrome (PCOS) on the follicular environment of maturing oocytes was assessed by Haouzi et al. (2012). A microarray-based approach followed by real-time PCR validation was employed to examine and compare the gene expression of CCs surrounding oocytes from six PCOS patients to CCs coming from women undergoing IVF for other indications. Interestingly, the expression patterns between the two groups were significantly different, and multiple genes were affected by PCOS. Examples included members of the growth factor family such as EGFR, EREG and AREG, and others known to regulate steroid metabolism such as CYP11A1, CYP1B1, CYP19A1 and CYP2B7P1. The authors postulated that the reduced oocyte competence seen in PCOS patients could be due to incorrect functioning of the transforming growth factor β and oestrogen receptors signalling cascades (Haouzi et al., 2012).

In recent years, several studies have examined follicular cells, especially CCs, in the hope of identifying biomarkers predictive of oocyte quality, embryo viability and establishment of a clinical pregnancy. One of the first reports describing a molecular CC signature specific to embryo quality and the establishment of a pregnancy was that of Assou et al. (2008). As with many of the gene expression studies previously discussed, a microarray approach was combined with real-time PCR, allowing an initial transcriptome-wide screening followed by robust validation of candidate genes. The microarray analysis revealed that CCs surrounding oocytes that led to live births had the majority of their differentially expressed genes up-regulated in comparison with CCs associated with oocytes that failed to produce a birth. The expression patterns of three CC genes could be used to predict which oocyte and corresponding embryo would result in a clinical pregnancy. These three genes were BCL2L11, regulating apoptosis, PCK1 (phosphoenolpyruvate carboxykinase 1), involved in gluconeogenesis and the transcription factor, NFIB. Embryos capable of implanting and leading to live births came

from oocytes whose surrounding CCs over-expressed BCL2L11 and PCK1 and under-expressed NFIB (Assou *et al.*, 2008). Although the results obtained during this study are promising, they were generated after double embryo transfers taking place on Day 3 of preimplantation development. Ideally, the three identified genes should be further validated in a single embryo transfer programme and in other IVF clinics (Assou *et al.*, 2008).

Gebhardt *et al.* (2011) chose to examine 13 genes in CC samples. These genes were ALDOA, LDHA, PFKP and PKM2, involved in the regulation of metabolism, AHR, GREM1, PTGS2 and STS, with roles in signalling, and HAS2, PTX3, TNFAIP6 and VCAN, involved in extracellular matrix formation, and they were selected as they were functioning in vital cellular processes. All were analysed via real-time PCR only. The aim of the study was to identify possible expression patterns associated with the oocyte's ability to lead to a live birth. Unlike Assou's investigation, this study involved a single embryo transfer, either on Day 3 or later on at the blastocyst stage. Interestingly, all 13 genes examined showed a tendency towards up-regulation in CCs from competent oocytes. It was concluded that four of the examined genes (VCAN, GREM1, PTGS2 and PFKP) had expression patterns indicative of oocytes capable of establishing a pregnancy. It should also be noted that variation in gene activity was not associated with embryo morphology either on Day 3 or Day 5, suggesting that information on viability obtained from evaluation of gene expression is independent of information obtained via morphological scoring (Gebhardt *et al.*, 2011).

Another investigation correlating CC gene expression with outcome analysed 11 genes (TRPM7, ITPKA, VCAN, SDC4, CAMK1D, STC1, STC2, EFNB2, PTHLH, CYP11A1 and HSD3B1) using quantitative real-time PCR (Wathlet *et al.*, 2012). As with Gebhardt's study, these genes were chosen because of their functioning in vital cellular process, such as the metabolism, steroidogenesis and signalling. Expression of five of the examined genes (TRPM7, ITPKA, STC2, CYP11A1 and HSD3B1) seemed to correlate with embryo grading criteria, such as the level of fragmentation on Day 3 and blastocyst development. As far as the establishment of pregnancies followed by live births was concerned, the expression of EFNB2, CAMK1D, STC1 and STC2 was found to be the most predictive. Multivariate models of CC expression profiles were created with positive and negative predictive values of >85% (Wathlet *et al.*, 2012). The results obtained during this investigation emphasized that gene expression-based viability analysis is independent of embryo morphology.

