Biomarkers of human oocyte developmental competence expressed in cumulus cells before ICSI: a preliminary study

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Abstract

Purpose To identify reliable genomic biomarkers expressed in cumulus cells that accurately and non-invasively predict the oocyte developmental competence and reinforce the already used morphological criteria.

Methods Eight consenting patients were selected for ovarian stimulation and ICSI procedures. Cumulus-oocyte complexes were transvaginally punctured and individually selected based on both good morphological criteria and high zona pellucida birefringence. Following ICSI, two 3-day embryos per patient were transferred. Pregnancy outcome was recorded and proven implantation was thereafter confirmed. Differential gene expression was assessed using two microarray platforms. Further real-time PCR validation, Ingenuity pathways analysis and intra-patient analysis were performed on 17 selected candidates. **Results** Seven genes were differentially ($p \leq 0.05$) associated to successful pregnancy and implantation. These biomarkers could be used to predict the oocyte developmental competence.

Conclusions These genomic markers are a powerful reinforcement of morphological approaches of oocyte selection.

Capsule Cumulus cells expressed some molecular biomarkers that discriminate the developmental competence of oocytes having similar and satisfactory morphological and ZP birefringence properties in human IVF.

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Their large-scale validation could increase pregnancy outcome and single embryo transfer efficiency.

Keywords Cumulus cells · Genomic biomarkers · Infertility · Oocyte developmental competence · Pregnancy · Zona pellucida birefringence

Introduction

Infertility is the biological inability of a couple to conceive a baby after one year of unprotected sexual intercourse. Its incidence is rising mainly in the developed countries. Assisted reproductive technologies (ART) offer interesting treatment possibilities to infertile couples by means of procedures that include the selection of the suitable gametes to fertilize and thereafter of the appropriate embryos to transfer [1]. This selection of high-quality embryos to transfer is still challenging in human ART. It was reported to be largely dependent on the oocyte quality acquired during the maturation process [2].

To clinically predict the oocyte developmental potential, several non-invasive parameters including early embryo (number and appearance of blastomeres, multinucleation, etc.) and oocyte morphology (oocyte diameter, an integral first polar body and a less granulated cytoplasm) were used [3, 4]. Additional criteria related to the oocyte and its neighboring cells were also used [5]. In this context, cumulus cells (CCs) appearance and number of layers were used [6–8]. Unfortunately, these morphological and microscopic criteria are subjective and lack enough accuracy and therefore weakly correlated with successful pregnancy [9]. To balance the weakness of these morphological criteria and increase the pregnancy outcome, clinicians have resorted to transferring more than one embryo. Conse-

quently, the incidence of multiple pregnancies, perinatal mortality as well as health problems for both the mother and the babies increased considerably [10–12].

In order to avoid these drawbacks on both the mother and the children, and reduce health care costs, elective single embryo transfer (eSET) is therefore a necessary approach [13, 14]. In addition to the reduction of multiple pregnancy rates, eSET is reported to provide several benefits not only associated to the safety of the mother and the offspring, but also at the socio-economic level. Indeed, a successful eSET requires the accurate selection of high-quality embryo to transfer [15–17].

It is established in most mammal species including human that embryo quality depends mainly on the competence of the oocyte selected for fertilization. This competence is the result of mutual interactions of the oocyte with its follicular environment [1, 2, 18, 19]. CCs are the somatic cells that occupy the immediate vicinity of the oocyte and represent the first interface of the oocyte with its environment [20]. CCs maintain a continuous and reciprocal relationship with the oocyte and are involved in the support and the chronology of oocyte maturation and competence acquisition [20–23]. They are also reported to be involved in the oocyte maturation, ovulation as well as the fertilization process [8, 24]. Given their importance in oocyte maturation and therefore embryo quality, CCs are usually used both in animal IVF and clinics as one of the main morphological criteria to assess the oocyte developmental potential [18, 25, 26]. Despite the interesting morphological criteria used including CCs morphology, there is still little correlation between these parameters and the pregnancy final outcome [7, 9]. Additional early approaches to reinforce these previous criteria were explored. Genomic biomarkers expressed in CCs are an interesting approach to non-invasively and quantitatively predict the oocyte developmental competence [27, 28].

Based on the previous reports, we used herein the morphological properties of CCs to reflect the oocyte quality. These morphologically good cumulus-oocyte complexes (COCs) were associated with their pregnancy outcome. We sought to identify quantitative biomarkers expressed in CCs that could accurately reflect the oocyte developmental potential. Finding differentially expressed genes associated with oocytes that lead to successful pregnancy would offer concise predictors to select high-quality oocytes and improve pregnancy rates in human ART.

