A non-invasive test for assessing embryo potential by gene expression profiles of human cumulus cells: a proof of concept study

S. Assou¹,²,³,†, D. Haouzi¹,²,³,†, K. Mahmoud⁴, A. Aouacheria⁵, Y. Guillemin⁵, V. Pantesco¹, T. Rème¹,³, H. Dechaud², J. De Vos¹,³ and S. Hamamah¹,²,³,⁶

¹CHU Montpellier, Institut de Recherche en Biothérapie, Hôpital Saint-Eloi, Montpellier F-34000, France; ²CHU Montpellier, Département de Médecine et Biologie de la Reproduction, Hôpital Arnaud de Villeneuve, Montpellier F-34000, France; ³INSERM, U847 ‘Développement embryonnaire précoce et cellules souches embryonnaires humaines’, Montpellier F-34000, France; ⁴Centre de FIV, Tunis, Tunisia; ⁵IBCP, Institut de Biologie et Chimie des Protéines, UMR 5086 CNRS / Université Claude Bernard Lyon 1, Lyon F-69367, France

Identification of new criteria for embryo quality is required to improve the clinical outcome of in vitro fertilization. The aim of this study was to determine the gene expression profile of cumulus cells (CC) surrounding the oocyte as biomarkers for embryo potential and to identify genes to be used as prognostic indicators of successful pregnancy. CC from single oocytes were analysed using DNA microarrays. Gene expression profiles of CC surrounding the oocyte associated with good embryonic quality and pregnancy outcome were computed. We observed that CC issued from oocytes that developed into embryos with a good morphology had differing gene expression profile according to the pregnancy outcome of the embryo. We demonstrated that the expression of BCL2L11, PCK1 and NFIB in CC is significantly correlated with embryo potential and successful pregnancy. These results were confirmed by quantitative RT–PCR. The gene expression profiling of human CC correlates with embryo potential and pregnancy outcome. BCL2L11, PCK1 and NFIB genes are proposed as biomarkers for predicting pregnancy. Our findings suggest a non-invasive approach, offering a new potential strategy for competent embryo selection. This approach should be validated in single-embryo transfer programmes.

Keywords: cumulus cells; microarray; embryo; non-invasive biomarkers

Introduction

In assisted reproductive technology (ART), pregnancy and birth rates following in vitro fertilization (IVF) attempts remain low. Indeed, 2 out of 3 IVF cycles fail to result in pregnancy (SART, 2004) and more than 8 out of 10 transferred embryos fail to implant (Kovalevsky and Patrizio, 2005). Subjective morphological parameters are still a primary criterion to select healthy embryos used for in IVF and ICSI (intracytoplasmic sperm injection) programmes. However, such criteria do not truly predict the competence of an embryo. Many studies have shown that a combination of several different morphologic criteria leads to more accurate embryo selection (Scott et al., 2000; Balaban and Urman, 2006; La Sala et al., 2008). Morphological criteria for embryo selection are assessed on the day of transfer, and are principally based on early embryonic cleavage (25–27 h post-insemination), the number and size of blastomeres on Day 2 or Day 3, fragmentation percentage and the presence of multi-nucleation in the 4- or 8-cell stage (Fenwick et al., 2002). However, a recent study has shown that the selection of oocytes for insemination does not improve outcome of ART as compared with the transfer of all available embryos, irrespective of their quality (La Sala et al., 2008). Hence, more than 50% of IVF-born babies are from multiple gestations (Reddy et al., 2007). Preterm deliveries that result from multiple pregnancies caused by ART are estimated to account for approximately $890 million of US health care costs annually (Bromer and Seli, 2008). There is a need to identify viable embryos with the highest implantation potential to increase IVF success rates, reduce the number of embryos for fresh replacement, lower multiple pregnancy rates and lower early pregnancy losses. For all these reasons, several biomarkers for embryo selection are currently being investigated (Pearson, 2006; Haouzi et al., 2008). As embryos that result in pregnancy differ in their metabolomic profiles compared with embryos that do not, some studies are trying to identify a molecular signature that can be detected by non-invasive evaluation of the embryo culture medium (Gardner et al., 2001; Brison et al., 2004; Sakkas and Gardner, 2005; Seli et al., 2007; Zhu et al., 2007).