As discussed above, analysis of the gene expression of CCs surrounding normal and aneuploid oocytes revealed two genes, SPSB2 and TP53I3, which might potentially serve as biomarkers of oocyte ploidy (Fragouli *et al.*, 2012). Given that chromosome abnormalities are responsible for the majority of embryo implantation failures and pregnancy losses, genes indicative of aneuploidy are also expected to show an association with other aspects of ART outcome, such as implantation, birth and miscarriage rates. To assess this possibility, the CC expression of SPSB2 and TP53I3 was quantified in 38 IVF cycles using real-time PCR. The experiments were conducted blindly with embryos transferred on the basis of standard morphological assessment, without any chromosome screening. Gene expression data were analysed retrospectively, revealing that both genes tended to be more active in CCs removed from oocytes which led to live births. The association was clearest for SPSB2, which approached statistical significance ($P = 0.054$) (Fragouli *et al.*, 2012). Experiments are under-way to further validate the

usefulness of TP53I3 and SPSB2 as non-invasive biomarkers of oocyte chromosomal status and clinical outcome.

The findings from the studies described in this section are summarized in Table II.

# Conclusion: the development and clinical application of non-invasive assays to assess oocytes and select embryos

The development of non-invasive oocyte assessments, based on the transcriptomic analysis of follicular somatic cells, is likely to be of clinical as well as of scientific value. In a clinical context, the accurate identification of oocytes that are both chromosomally normal and competent to support early preimplantation development could revolutionize IVF, leading to higher success rates and decreasing the risks of miscarriage and children affected by aneuploid syndromes. From a scientific perspective, a non-invasive assay of GCs and CCs could reveal processes that malfunction in suboptimal follicles and improve our understanding of the inter-play between extrinsic and intrinsic factors influencing oocyte development. This knowledge could in turn be used clinically, leading to improvement in *in vitro* oocyte maturation protocols, or the individualization of COS protocols.

The data from all of the reviewed investigations suggest that competent oocytes develop in a follicular environment in which processes such as steroidogenesis, cell−cell communication and signalling, metabolism and transport are active. However, they also clearly show that the transcriptomic behaviour of follicular cells is influenced by a variety of factors. These include the stage of maturation of the developing oocyte (Ouandaogo *et al.*, 2011; Feuerstein *et al.*, 2012), the type of COS employed during ART (Adriaenssens *et al.*, 2010; Gerasimova *et al.*, 2010; de los Santos *et al.*, 2012), the female age (McReynolds *et al.*, 2012; Pacella *et al.*, 2012) and the chromosome constitution of the enclosed oocyte (Fragouli *et al.*, 2012).

In order to develop methods for the identification of viable oocytes based upon CC or GC gene expression analysis, several difficulties need to be overcome. One challenge is the way that viability is defined and measured. The vast majority of IVF laboratories base embryo selection on a set of morphological criteria. However, morphology is a relatively poor guide to viability and unable to predict aneuploidy. A more definitive measure of viability is the establishment of a sustained clinical pregnancy. Interestingly, among investigations which assessed the transcriptomes of follicular cells in relation to the establishment of a clinical pregnancy, it was evident that oocytes leading to live births tend to be surrounded by follicular cells that are more transcriptionally active, displaying high levels of mRNA for most of the genes tested (e.g. Assou *et al.*, 2008; Gebhardt *et al.*, 2011; Fragouli *et al.*, 2012).

Another issue is the fact that studies carried out by different research groups have tended to identify different candidate genes. The genes proposed as potential biomarkers of oocyte competence show little overlap between different investigations. Among the data sets discussed in the current review, there were two candidate genes whose differential expression was observed in at least three studies. These were CYP11A1 which is involved steroidogenesis (Kõks *et al.*, 2010; Grøndahl *et al.*, 2012; McReynolds *et al.*, 2012; Wathlet *et al.*, 2012) and VCAN which