Besides the oocyte and CCs morphology, zona pellucida (ZP) birefringence is another morphological criterion that was also used in our study to reinforce our selection of high-quality oocytes. In fact, the inner immediate layers of CCs are connected with the ZP, which is a thick protective layer that surrounds the oocyte. Many studies have

demonstrated a positive correlation between ZP thickness and oocyte developmental competence, successful embryo implantation and subsequent pregnancy rates [29–31]. To prevent any user bias and to increase objectivity and repeatability, ZP thickness and homogeneity measurements in Dr. Montag's lab were automated [29, 32].

The goal was therefore to define a preliminary list of genomic markers expressed differentially in the CCs of oocytes with high ZP birefringence (HZB) and associated with successful pregnancy. These molecular biomarkers should offer a powerful tool to considerably improve oocyte selection, and therefore increase the pregnancy outcomes and diminish multiple pregnancy incidences. Ultimately, once the large-scale validation of these biomarkers is achieved, efficient single embryo transfer could be envisioned.

Materials and methods

Patient selection

Eight consenting patients ($n=8$) were selected for this study at the IVF clinic of the University of Bonn Medical School. ICSI (intracytoplasmic sperm injection) was the recommended procedure for all the patients.

Ovarian stimulation and cumulus-oocyte complex (COC) retrieval

Ovarian stimulation with the administration of the gonadotrophin releasing hormone agonist (GnRHa) triptorelin acetate (Decapeptyl (0.1 mg/day), Ferring, Germany) was started on the 22nd day of the preceding menstrual cycle. Daily administration of the human menopausal gonadotrophin (HMG; Menogon, Organon) and/or follicular stimulating hormone (FSH; Gonal-F, Serono) was carried out 12 to 15 days later. The HMG/FSH (225 IU) dose was adjusted through transvaginal ultrasound monitoring of the patient's individual response, mainly follicular size and oestradiol levels. When some follicles of the ovulatory wave were larger than 18 mm in diameter, human chorionic gonadotrophin (HCG; 10,000 IU) was administered and 36 to 38 h later, COCs were transvaginally punctured. All the protocols used herein were approved by the institutional review board of the University of Bonn Medical School.

Cumulus cells collection and zona birefringence analysis

Following follicular aspiration, collected COCs were immediately washed in HEPES-buffered medium (Cook, Brisbane, Australia) and individually cultured in fertilization media (Cook) for 2 h. Incubation was performed in a

mini-incubator (Minc, Cook) using pre-mixed gas with low oxygen (6% CO₂, 5% O₂, 89% N₂) at 37°C. Each COC was put in a dish containing HEPES-buffered medium under oil. CCs were dissected using a sterile scalpel and transferred immediately into a sterile tube and stored at -80°C for further analysis.

Next, a hyaluronidase treatment to remove the remaining cumuli was performed and denuded oocytes were individually incubated at 37°C in 5-μL droplets of fertilization medium covered with mineral oil in a glass-bottom dish (Willco, Wells BV dish, MTG, Altdorf, Germany) for 1 to 2 h. Oocytes with vacuolization were excluded and neither used for zona imaging nor for ICSI. Prior to switching to the birefringence analysis mode to assess the zona score, immature oocytes (absence of the first polar body when scanned by conventional light microscopy) were also removed. As described previously by Dr. Montag's team [29], unfertilized MII oocytes were classified based on their inner zona layer birefringence measurement using an automatic module Octax polairAide™ (Octax ICSI Guard™, OCTAX Microscience GmbH, Altdorf, Germany) connected to a polarization imaging software (OCTAX Eyeware™). Zona score was therefore automatically and non-invasively measured in real time based on the intensity and the uniformity of the birefringence at 180 measuring points of the inner zona layer. The temperature of the heated plate was linked to a calibrated sensor to maintain 37.0±0.5°C in the medium droplet during microscopic observation.

MI I oocytes with an irregular and/or low birefringence distribution in the inner zona layer were classified as low zona birefringence (LZB). However, those with a high intensity and uniform birefringent inner zona layer were classified as high zona birefringence (HZB) [29].