Genomics are also providing vital knowledge of genetic and cellular function during embryonic development (McKenzie et al., 2004) and Feuerstein et al. (2007) have reported that the expression of several genes in cumulus cells (CC), such as cyclooxygenase 2 (COX2), was indicative of oocyte and embryo quality. Gremlin 1 (GREM1),
hyaluronic acid synthase 2 (HAS2), steroidogenic acute regulatory protein (STAR), stearoyl-coenzyme A desaturase 1 and 5 (SCD1 and 5), amphiregulin (AREG) and pentraxin 3 (PTX3) have also been shown to be positively correlated with embryo quality (Zhang et al., 2005). More recently, the expression of glutathione peroxidase 3 (GPX3), chemokine receptor 4 (CXC4), cyclin D2 (CCND2) and catenin delta 1 (CTND1) in human CC have been shown to be inversely correlated with embryo quality, based on early-cleavage rates during embryonic development (van Montfoort et al., 2008). But, despite the fact that early cleavage has been shown to be a reliable biomarker for predicting pregnancy (Lundin et al., 2001; van Montfoort et al., 2004; Yang et al., 2007), gene expression profiles of CC had not been studied with respect to pregnancy outcome.

The aim of this study was to determine the gene expression profile of CC as biomarkers of embryo potential and to identify genes to be used as a prognostic indicators of successful pregnancy.

Materials and methods

Patients and IVF treatment

In this retrospective study, normo-responder patients (n = 30) aged 30.9 ± 2.5 years and referred to our centre for ICSI for male infertility factor were studied. This study has received institutional review board approval. Patients were stimulated with a combination of GnRH agonist or antagonist with recombinant FSH (GonalF, Puregon; respectively of Merck-Serono and Organon) or with hMG (Menopur, Ferring). Ovarian response was evaluated by serum estradiol level and ultrasound examination to monitor follicle development. Retrieval of oocytes was performed 36 h after hCG administration (5000 IU), under ultrasound guidance.

Assessment of embryo quality

On Day 2 and 3 post-microinjection, the quality parameters of individually cultured embryos were evaluated using the number of blastomeres and the degree of fragmentation as criteria (grade 1–2: equally sized blastomeres and 0–20% fragmentation, grade 3–4: nonequally sized blastomeres and >20% fragmentation. A top-quality embryo was defined on Day 3 as 6–8 cells, equally sized blastomeres and no fragmentation. One or two embryos were transferred on Day 3 after oocyte retrieval. Clinical pregnancy was evaluated 2 and 6 weeks after embryo transfer based respectively on serum beta-hCG and ultrasound examination (presence of gestational sac with heart beat).

Cumulus cells

All CC samples were frozen on oocyte collection day. Then, one to three CC samples per patient were randomly selected for microarray analysis. A total of 50 CC samples were collected from 50 single oocytes and analysed individually: 34 CC from grade 1–2 embryos (n = 20 patients), 11 CC from grade 3–4 embryos (n = 10 patients) and 5 CC from unfertilized oocytes (n = 5 patients) (Table I). The data analysis was performed under double blind conditions in which pregnancy outcome was disclosed only after microarrays were hybridized. Regarding pregnancy outcome, the 45 CC from fertilized oocytes included 16 CC from grade 1–2 embryos that did not result into pregnancy (n = 9 patients), 18 CC associated with a positive pregnancy outcome (n = 11 patients) and 11 CC from grade 3–4 embryos that were not transferred. CC were stripped immediately following oocyte recovery (<4 h post-hCG administration). CC were mechanically removed and washed in culture medium and immediately frozen at −80°C in RLT RNA extraction buffer (RNeasy kit, Qiagen, Valencia, CA, USA) before RNA extraction.

Granulosa cells

An independent group of normo-responder patients (n = 8) aged 34.8 ± 3.2 years referred for ICSI programme for male infertility factor was selected for granulosa cells collection (Eight samples). Immediately after oocyte recovery, follicular fluids from matures follicles (>17 mm) of the same patient were pooled, after removal of the cumulus–oocyte complex and diluted in 1/3 volume of HBSS solution (BioWhittaker) in 50 ml batches, representing one sample. Granulosa cells purification was adapted from the protocol by Kolena et al. (1983). Following a 20 min centrifugation at 500g in swinging buckets, granulosa cells were collected on a Ficol cushion (12 mL Lymphocyte separation medium, BioWhittaker). They were successively washed in HBSS and PBS, incubated 5 min in blood lysis buffer (KHCO3 10 mM, NH4Cl 150 mM, EDTA 0.1 mM) to remove red blood cells, counted and pelleted in PBS before lysis in RLT buffer (Quiagen) and storage at −80°C. The number of follicular puncture and the number of purified granulosa cells ranged from 6 to 12 and from 2 × 10^6 to 9 × 10^6, respectively.