**Table II** Candidate biomarkers for COS response and the selection of oocytes and embryos.

| Study | Methods used | Selection measure | Genes | Function | Expression pattern |
|---|---|---|---|---|---|
| Gerasimova et al. (2010) | Real-time PCR | Response to COS using FSH | FSHR and variants | G protein-coupled receptor | Associated with very low and very high response to FSH |
| Adriaenssens et al. (2010) | Real-time PCR | COS via HP-hMG or rFSH | SPROUTY4<br>SDC4 | EGF signalling<br>Transforming growth factor beta signalling | Up-regulated in rFSH COS<br>Down-regulated in rFSH COS |
| Assou et al. (2008) | Microarray and real-time PCR | Clinical outcome | BCL2L11<br>PCK1<br>NFIB | Apoptosis<br>Gluconeogenesis<br>Transcription factor | Up-regulated in CCs from oocytes leading to live births<br>Up-regulated in CCs from oocytes leading to live births<br>Down-regulated in CCs from oocytes leading to live births |
| Gebhardt et al. (2011) | Real-time PCR | Embryo morphology and clinical outcome | VCAN<br>PTGS2<br>GREM1<br>PFKP | Extracellular matrix<br>Signalling<br>Signalling<br>Metabolism | Up-regulated in CCs from oocytes leading to live births<br>Up-regulated in CCs from oocytes leading to live births<br>Trended to positive correlation with birthweight<br>Positive correlation with birthweight |
| Wathlet et al. (2012) | Real-time PCR | Embryo morphology and clinical outcome | TRPM7<br>ITPKA<br>STC2<br>CYP11A1<br>HSD3B1<br>EFNB2<br>CAMK1D<br>STC1<br>STC2 | LH response<br>$Ca^{2+}$ regulation<br>Steroidogenesis<br>Steroidogenesis<br>Steroidogenesis<br>Transmembrane protein receptor<br>Serine/threonine kinases<br>Steroidogenesis<br>Steroidogenesis | Predictive of embryo moprhology<br>Predictive of embryo moprhology<br>Predictive of embryo moprhology<br>Predictive of embryo moprhology<br>Predictive of embryo moprhology<br>Predictive of pregnancy<br>Predictive of pregnancy<br>Predictive of pregnancy<br>Predictive of pregnancy |
| Fragouli et al. (2012) | Microarray and real-time PCR | Oocyte chromosome status and clinical outcome | SPSB2 | Ubiquitination | Trended to be up-regulated in CCs from oocytes leading to live births |
| Iager et al. (2012) | Microarray and real-time PCR | Clinical outcome | SCL2A9<br>NR2F6<br>ARID1B<br>FAM36A<br>GPR137B<br><br>ZNF132<br>DNAJC15<br>RHBDL2<br>MTUS1<br>NUP133<br>ZNF93 | Glucose homeostasis<br>Orphan nuclear receptor<br>Cell cycle control<br>Mitochondrial membrane<br>G-protein-coupled receptor (GPCR) integral membrane protein<br>DNA-binding subunit of transcription factors<br>Mitochondria/heat shock binding<br>Intermembrane protease<br>Not specified<br>Nucleocytoplasmic transport activity<br>DNA-binding subunit of transcription factors | Up-regulated in CCs from oocytes leading to live births<br>Up-regulated in CCs from oocytes leading to live births<br>Up-regulated in CCs from oocytes leading to live births<br>Up-regulated in CCs from oocytes leading to live births<br>Up-regulated in CCs from oocytes leading to live births<br><br>Up-regulated in CCs from oocytes leading to live births<br>Down-regulated in CCs from oocytes leading to live births<br>Down-regulated in CCs from oocytes leading to live births<br>Down-regulated in CCs from oocytes leading to live births<br>Down-regulated in CCs from oocytes leading to live births<br>Down-regulated in CCs from oocytes leading to live births |

CC, cumulus cells; COS, controlled ovarian stimulation; EFG, epidermal growth factor; HP-hMG, highly purified human menopausal gonadotrophin.

is involved in extracellular matrix formation (Gebhardt *et al.*, 2011; Grøndahl *et al.*, 2012; McReynolds *et al.*, 2012). Up-regulation of both these genes was associated with oocytes which developed into blastocysts, implanted and led to live births. Conversely, both these genes were down-regulated in CCs from oocytes from older women, which could in part explain the increasing difficulty in achieving a live birth with advancing female age.

The lack of uniformity between the genes highlighted by different groups may simply indicate that there are many clinically relevant genes expressed by CCs, with different studies happening upon different candidates. However, it might also be a warning that CC gene expression is affected by multiple variables, which could be timing-, patient-, clinic-, treatment- or aetiology specific. It should be noted that most of the investigations described results from a single IVF clinic. Hence, the observed GC or CC transcriptomic signature and/or candidate genes might only be relevant to the patient group and COS protocols of the clinic where the research was carried out. The possibility that findings from transcriptomic studies might not be transferrable from one laboratory to another is reminiscent of difficulties that affected attempts to introduce non-invasive embryo assessment based upon metabolomic profiling (Hardarson *et al.*, 2012).