Intracytoplasmic sperm injection (ICSI), embryo culture and transfer

All media used for oocyte retrieval, denuding, ICSI treatment and subsequent culture were of pharmaceutical grade, free of phenol red and provided by Cook company (Fertilization, Cleavage, Gamete, PVP, Hyaluronidase, Culture oil). The selection of patients for ICSI treatment was based on the diagnosis of male factor infertility due to reduced sperm quality (mainly morphological abnormalities). ICSI was the recommended and the most efficient approach in this case to prevent male factor effect [33]. In a collaborative approach, all patients underwent an extensive andrological, gynaecological and cytogenetic examination prior to ICSI to avoid any other bias.

ICSI was performed within 1 h after zona imaging. Oocytes were kept in the same order as during zona imaging and subsequently cultured individually in 30-μL

medium droplets under oil. The spermatozoa ejaculate was first diluted by a mini-swim-up technique, then washed and the final sperm pellet was resuspended in 20–50 mL of fertilization medium and stored in a CO₂ incubator. A few microliters of the motile sperm suspension were placed into a central polyvinylpyrrolidone (PVP) droplet in the injection dish where their morphological criteria were quickly checked. ICSI was carried out on the heated stage of an inverted microscope (DMIRB; Leica, Bensheim, Germany) equipped with microinjection devices for holding the oocyte and sperm injection (Narishige, Tokyo, Japan). All MII oocytes were fertilized by ICSI. According to the German law, the culture of fertilized oocytes should not go beyond the 2 pronuclei stage (2PN) except the two embryos selected for transfer. Eighteen (18) hours following ICSI, some morphological criteria related to successful fertilization were therefore checked herein including the extrusion of the second polar body and visible and normal 2PN. In fact, oocytes with two pronuclei (2PN) of equal size in close proximity and centrally located within the ooplasm were considered as successfully fertilized. Among the fertilized oocytes with these satisfactory criteria, the two top zona score oocytes were selected and cultured in cleavage medium up to the time of transfer on day 3, whereas the supernumerary oocytes were cryopreserved. Transvaginal intrauterine embryo transfer was performed in 30 μL of culture medium using a Sydney IVF catheter (COOK, Brisbane, Australia) as described previously [29]. Progesterone vaginal suppositories (200 mg/day) were used twice a day to support the luteal phase support. This treatment began on the day following the HCG administration. Pregnancy was assessed first through a positive HCG test at day 14 after transfer and then a higher value 2 days later. Proven implantation and pregnancy were thereafter confirmed by ultrasonic detection of gestational sacs and a positive heart beat (viable embryo) 3 weeks later.

Patient groups

Based on the pregnancy results, individual cumulus cells from the eight ($n=8$) patients were divided into two main groups to explore *in vivo* genomic markers expressed in CCs and associated with oocyte competence, embryo quality as well as pregnancy. The CCs of the zona good oocytes with successful pregnancy (ZGP) was the first (positive) group. It included 8 cumuli of individual oocytes (from 4 patients) that led to pregnancy. Following the transfer of two HZB fertilized oocytes for each patient, only one gave heterozygous twins. However, each one of the three (3) remaining patients got a single embryo. On the other hand, the second (negative) group contained 6 individual cumuli of individual oocytes (again from 4 different patients) with zona good score but an unsuccessful

pregnancy (ZGNP). While ZGP represented the positive group, ZGNP was the negative one.

Custom-made cDNA microarray preparation

Four suppressive subtractive cDNA hybridizations (SSH) previously achieved in our lab were printed on our custom-made microarray. Differentially expressed cDNAs in both cumulus and granulosa cells associated to *in vivo* competent oocytes [34–36] were amplified, purified, sequenced, and identified by BLAST analysis against the cDNA Library Manager Program (Genome Canada bioinformatics, Quebec, Canada). SSH products and negative and positive controls were dissolved in equal volumes of dimethyl sulfoxide (DMSO) and H₂O and spotted in duplicate on different locations on GAPSII glass slides (Corning, Corning, NY) using VersArray Chip WriterPro robot (Bio-Rad, Mississauga, Canada) as detailed elsewhere [36]. UV rays served to cross-link the oligonucleotides before the Terminal Deoxynucleotidyl Transferase quality control Assay (GE healthcare, Quebec, Canada).

Total RNA extraction

The CCs samples of each oocyte in both experimental groups were subjected to total RNA extraction using the PicoPure RNA Isolation Kit (Arcturus, Molecular Devices Analytical Technologies, Sunnyvale, CA) according to the manufacturer's instructions and resuspended in 30 µL of Elution buffer (EB) provided in the kit. The concentration and quality of the RNA were assessed using the Agilent 2100 bioanalyzer (Agilent Technologies, Waldbronn, Germany) according to the manufacturer's protocol.