Complementary RNA preparation and microarray hybridization

CC and granulosa cells RNA was extracted using the micro RNeasy Kit (Qiagen). The total RNA quantity was measured with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE, USA) and RNA integrity was assessed with an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA). cRNA was prepared with two rounds of amplification according to the manufacturer’s protocol ‘double amplification’ (Two-Cycle cDNA Synthesis Kit, Invitrogen) starting from total RNA (ranging from 70 to 100 ng). cRNA obtained after the first amplification ranged from 0.1 to 1.9 μg/μl and after the second amplification ranged from 1.6 to 4.5 μg/μl.

Labelled fragmented cRNA (12 μg) was hybridized to oligonucleotide probes on an Affymetrix HG-U133 Plus 2.0 array containing 54 675 sets of oligonucleotide probes (‘probeset’) which correspond to ~30 000 unique human genes or predicted genes. Each cumulus and granulosa sample was put individually on a microarray chip.

Data processing

Scanned GeneChip images were processed using Affymetrix GCOS 1.4 software to obtain an intensity value and a detection call (present, marginal or absent) for each probeset, using the default analysis settings and global scaling as first normalization method, with a trimmed mean target intensity value (TGT) of each array arbitrarily set to 100. Probe intensities were derived using the MAS5.0 algorithm. This algorithm also determines whether a gene is expressed with a defined confidence level or not (‘detection call’).

Table 1. The characteristics of CC samples in this study.

<table>
<thead>
<tr>
<th></th>
<th>30 patients</th>
<th>5 patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>45 CC</td>
<td>5 CC</td>
</tr>
<tr>
<td></td>
<td>G1/2 (34 CC)</td>
<td>G1/2 (11 CC)</td>
</tr>
<tr>
<td></td>
<td>P+</td>
<td>P−</td>
</tr>
<tr>
<td>Chips no.</td>
<td>18</td>
<td>16</td>
</tr>
<tr>
<td>Patients no.</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>CC no.</td>
<td>18</td>
<td>16</td>
</tr>
</tbody>
</table>

CC, cumulus cells; P+, cumulus cells from embryos with positive pregnancy outcome; P−, cumulus cells from embryos without pregnancy outcome; G1/2, cumulus cells from grade 1–2 embryos; G3/4, cumulus cells from grade 3–4 embryos; NT, no transfer.
This ‘call’ can either be ‘present’ (when the perfect match probes are significantly more hybridized than the mismatch probes, \( P < 0.04 \)), ‘marginal’ (for \( P > 0.04 \) and \( P < 0.06 \)) or ‘absent’ (\( P > 0.06 \)). The microarray data were obtained in our laboratory in agreement with the Minimal Information about a Microarray Experiment (MIAME) recommendations (Brazma et al., 2001).

### Data analysis and visualization

Significant Analysis of microarrays (SAM) (Tusher et al., 2001) ([http://www-stat.stanford.edu/~tibs/SAM/](http://www-stat.stanford.edu/~tibs/SAM/)) was used to identify genes whose expression varied significantly between sample groups. SAM provides median or mean fold change (FC) values and a false discovery rate (FDR) confidence percentage based on data permutation (mean FC >2 and FDR <5%). Array analysis allowing the comparison of gene expression profile between CC samples and granulosa cell samples is first based on the significant RNA detection (detection call ‘present’ or ‘absent’) and then, submitted to a SAM to identify genes whose expression varied significantly between sample groups. To perform the comparison of gene expression profile between CC samples according embryonic quality and/or pregnancy outcome, a non-supervised selection of probesets using a variation coefficient (CV ≥40%) and a absent/present ‘detection call’ filter was performed before the SAM. To compare profile expression of CC from altered (grade 3–4) and good (grade 1–2) embryonic development, or from embryos leading, or not, to a pregnancy, we performed an unsupervised classification with both principal component analysis (PCA) and hierarchical clustering (Eisen et al., 1998; de Hoon et al., 2004). The PCA involved original scripts based on the R statistics software through the RAGE web interface ([http://rage.montp.inserm.fr](http://rage.montp.inserm.fr)) (Reme et al., 2008). Hierarchical clustering analysis based on the expression levels of varying probes was performed with the CLUSTER and TREEVIEW software packages. To uncover functional biological networks and top canonical pathways, we imported gene expression signatures into the Ingenuity Pathways Analysis (IPA) Software (Ingenuity Systems, Redwood City, CA, USA).