Two recent investigations have sought to overcome laboratory to laboratory variation by assessing the transcriptome of CCs obtained from oocytes generated by patients having treatment at several different clinics. The first was our study of CC gene expression in relation to oocyte ploidy (Fragouli *et al.*, 2012), which involved CCs from three unrelated IVF clinics in the USA. The other was by Iager *et al.* (2012) who carried out a retrospective analysis of CCs obtained from patients undergoing IVF procedures in three clinics, situated in Chile and the USA. Analysis took place via microarray and real-time PCR with the aim of identifying a characteristic CC gene expression profile predictive of live birth. Twelve genes were defined as being indicative of pregnancy outcome, with the resulting model having a 78% accuracy and an 81% positive predictive value (Iager *et al.*, 2012).

To conclude, multiple investigations have contributed valuable data, indicating that the analysis of gene expression in follicular cells can reveal scientifically and clinically useful information. However, before transcriptomic tests can be widely applied, further studies are necessary. One of the main issues to be addressed is the lack of biomarker uniformity among studies. An initial step towards this could perhaps be the construction of lists outlining the expression of well-validated genes, according to different variables, such as patient pathology, COS protocol, oocyte maturity or clinical outcome. This could ensure that the value of the available transcriptomic data is maximized. Further research is needed to confirm that the genes analysed are not influenced by patient- clinic- or treatment-associated variables. Additionally, well controlled clinical trials (ideally large, multicentre and randomized) are needed so as to quantify the true clinical benefit of oocyte selection based upon transcriptomic analysis of CCs and GCs. In order for such trials to be successful, they will have to include clearly established expression thresholds suitable for the selection of oocytes and embryos. The development of non-invasive oocyte assays is challenging, and even though a wealth of transcriptomic data exists, most genes considered as candidates for the assessment of oocyte quality have not been independently verified. The huge potential benefits of such assays, however, make them a goal well worth pursuing.

## References

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, Mahmoud K, Aouacheria A, Guillemin Y, Pantesco V, Rème T, Dechaud H, De Vos J, Hamamah S. A non-invasive test for assessing embryo potential by gene expression profiles of human cumulus cells: a proof of concept study. *Mol Hum Reprod* 2008;**14**:711–719.

Barrett SL, Albertini DF. Cumulus cell contact during oocyte maturation in mice regulates meiotic spindle positioning and enhances developmental competence. *J Assist Reprod Genet* 2010;**27**:29–39.

Chian RC, Lim JH, Tan SL. State of the art in vitro oocyte maturation. *Curr Opin Obstet Gynecol* 2004;**16**:211–219.

Cohen J, Wells D, Munné S. Removal of 2 cells from cleavage stage embryos is likely to reduce the efficacy of chromosomal tests that are used to enhance implantation rates. *Fertil Steril* 2007;**87**:496–503.

de los Santos MJ, García-Láez V, Beltrán-Torregrosa D, Horcajadas JA, Martínez-Conejero JA, Esteban FJ, Pellicer A, Labarta E. Hormonal and molecular characterization of follicular fluid, cumulus cells and oocytes from pre-ovulatory follicles in stimulated and unstimulated cycles. *Hum Reprod* 2012;**27**:1596–1605.

Dohlman HG, Thorner J. RGS proteins and signaling by heterotrimeric G proteins. *J Biol Chem* 1997;**272**:3871–3874.

Edson MA, Nagaraja AK, Matzuk MM. The mammalian ovary from genesis to revelation. *Endocr Rev* 2009;**30**:624–712.

Eppig JJ. Intercommunication between mammalian oocytes and companion somatic cells. *Bioessays* 1991;**13**:569–574.

Eppig JJ. Oocyte control of ovarian follicular development and function in mammals. *Reproduction* 2001;**122**:829–838.

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.

Feuerstein P, Puard V, Chevalier C, Teusan R, Cadoret V, Guerif F, Houlgatte R, Royere D. Genomic assessment of human cumulus cell marker genes as predictors of oocyte developmental competence: impact of various experimental factors. *PLoS One* 2012;**7**:e40449.

Fragouli E, Wells D, Delhanty JD. Chromosome abnormalities in the human oocyte. *Cytogenet Genome Res* 2011;**133**:107–118.

Fragouli E, Wells D, Iager AE, Kayisli UA, Patrizio P. Alteration of gene expression in human cumulus cells as a potential indicator of oocyte aneuploidy. *Hum Reprod* 2012;**27**:2559–2568.