Messenger RNA linear amplification

Based on RNA concentrations of each individual CC (biological replicate), 10 ng of total RNA were pooled for each experimental group (pool of 8 and 6 replicates respectively for the pregnant and the non-pregnant groups) for amplification using 2-round *in vitro* transcription (IVT) following the instructions of the RiboAmp^{plus} RNA Amplification kit (Arcturus, Molecular Devices Analytical Technologies). Briefly, RNA was first reversed transcribed with the incorporation of a primer containing a T7 RNA polymerase promoter sequence (RiboAmp primer A). Double-stranded cDNA was then synthesized, column-purified and used as a template to drive the first 6-h round of the T7-polymerase IVT. One microliter of this elution was used for NanoDrop (NanoDrop Technologies, Wilmington, DE) quantification of the first round yield, whereas the rest served as a template for the second round. Similarly to round 1, the second linear amplification round

was carried out according to the kit recommendations and the resulting RNA was column-purified and eluted in 30 µL of RNA eluted buffer (RE). The final RNA amplification yield was quantified by spectrophotometry at 260 nm using the NanoDrop ND-1000 (NanoDrop Technologies) as before.

Messenger RNA indirect labelling

Amplified messenger RNA of each group (ZGP vs. ZGNP) was divided into 2 sub-replicates per chip type (Fig. 1) to get a dye-swap design and labelled using the Universal Linkage System (ULSTM) aRNA Fluorescent Labelling Kit (KREATECH Biotechnology, Amsterdam, The Netherlands) according to the manufacturer's instructions. Briefly, for each sub-replicate, 2.5 µg of amplified RNA was labelled by incubation with 2.5 µL of Cy5/DY647-ULS or Cy3/DY547-ULS dyes, and 2 µL of 10x labelling solution in a 20-µL total volume at 85°C for 15 min. Unbound dye was then removed, as recommended, using the KREApure columns provided in the kit. Labelled RNA was quantified on the NanoDrop ND-1000 (NanoDrop Technologies). Each probe of the ZGP group was mixed with its corresponding one in the ZGNP group in equimolar proportions before hybridization.

Hybridization design

Custom-made cDNA microarray hybridizations

Two hybridizations were performed in a dye-swap design (Fig. 1) on our custom-made array. Hybridizations were performed in the ArrayBooster using the Advacard AC3C (The Gel Company, San Francisco, CA) for 18 h at 50°C using Slide Hyb#1 (Ambion, Austin, TX). The slides were then washed, spin-dried, scanned and analyzed using the ChipReader and ArrayPro Analyzer software (Media Cybernetics, San Diego, CA)[36].

Hybridizations using the OneArray 30 K 60-mer oligo microarray

In order to achieve an extensive candidate search, two additional hybridizations in a dye-swap design (Fig. 1) were performed using two One Array chips (Phalanx Biotech, Palo Alto, CA). It consisted of a 60-mer sense-strand polynucleotide microarray that contains 30,968 of highly sensitive and specific human probes and 1,082 experimental control probes. After a prehybridization step of 10 min at 60°C, the hybridization protocol was performed according the DNA microarray user guide available at http://www.phalanxbiotech.com/Support/Usr_Gd_combo.pdf and using the recommended hybridization and washing buffers.

Fig. 1 Summary of the experimental design used

Hybridization data analysis

Following hybridization, both microarray slides were scanned using the VersArray ChipReader 3.1 System (Bio-Rad, Mississauga, Canada) and analyzed using the ArrayPro Analyzer software (Media Cybernetics, Bethesda, MD). Raw microarray data were first Loess-normalized and corrected for background as described elsewhere [36]. Ratio of net fluorescence intensities of our dye-swap experiments between positive (pregnant) and negative (non-pregnant) groups was analysed using the free-software National Institute on Aging (NIA) Array Analysis Tool (Baltimore, MD) developed at NIA [37] at FDR=5% and a minimum cut-off limit of 2.25. Since each clone was printed twice on our slide [34], two additional technical sub-replicates that emerged from this design were taken into account during statistical analysis. Two lists of more than two-fold change in both over-expressed and under-expressed clones in the ZGP group compared to ZGNP were generated for subsequent analysis to define suitable markers expressed in CCs and associated with good-quality oocytes.