### Ingenuity pathway analysis

Genes were analysed using IPA software (Ingenuity Systems). Those genes with known gene symbols and their corresponding expression values were uploaded into the software. Each gene symbol was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. Networks of these genes were algorithmically generated based on their connectivity and assigned a score. The score is a numerical value used to rank networks according to how relevant they are to the genes in the input dataset but may not be an indication of the quality or significance of the network. The score takes into account the number of focus genes in the network and the size of the network to approximate how relevant this network is to the original list of focus genes. The network identified is then presented as a graph indicating the molecular relationships between genes/gene products. Genes are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). The intensity of the node colour indicated the degree of up-regulation. Canonical pathways analysis identified the pathways, from the IPA library of canonical pathways, which were most significant to the input data set. The significance of the association between the data set and the canonical pathway was determined based on two parameters: (i) A ratio of the number of genes from the data set that map to the pathway divided by the total number of genes that map to the canonical pathway and (ii) A "P"-value calculated using Fisher’s exact test determining the probability that the association between the genes in the data set and the canonical pathway is due to chance alone.

### Quantitative RT–PCR analyses

For quantitative reverse transcriptase–polymerase chain reaction (qRT–PCR) analysis, 10 CC samples used in the microarray experiments were selected according to their pregnancy outcome (5 CC samples associated with a negative outcome and 5 to a positive outcome corresponding to 10 patients). Labelled cRNA (1 \( \mu \)g) from the patient was used to generate first-strand cDNA. These cDNAs (5 \( \mu \)l of a 1/10 dilution) were used for real-time quantitative PCR reactions according to the manufacturer’s recommendations (Applied Biosystems). The 20 \( \mu \)l reaction mixture consisted of cDNA (5 \( \mu \)l), 1 \( \mu \)M of primers and 10 \( \mu \)l of TaqMan Universal PCR Master Mix (Applied Biosystems). The amplification was measured during 40 cycles with an annealing temperature at 60°C. The amount of PCR product produced in every cycle step of the PCR reaction is monitored by TaqMan probe. A threshold is set in the exponential phase of the amplification curve, from which the cycle number (‘CT’ for ‘cycle threshold’) is read off. The Ct value is used in the calculation of relative mRNA transcript levels. Effectiveness (E) of the PCR was measured. This effectiveness is obtained by a standard curve corresponding to the primers used. Quantitative RT–PCR was performed using the ABI Prism 7000 sequence detection system (Applied Biosystems) and normalized to PGK1 for each sample using the following formula: 

\[
E_{\text{sample}} = E_{\text{control}} \times \frac{C_{\text{PGK1(sample)}}}{C_{\text{PGK1(control)}}}
\]

\( 
C_{\text{PGK1}} = \Delta C_{\text{T}} = \text{Ct (control) - Ct (sample)}
\]

Gene expression profile of human cumulus cells and embryo potential

### Results

#### Gene expression profile of CC according to embryo outcome

To identify a gene expression profile in CC that correlated with embryo outcome, we established a gene expression signature for each outcome category: CC of unfertilized oocytes, CC from oocytes that resulted in embryo development but extensive fragmentation (grade 3–4) and CC from oocytes that resulted in embryo development with no or limited fragmentation (grade 1–2). Granulosa cells samples were taken as a reference tissue (control). Indeed, granulosa cells are cells closely related to CC as opposed to other adult tissues. The use of this reference tissue lowered the number of differentially expressed genes related to crude lineage differences, thus facilitating the identification of subtle variation in the CC/oocyte interaction. A SAM analysis showed that 2605 genes were up-regulated in the unfertilized group, 2739 in the grade 3–4 group and 2482 in the grade 1–2 group with a FDR <5%. Conversely, 4270, 4349 and 4483 genes were down-regulated, respectively. These lists of genes were then intersected to determine their overlap (Fig. 1). While 449 up- and 890 down-expressed genes were in common in all three groups, each category displayed a specific gene expression profile. Interestingly, 860 up-regulated genes, including for example Galanin and Gap Junction A5 (GJAS), and 1416 down-regulated genes, including HLA-G and EGR1 were specifically modulated in CC associated with a good morphological embryonic quality. It must be noted that although the grade 1–2 group displayed a strong gene expression profile, this group was heterogeneous regarding to pregnancy outcome and included 18 CC samples associated with embryos that resulted in pregnancy (including four twin pregnancies) but also 16 CC samples associated with embryos that failed to give rise to pregnancy.