Gebhardt KM, Feil DK, Dunning KR, Lane M, Russell DL. Human cumulus cell gene expression as a biomarker of pregnancy outcome after single embryo transfer. *Fertil Steril* 2011;**96**:47–52.

Gerasimova T, Thanasoula MN, Zattas D, Seli E, Sakkas D, Lalioti MD. Identification and in vitro characterization of follicle stimulating hormone (FSH) receptor variants associated with abnormal ovarian response to FSH. *J Clin Endocrinol Metab* 2010;**95**:529–536.

Gilchrist RB, Lane M, Thompson JG. Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. *Hum Reprod Update* 2008;**14**:159–177.

Gittens JE, Barr KJ, Vanderhyden BC, Kidder GM. Interplay between paracrine signaling and gap junctional communication in ovarian follicles. *J Cell Sci* 2005; **118**:113–122.

Gondos B, Westergaard L, Byskov AG. Initiation of oogenesis in the human fetal ovary: ultrastructural and squash preparation study. *Am J Obstet Gynecol* 1986; **155**:189–195.

Grøndahl ML, Eilsø Nielsen M, Dal Canto MB, Fadini R, Rasmussen IA, Westergaard LG, Kristensen SG, Yding Andersen C. Anti-Mullerian hormone remains highly expressed in human cumulus cells during the final stages of folliculogenesis. *RBM Online* 2011;**22**:389–398.

Grøndahl ML, Yding Andersen C, Bogstad J, Borgbo T, Hartvig Boujida V, Borup R. Specific genes are selectively expressed between cumulus and granulosa cells from individual human pre-ovulatory follicles. *Mol Hum Reprod* 2012;**18**:572–584.

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;**3**:1118–1127.

Hamel M, Dufort I, Robert C, Léveillé MC, Leader A, Sirard MA. Genomic assessment of follicular marker genes as pregnancy predictors for human IVF. *Mol Hum Reprod* 2010;**16**:87–96.

Haouzi D, Assou S, Monzo C, Vincens C, Dechaud H, Hamamah S. Altered gene expression profile in cumulus cells of mature MII oocytes from patients with polycystic ovary syndrome. *Hum Reprod* 2012;**27**:3523–3530.

Hardarson T, Ahlström A, Rogberg L, Botros L, Hillensjö T, Westlander G, Sakkas D, Wikland M. Non-invasive metabolomic profiling of Day 2 and 5 embryo culture medium: a prospective randomized trial. *Hum Reprod* 2012;**27**:89–96.

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

Hsieh M, Zamah AM, Conti M. Epidermal growth factor-like growth factors in the follicular fluid: role in oocyte development and maturation. *Semin Reprod Med* 2009;**27**:52–61.

Huang Z, Fragouli E, Wells D. Biomolecules of human female fertility—potential therapeutic targets for pharmaceutical design. *Curr Pharm Des* 2012;**18**:310–324.

Hutt KJ, Albertini DF. An oocentric view of folliculogenesis and embryogenesis. *RBM Online* 2007;**14**:758–764.

Iager AE, Kocabas AM, Otu HH, Ruppel P, Langerveld A, Schnarr P, Suarez M, Jarrett JC, Conaghan J, Rosa GJ *et al.* Identification of a novel gene set in human cumulus cells predictive of an oocyte's pregnancy potential. *Fertil Steril* 2012; First published 29 November 2012. doi:pii:S0015-0282(12)02385-0. 10.1016/j.fertnstert.2012.10.041 [Epub ahead of print].

Johnson MH. Ovarian function in the adult. In: Johnson MH, Everitt J (eds). *Essential Reproduction*, 6th edn. Oxford, UK: Blackwell Scientific Ltd, 2007, 82–91.

Kawamura K, Cheng Y, Kawamura N, Takae S, Okada A, Kawagoe Y, Mulders S, Terada Y, Hsueh AJW. Pre-ovulatory LH/hCG surge decreases C-type natriuretic peptide secretion by ovarian granulosa cells to promote meiotic resumption of pre-ovulatory oocytes. *Hum Reprod* 2011;**26**:3094–3101.

Kõks S, Velthut A, Sarapik A, Altmäe S, Reinmaa E, Schalkwyk LC, Fernandes C, Lad HV, Soomets U, Jaakma U *et al.* The differential transcriptome and ontology profiles of floating and cumulus granulosa cells in stimulated human antral follicles. *Mol Hum Reprod* 2010;**16**:229–240.