Functional genomic analysis including network mapping and molecular pathways of both overexpressed and under-expressed genes within the two patient groups (ZGP vs. ZGNP) was achieved using the Ingenuity Pathways Analysis software (IPA) [38]. Briefly, the candidate genes were uploaded with their official name and fold change into the IPA. Using its web database based on previous studies, IPA is able to automatically find the potential connections between the uploaded genes and to classify them into networks (using

numerical scores) according to the molecular pathways involved and the quality of the gene relationships already established. Each network is therefore composed of selected genes from the uploaded list linked together and with other molecules (suggested by the software), and mapped in a whole signalling pathway. Each molecular relationship among the network members is represented in a conventional mapping that allows identification and therefore interpretation of the type of interactions.

The use of IPA analysis in our study aims at discovering some gene networks and exploring the possible interactions between the genomic biomarkers differentially expressed in CCs and associated to oocytes with successful pregnancy.

Real-Time PCR validation

Equal amounts of total RNA were taken from each replicate (non-amplified original material) of individual CCs of each patient group. To denature the RNA and remove secondary structures, the RNAs were heated at 65°C for 5 min and then quenched rapidly on ice for at least 2 min. Samples were then reversed transcribed using the SensiScript reverse transcriptase kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's recommendations. Real-time PCR was performed on the 17 selected candidates from both hybridizations of our custom-made cDNA array and the 60-mer oligonucleotide OneArray chip in Light-Cycler capillaries (Roche Applied Science, Mannheim, Germany) using the LightCycler FastStart DNA Master SYBR Green I (Roche) as detailed elsewhere [36].

Candidate selection for QPCR was based on their fold change and their redundancy within our libraries. For each candidate, a specific set of primers was designed using the NCBI's primer-blast software and the candidates' specific sequences (NCBI) (Table 1). Additionally, three housekeeping genes, ACTB (β -actin), GAPDH, and PPIA, were quantified and used in GeNorm normalization. The two most stable housekeeping genes (ACTB and PPIA; $P > 0.05$) had an M value=1.17 which is less than the 1.5 value recommended by the GeNorm software. These two housekeeping genes were therefore used in both groups as the suitable control genes for quantitative real-time PCR (QPCR) data normalization. The real-time PCR product specificity of each candidate was confirmed by sequencing

to validate the amplification of the appropriate product as well as by analysis of the Lightcycler melting curve (Roche). Each gene mRNA expression level was then divided by its normalization factor and log-transformed. A *t*-test to compare gene expression levels between both groups was then performed using the GraphPad Prism 5 software (GraphPad Software, San Diego, CA) at $\alpha=0.05$.

Intra-patient profound analysis

In order to discriminate between the positive samples (associated to successful pregnancy) and the negative ones within each patient (two embryos were transferred per patient), we used the principal component analysis (PCA)

Table 1 Sequences of specific primers of candidates used in real time PCR quantification