#### Gene expression profile of CC according to pregnancy outcome

CC samples were therefore compared according to the pregnancy outcome. A SAM analysis delineated a ‘pregnancy outcome’ list of 630 genes that varied significantly (FDR <5%) between the two groups of patients (pregnancy versus no pregnancy). PCA and hierarchical clustering confirmed that this 630 genes list indeed segregated a majority of CC samples associated with pregnancy from those associated with no pregnancy (Fig. 2A and B). Of note, genes from the ‘pregnancy outcome’ list were predominantly up-regulated in samples associated with a good outcome. The ‘pregnancy outcome’ expression signature was particularly marked in a subgroup of 10 CC samples from embryos associated with the ‘pregnancy’ group.
Functional annotation of the pregnancy outcome gene list

To investigate biological processes correlated to embryo achieving pregnancy, Ingenuity and Pubmed databases were used to annotate the 630 genes from the ‘pregnancy outcome’ gene list. Among genes whose overexpression is associated with pregnancy, the most significantly overrepresented pathways were ‘oxidative stress’, ‘TR/RXR activation’, ‘G2/M transition of the cell cycle’, ‘xenobiotic metabolism’ and ‘NFKappaB’ signalling (Fig. 2C). Among these pathways, the most representative genes were interleukins, chemokines, adaptator proteins and kinases: IL1Beta (x4.5 in pregnancy samples versus no pregnancy, \( P = 0.001 \)), IL16 (x4.8, \( P = 0.001 \)), IL8 (x2.6, \( P = 0.007 \)), IL1RN (x2.1, \( P = 0.0051 \)), IL17RC (x3.6, \( P = 0.001 \)), TIRAP (x8.0, \( P = 0.001 \)), CXCL12 (x3.1, \( P = 0.001 \)), CCR5 (x2.6, \( P = 0.0051 \)) and PCK1 (x3.4, \( P = 0.001 \)). Strikingly, numerous genes involved in the regulation of apoptosis were significantly modulated in CC samples from oocytes resulting in a pregnancy. These genes were BCL2L11 (x6.9, \( P < 0.001 \)), CRADD (x2, \( P = 0.0036 \)), NEMO (x4.6, \( P < 0.001 \)), BCL10 (x3.1, \( P < 0.001 \)), SERPINB8 (x9.1, \( P < 0.001 \)) and TNFSF13 (x2.5, \( P = 0.0038 \)).
On the other hand, genes associated with no pregnancy were correlated with the following pathways: G2/M DNA damage and checkpoint regulation of the cell cycle, ‘Sonic hedgehog’, ‘IGF-1’, ‘complement system’ and ‘Wnt/Beta-catenin’ signalling (Fig. 2D). Representative genes correlated with no pregnancy included NFIB (x0.3, $P_{0.001}$), MAD2L1 (x0.4, $P_{0.001}$) and IGF1R (x0.4, $P_{0.001}$).

Candidate genes expressed in CC for embryo potential

The SAM analysis of CC according to pregnancy outcome identified genes that are candidate biomarkers for embryo potential that would differentiate between oocytes that produced embryos resulting in a pregnancy versus those that did not result in pregnancy based on CC analysis. Representative genes from the pregnancy outcome gene list were selected and qRT–PCR used to confirm independently the microarray data. We analysed the differential expression of two up-regulated genes (BCL2L11 and PCK1) and one down-regulated gene (NFIB) between CC from grade 1–2 embryos without positive pregnancy and CC from grade 1–2 embryos achieving pregnancy (Fig. 3). The qRT–PCR mean transcripts levels were $1.1 \pm 0.9$ versus $0.4 \pm 0.3$ (pregnancy versus no pregnancy) for BCL2L11 ($P = 0.25$, NS), $11.6 \pm 8.8$ versus $4.0 \pm 2.5$ for PCK1 ($P = 0.22$, NS) and $0.4 \pm 0.3$ versus $0.9 \pm 0.4$ for NFIB ($P = 0.16$, NS).

Figure 3: Quantitative RT–PCR confirmation.