Kuang Z, Lewis RS, Curtis JM, Zhan Y, Saunders BM, Babon JJ, Kolesnik TB, Low A, Masters SL, Willson TA *et al.* The SPRY domain-containing SOCS box protein SPSB2 targets iNOS for proteasomal degradation. *J Cell Biol* 2010;**190**:129–141.

Lee JH, Kang Y, Khare V, Jin ZY, Kang MY, Yoon Y, Hyun JW, Chung MH, Cho SI, Jun JY *et al.* The p53-inducible gene 3 (PIG3) contributes to early cellular response to DNA damage. *Oncogene* 2010;**29**:1431–1450.

Matzuk MM, Burns KH, Viveiros MM, Eppig JJ. Intercellular communication in the mammalian ovary: oocytes carry the conversation. *Science* 2002;**21**:2178–2180.

McReynolds S, Dzieciatkowska M, McCallie BR, Mitchell SD, Stevens J, Hansen K, Schoolcraft WB, Katz-Jaffe MG. Impact of maternal aging on the molecular signature of human cumulus cells. *Fertil Steril* 2012;**98**:1574–80.e5.

Mehlmann L. Stops and starts in mammalian oocytes: recent advances in understanding the regulation of meiotic arrest and oocyte maturation. *Reproduction* 2005; **130**:791–799.

Noma N, Kawashima I, Fan HY, Fujita Y, Kawai Y, Tomoda Y, Mihara T, Richards JAS, Shimada M. LH-induced neuregulin 1 (NRG1) Type III transcripts control granulosa cell differentiation and oocyte maturation. *Molec Endocrinol* 2011; **25**:104–116.

Ouandaogo ZG, Haouzi D, Assou S, Dechaud H, Kadoch IJ, De Vos J, Hamamah S. Human cumulus cells molecular signature in relation to oocyte nuclear maturity stage. *PLoS One* 2011;**6**:e27179.

Pacella L, Zander-Fox DL, Armstrong DT, Lane M. Women with reduced ovarian reserve or advanced maternal age have an altered follicular environment. *Fertil Steril* 2012;**98**:986–94.e1-2.

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.

Richards J. New signaling pathways for hormones and cyclic adenosine 3′,5′-monophosphate action in endocrine cells. *Mol Endocrinol* 2001;**15**: 209–218.

Robker RL, Akison LK, Russell DL. Control of oocyte release by progesterone receptor-regulated 1165 gene expression. *Nucl Recept Signal* 2009;**7**:e012.

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

Shimada M, Hernandez-Gonzalez I, Gonzalez-Robayna I, Richards JS. Induced expression of pattern recognition receptors in cumulus oocyte complexes: novel evidence for innate immune-like functions during ovulation. *Mol Endocrinol* 2006a; **20**:3228–3239.

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

Sutton ML, Gilchrist RB, Thompson JG. Effects of in-vivo and invitro environments on the metabolism of the cumulus-oocyte complex and its influence on oocyte developmental capacity. *Hum Reprod Update* 2003;**9**:35–48.

Sutton-McDowall ML, Gilchrist RB, Thompson JG. The pivotal role of glucose metabolism in determining oocyte developmental competence. *Reproduction* 2010;**139**:685–695.

Tsafriri A, Pomerantz SH. Oocyte maturation inhibitor. *Clin Endocrinol Metab* 1986; **15**:157–170.

Vasudevan S, Seli E, Steitz JA. Metazoan oocyte and early embryo development program: a progression through translation regulatory cascades. *Genes Dev* 2006; **20**:138–146.

Wathlet S, Adriaenssens T, Segers I, Verheyen G, Janssens R, Coucke W, Devroey P, Smitz J. New candidate genes to predict pregnancy outcome in single embryo transfer cycles when using cumulus cell gene expression. *Fertil Steril* 2012; **98**:432–439.

Yeo CX, Gilchrist RB, Lane M. Disruption of bidirectional oocyte–cumulus paracrine signaling during in vitro maturation reduces subsequent mouse oocyte developmental competence. *Biol Reprod* 2009;**80**:1072–1080.

Zhang M, Su YQ, Sugiura K, Xia G, Eppig JJ. Granulosa cell ligand NPPC and its receptor NPR2 maintain meiotic arrest in mouse oocytes. *Science* 2010;**330**:366–369.

Zuccotti M, Merico V, Cecconi S, Redi CA, Garagna S. What does it take to make a developmentally competent mammalian egg? *Hum Reprod Update* 2011; **17**:525–540.