Gene Name	Primer set (5'-3')	Genbank	Unigene	Annealing temperature	Fluorescence acquisition temperature
CALU	Up 5'- ACAAGGATGGAGACCTCATTGCC -3' low 5'- TGCTCTCGCTCTGTCTTTACCC -3'	AF013759	Hs.7753	62	80
DPP8	Up 5'- GCTGCCTGCTCCAAGTGATTTCAA -3' low 5'- GCAAGAATGTGAGTAGCCACGGT -3'	NM_197960.2	Hs.591106	61	83
HIST1H4C	Up 5'- CCATCGTAAGGTGCTCCG -3' low 5'- TTGGCGTGCTCCGTATAGGT -3'	NM_003542	Hs.46423	60	81
PKN2	Up 5'- ACAAGCCTGATACTCCTCAGTCAG -3' low 5'- GCAACCCAAGAACTACACAAGCAG -3'	NM_006256.2	Hs.440833	61	81
PSMD6	Up 5'- TTCCAGCAGTTCGGCAGTATCTGT -3' low 5'- TTCCACACCAACACCAAACGCTTC -3'	BC000630	Hs.152536	61	81
RPL9	Up 5'- TGAAGGGACGCACAGTTATCGTGA -3' low 5'- AAGCAACACCTGGTCTCATCCGAA -3'	BC066318	Hs.719072	60	83
SYT11	Up 5'- ACCCTGTGTTTGACGAGACCTTCA -3' low 5'- CATCTTCGGCAAGTGTCTGGCTTT -3'	BC039205	Hs.32984	63	87
THOC2	Up 5'- GGTAATCTTTCAGGAAGGTGGAGA -3' low 5'- GCTGATGTCATCCCAGACTTTG -3'	NM_001081550.1	Hs.592243	60	81
TOM1	Up 5'- CAATCTCAACAATGTGTTCTGCG -3' low 5'- TACCTCTTTCCGTTGGTCAGCC -3'	NM_001135732	Hs.474705	60	77
TUG1	Up 5'- CTTTCAGATCAGCAGGACAGTTGG -3' low 5'- GGGAGTTGTTACAAGATGGAACGG -3'	NR_002323.1	Hs.554829	62	80
AR	Up 5'- AACCCTATTTCCCCACCCAG -3' low 5'- GCTCTCTAAACTTCCCGTGGCA -3'	NM_000044.2	Hs.496240	59	83
CALM1	Up 5'- TACTTCGTGTGCTCCGACCCAT -3' low 5'- AGTCCACAGCCACAGCCTACTC -3'	BC007965	Hs.282410	60	84
NRP1	Up 5'- ACCTGAAACCCAGTGCCAGAA -3' low 5'- TGTTGTTGCGGTTGTCAGCAGT -3'	NM_003873.5	Hs.131704	60	84
CHD9	Up 5'- ACCAGCCTCGTCAATTTCCCAA -3' low 5'- CATCTCCTGCAAGTCTCGTTCCA -3'	NM_025134.4	Hs.622347	59	80
SPHKAP	Up 5'- GCAGCGATTTGCCTTGACAAC -3' low 5'- TTAAAGCTCAGGGTGCTCGTCC -3'	NM_001142644.1	Hs.436306	58	85
CHGB	Up 5'- CAACTGGACCAGCTCCTTCAC -3' low 5'- GCACAGTCATTGTCATAAGCATGT -3'	NM_001819.2	Hs.516874	58	85
UBQLN1	Up 5'- CAGTGATACATTGGCTGACTCTGG -3' low 5'- GCCTCCACCGTAACCTTTGTACTT -3'	BC017289	Hs.9589	62	82
PPIA	Up 5'- TGCTGGACCCAACACAAATGGTTC -3' low 5'- TGGTGATCTTCTTGCTGGTCTTGC -3'	NM_021130.3	Hs.356331	60	84
ACTB	Up 5'- CGTGACATTAAGGAGAAGCTGTGC -3' low 5'- CTCAGGAGGAGCAATGATCTTGAT -3'	NM_001101	Hs.520640	59	89

on normalized gene expression levels of the 7 significant genes revealed by QPCR. PCA is a powerful method that allows the accurate projection of a high-dimensional data set onto a new space with reduced dimensions for easy visualization and interpretation. Mathematically, the new space axes, called principal components (PC), are determined successively by order of importance according to the amount of variance they represent in the original data. Each PC is therefore a linear combination of the levels of expression of the measured genes. Based on the maximal Euclidean distance between the positive and negative samples, PCA was used to determine the true positive sample associated with successful pregnancy among the two transferred embryos as described elsewhere [39].

Using PCA, the true positive sample (associated with successful pregnancy) in each patient was determined. Binary logistic regression was thereafter used to establish a preliminary predictive model that links the pregnancy probability (successful pregnancy=1 vs. failure pregnancy=0) to the expression level of each biomarker gene measured in CCs. This preliminary model allows the determination of the relative importance of our biomarker genes in the pregnancy success probability at $\alpha=0.05$

Results

Microarray data analysis

CCs biomarker candidates were selected using two different platforms. The first was a custom-made microarray platform obtained by suppressive subtractive hybridizations of cDNA sequences, whereas the second was the OneArray commercial arrays. Following microarray experiment analysis, candidate gene selection was achieved based on the microarray results from the dye-swap of both our custom-made cDNA array and the OneArray slides. By comparing the positive clone lists from the two different groups, two main categories of candidates were selected based on their fold change (fold >2, FDR=5%). The first category corresponds to the competence markers and includes **260** candidate genes (69 from our library and 191 from OneArray) that were differentially expressed in the CCs of pregnant patients compared to the non-pregnant group. Conversely, the second group contains **231** potential incompetence markers (29 in our library and 202 from OneArray) that were underexpressed in the CCs of pregnant patients compared to the non-pregnant. These candidates are potential negative indicators of oocyte quality.

The selected differentially expressed candidates revealed by our analysis were then ordered according to their redundancy in different libraries, their signal intensities (Fig. 2) and their recurrence inside the same library. Given

Fig. 2 Schematic representation of the differentially expressed genes in CCs of ZGP oocytes compared to the ZGNP group according to both their fold change and the microarray platform

the importance of the cross-dialog between CCs and oocyte to fulfil maturation, it was expected that these candidates would likely be potential indicators associated with the molecular process of oocyte competence.