Box-and-whisker plots comparing the expression level of NFIB, PCK1 and BCL2L11 in CC from the positive pregnancy outcome group and negative pregnancy outcome group. The signal intensity for each gene is shown on the y axis as arbitrary units determined by the GCOS 1.2 software (Affymetrix) or by quantitative real-time qRT–PCR analysis (five samples in each group). Data were normalized using PGK1 expression.
Discussion

In most mammalian species including human, the CC which surrounds the oocyte are still present at the time of fertilization in the oviduct and remain until embryonic implantation. Extracellular matrix remodelling within and around the cumulus probably plays a key role in both of these steps. In this respect, we identified three promising genes expressed in CC that could be biomarkers for embryo potential and pregnancy outcome: BCL2L11, PCK1 and NFIB.

Some recent studies have suggested non-invasive methods for oocyte and embryo viability by analysing human follicular fluid or culture media (Gardner et al., 2001; Brison et al., 2004; Sakkas and Gardner, 2005; Seli et al., 2007; Zhu et al., 2007). However, an important technical drawback is the number of metabolites and the concentration that each metabolite must reach to be detected.

Therefore, a few studies in human species have been conducted by studying CC or granulosa cells to identify biomarkers for oocyte quality and competence (Zhang et al., 2005; Hamel et al., 2008), or for early embryo development (McKenzie et al., 2004; van Montfoort et al., 2008) (Table II). Here we present a retrospective study that is the first one performing gene expression profiles of CC with respect to embryo quality and pregnancy outcome.

In our study, we demonstrated that genes expression profile of CC which surrounds oocyte correlated to different outcomes, allowing the identification of a specific expression signature of embryos developing towards pregnancy. Interestingly, genes in cumulus samples resulting in a successful pregnancy were predominantly up-regulated, including BCL2L11 and PCK1, respectively involved in apoptosis and gluconeogenesis. Some genes were nevertheless found down-regulated in CC associated with a good pregnancy outcome, such as the transcription factor NFIB. Related to a possible impact of hormonal treatment under COS, we compared the 26 CC after GnRH agonist with recombinant FSH or hMG to the 19 CC under GnRH antagonist with recombinant FSH or hMG. There were 16 genes significantly modulated (FDR <5%).

Abnormal cells are normally removed by apoptosis, a cell death programme, which is a physiological process in embryos (Hauouzi et al., 2008). Many proteases involved in apoptotic processes have been well characterized, principally the caspases, which are key executioners of apoptosis. There are two major pathways of caspase activation: the extrinsic pathway mediated by death receptor activation, and the intrinsic pathway triggered by cellular stress or DNA damage. BCL2 family proteins, which include pro- and anti-apoptotic members, are critical regulators of the intrinsic pathway. The balance between pro- and anti-apoptotic members is a determining factor controlling cell survival or death decision. A strong incidence of CC apoptosis has been correlated with poor oocyte quality and low pregnancy rate (Lee et al., 2001). Recently, six up-regulated pro-apoptotic genes and two down-regulated anti-apoptotic genes were reported in CC from oocytes resulting in a non-early cleavage embryo (van Montfoort et al., 2008).

In the present study, we observed a significant differential expression of several genes involved in apoptotic pathways, including one BCL2 family member, BCL2L11, CRADD protein, BCL10, NEMO and a serpin. BCL2L11, also known as BIM, belongs to the BH3-only proteins, a pro-apoptotic subgroup of the BCL2 family that contains only one of the BCL2 homology regions (BH3). This BH3-only protein promotes apoptosis by inhibiting anti-apoptotic members such as BCL2 and/or directly activating the pro-apoptotic BAX and BAK proteins. Interestingly, BCL2L11 gene expression was up-regulated in CC correlated to a pregnancy outcome. These results suggest that BCL2L11, which may participate in the apoptotics of CC, is induced during oocyte development. A high level of BCL2L11 may sign this physiological process and therefore be a biomarker for optimal embryonic development.

Recently, it was demonstrated in a mouse model that nutritional support from CC is essential for growth and development of the mouse oocyte (Sugiura et al., 2007; Su et al., 2008). Evidence for the existence of a ‘dialogue’ between metabolic pathways of the CC and the oocyte, allowing the development of both cell types, has been documented. These metabolic pathways involve both glycolysis and cholesterol biosynthesis (Sugiura et al., 2007; Su et al., 2008).