Real-time PCR analysis and biomarkers determination

In order to validate both our positive and negative genes lists, 17 candidates (14 overexpressed genes and 3 underexpressed genes) were chosen for subsequent validation by quantitative real-time PCR. The QPCR validation was achieved on the CCs tissues of the two ZGP and ZGNP groups (positive and negative groups). Seven (7) candidates among the 17 assessed were statistically significant following QPCR analysis, which represents a proportion of more than 41%. In fact, among the 14 positive candidates selected, 6 positive markers of oocyte quality and successful pregnancy were statistically significant between pregnant and non-pregnant patient groups. These candidates are DPP8 ($p=0.0441$), HIST1H4C ($p=0.0482$), UBQLN1 ($p=0.0236$), CALM1 ($p=0.05$), NRP1 ($p=0.0107$) and PSMD6 ($p=0.0412$) (Fig. 3a).

As for the 3 underexpressed markers assessed, only TOM1 ($p=0.0126$) was confirmed as a negative marker differentially expressed in the CCs of the non-pregnant patient group. TOM1 is therefore an interesting incompetence marker that was highly significant following the QPCR validation (Fig. 3b).

Fig. 3 Real-time PCR analysis of differentially expressed genes in individual CCs of ZGP group versus ZGNP. **a**, positive gene markers associated with pregnancy. **b**, negative markers associated with

pregnancy failure. Candidates were ranked according to their *p*-values, which were determined following a *T*-test analysis achieved on normalized data at $\alpha=0.05$

Intra-patient analysis and positive embryo determination

This profound statistical analysis was achieved only on CCs from patients who got a single embryo after transferring two HZB fertilized oocytes. The objective was to distinguish the embryo that led to successful pregnancy among the two HZB transferred embryo. Following PCA analysis and eigenvalues determination, five uncorrelated PCs were reported. The first principal component F1 accounted for 78.15% of the data total variability, while the F2 accounted for 17.95%. The representation of our six CCs zona good

(ZG) samples (two samples for each patient) in the new space including just F1 and F2 represented 96.10% of the total variability (Fig. 4). This representation allowed us to discriminate the true positive embryo associated with successful pregnancy from the two embryos transferred.

Pregnancy prediction model

The logistic regression was used to explore mathematical relationships between diverse input variables and an output explained variable that takes values between 0 and 1. If the

Fig. 4 Bi-dimensional PCA representation of the six CCs samples to distinguish the positive embryo that led to successful pregnancy among the two transferred for each patient. For each patient and using the Euclidian distance and the origin (negative control), the nearer embryo is the false positive (FP, associated with pregnancy failure), while the farther is the true positive (TP, associated with successful pregnancy). If the distances have opposite signs, the embryo with positive Euclidian distance is automatically the true positive. (F1, F2), the two main PCA axes. (ZG), cumulus cells of oocytes with high zona score; (TP), true positive; (FP), false positive; (1, 2, 3), patient number

out variable has just two potential outcome (“successful pregnancy” or “failure”, *i.e.*, 1 or 0), the appropriate model is the binary logistic regression (BLR). We used BLR to predict the probability of occurrence of pregnancy using the normalized expression levels of the significant genomic biomarkers and their pregnancy outcome according to the PCA analysis described before. Given the high correlation between most of our gene candidates ($r^2 > 0.95$ between 4 genes), only two biomarker genes (HIST1H4C and NRP1) were maintained in the predictive model whose determination coefficient ($R^2 = 0.75$) was very satisfactory. The two genes’ coefficients (slope) as well as the constant term were very significant ($P < 0.0001$) following the χ^2 test at the 95% confidence level (Fig. 5). This simplified model was established using the minimum number of gene markers that were significant. It is to note that this model required further validation step (with a large group of patients receiving SET) in order to be useful in clinical applications.

Gene network analysis

Genomic analysis of network mapping and molecular pathways of the differentially expressed genes using the Ingenuity Pathways Analysis (IPA) software was performed to investigate potential signalling pathways and biological processes driven by our gene predictors of pregnancy. Two networks were selected for deep analysis and exploration of gene

candidates’ interactions. The first network included in particular the candidates validated by QPCR (Fig. 6). Most of the 17 modulated candidates were involved in diverse molecular and cellular functions including intercellular signalling and cell cycle (AR, UBQLN1, PKN2), cell assembly and interactions (NRP1, TOM1, PKN2), molecular and vesicle trafficking (CHGB, TOM1, SYT11), protein folding and/or apoptosis (CALU, PSMD6, DPP8), calcium-dependent pathways (CALM1, CALU), gene expression and cell differentiation (HIST1H4C, RPL9, THOC2).