Interestingly, in our study, we observed an up-regulation of PCK1 mRNA in CC from oocytes that produce embryos resulting in a pregnancy. This gene is a main control point for the regulation of gluconeogenesis and catalyses the formation of phosphoenolpyruvate from oxaloacetate. Interestingly, the majority of genes involved in the network around the PCK1 gene were up-regulated (Fig. 4B).

Nuclear factor I (NFI) consists of a family of four genes that each gives rise to multiple isoforms via alternative splicing (NFIA, NFIB, NFIC and NFIX). The NFI proteins play an important role in regulating tissue-specific gene expression during mammalian embryogenesis (Steele-Perkins et al., 2005). Interestingly, NFIB gene expression was down-regulated as confirmed by qRT–PCR, in CC associated with a pregnancy.

Other genes were found differentially expressed between CC with different pregnancy outcome. Among them, additional factors involved in apoptosis, such as BCL10, an anti-apoptotic gene found up-regulated in the pregnancy group. The protein encoded by this gene contains a caspase recruitment domain (CARD), and has been shown to both induce apoptosis and to activate NFKappaB. BCL10 is reported to interact with other CARD domain-containing proteins, which are thought to function as upstream regulators in NFκB signalling (Fig. 4A). Moreover, CRADD and IKBKG/FIP3/NEMO gene expression were also up-regulated in CC from oocytes that produce embryos resulting in a pregnancy. The proteins encoded by the CRADD and NEMO genes are respectively a death domain (CARD/
DD)-containing protein and the regulatory subunit of IKK (IKappaB kinase). IKK signalling leads to the activation of NFKappaB, a transcription factor involved in protection against apoptosis (Courtois and Smahi, 2006). Moreover, the TNFSF13 gene was an up-regulated member of the network around the BCL2L11 gene (Fig. 4A). We previously identified TNFSF13 as highly produced by CC (Assou et al., 2006). The protein encoded by this gene is a member of the tumour necrosis factor (TNF) ligand family. Initially, this protein and its receptor, TNFRSF17, were found to be important for B cell development (Stein et al., 2002). In vitro experiments suggested that this protein may be able to attenuate apoptosis and to increase NFKappaB activation (He et al., 2004).

In conclusion, we found a differential gene expression between human CC from oocytes resulting in different pregnancy outcome from patients referred for ICSI for male infertility. This analysis is a novel concept, and provides new biomarker candidates to assess the embryo potential for pregnancy outcome, such as BCL2L11, PCK1 and NFIB. The relationship between CC apoptosis and the developmental competence of the corresponding oocytes are worthy of further investigation. Before any routine use, a large cohort of CC

![Figure 4: Top-ranked networks evidenced by Ingenuity Pathway software.](image1.png)

*Figure 4:* Top-ranked functional networks obtained from up-regulated genes from the ‘pregnancy outcome’ 1–2 signature were found to aggregate around BCL2L11 (A) and PCK1 (B) genes. The red colour intensity of the node indicated the degree of up-regulation. Un coloured genes were not identified as differentially expressed in our experiment and but were integrated into the network for computational grounds. In each network, edge types are indicative: a plain line indicates direct interaction, a dashed line indicates indirect interaction; a line without arrowhead indicates binding only, a line finishing with a vertical line indicates inhibition; a line with an arrowhead indicates ‘acts on’.

![Figure 5: Envisioned use of a genomic analysis of CC (‘G-test’) to identify the CC with the best gene expression profile according to pregnancy outcome.](image2.png)

*Figure 5:* Envisioned use of a genomic analysis of CC (‘G-test’) to identify the CC with the best gene expression profile according to pregnancy outcome.
should be analysed by using microarrays and validated by qRT–PCR. Our results suggest that analysis of CC surrounding the oocyte can be a non-invasive approach for embryo selection. We envision the possibility to collect CC immediately after oocyte retrieval, analyse the CC with a genomic test (G-test) to assess the potential of the embryo and select the embryo for fresh replacement based on the G-test results (Fig. 5). As we did double-embryo transfer, the validation of new biomarkers such as BCL2L11, PCK1 and NFIB genes should be tested prospectively in single-embryo transfer programmes.

**Acknowledgements**

We thank the University-Hospital of Montpellier and IRB for support and the ART team for their assistance during this study. The public repository accession number for microarray data will be available from the authors.

**Funding**

This work was supported by Ferring, Merck-Serono and Schering-Plough Pharmaceuticals.

**References**


Submitted on August 15, 2008; resubmitted on November 6, 2008; accepted on November 6, 2008.