The second pathway shows a custom-made network that reflects the complexity of the signal transduction pathways in CCs (P38 MAPK, PI3K and PLC pathways). At this physiological period just prior to ovulation, CCs have reached full expansion marked by cell distension and selective gap-junction down-regulation (AR, NRP1, CAV1, PTX3, and GJA1). In order to prepare ovulation as an inflammatory-like process, many candidates were reported herein to be regulated including caspases, and chaperone proteins (HSPD1). Most of these pathways occurred downstream the preovulatory gonadotropins stimulations (LH/hCG) (Fig. 7).

Discussion

Despite several morphological criteria used, prediction of oocyte developmental competence is still challenging. Finding quantitative biomarkers that could accurately predict oocyte quality and subsequent pregnancy would be a valuable tool to improve the clinical outcomes of IVF and ICSI procedures. CCs are the site of expression of gene sets that are correlated to the oocyte maturation status and could serve to predict the pregnancy outcome [40, 41]. Moreover, CCs maintain a close relationship with the oocyte during ovulation, fertilization and even until early embryo development [42–44]. In addition to the effect of gonadotropins on CCs, it was also established that their functions are under the control of oocyte-secreted factors [45–47]. These properties offer CCs key roles in the competence acquisition process and could therefore be the site of expression of valuable markers that reflect oocyte competence. The main goal of this work was the identification of genomic markers expressed in CCs and associated with high developmental potential oocytes. CCs were harvested just prior to ICSI to ensure that the CCs at this stage are the most reflective of oocyte quality, and therefore express suitable markers to investigate.

To find valuable biomarkers that provide added value to the selection criteria already used, all the selected COCs

Fig. 5 Equation of the pregnancy prediction model. (P), probability of successful pregnancy

$$Pregnancy = \begin{cases} 1 & \text{(i.e. pregnant) with a probability } P = \frac{1}{1 + \exp(-(12347,135 + 46417186,605 * HIST1H4C - 3397039537,268 * NRP1))} \\ 0 & \text{(i.e. pregnancy failure) with a probability } (1 - P) \end{cases}$$

Fig. 6 Ingenuity pathways network generated from the QPCR validated candidates in human CCs prior to ICSI. Overexpressed genes (red) are RPL9 (ribosomal protein L9), UBQLN1 (ubiquilin 1), CALM1 (calmodulin 1), AR (androgen receptor), NRP1 (neuropilin 1), PKN2 (protein kinase N2), SYT11 (synaptotagmin XI), HIST1H4C (histone cluster 1, H4c), CALU (calumenin) and PSMD6 (proteasome (prosome, macropain) 26 S subunit, non-ATPase, 6). Underexpressed genes (green) are TOM1 (target of myb1 (chicken))

and CHGB (chromogranin B). Other genes: TMF1 (TATA element modulatory factor 1), CDC2L1 and CDC2L2 (cell division cycle 2-like 1 and 2), PXN (paxillin), CKAP4 (cytoskeleton-associated protein 4), HSPA13 (heat shock protein 70 kDa family, member 13), HERC3 (hect domain and RLD 3), Akt (protein kinase B), Hat (histone acetylase or acetyltransferase), PDK1 (pyruvate dehydrogenase kinase, isozyme 1), UBE3C (ubiquitin protein ligase E3C), and SELENBP1 (selenium binding protein 1)

had good morphological properties as well as a high ZP score. Although morphological criteria are widely used in IVF clinics, several studies have supported a significant improvement of pregnancy outcome in oocytes with high and uniform ZP birefringence [29, 30, 32, 48]. Using similar and good morphological and ZP criteria, the individual CCs were classified based on the pregnancy outcome of their oocytes. Two groups of pooled CCs samples were analysed: ZGP (8 CCs of the pregnant group) and ZGNP (6 CCs of the non-pregnant group).

Hybridizations were performed using two different microarray platforms. The first was a custom-made microarray platform obtained by suppressive subtractive hybridizations of cDNA sequences (200 to 1000 bp) differentially expressed in follicular cells (cumulus and granulosa cells) of oocytes that successfully achieved embryo development [34]. The second microarray platform was the OneArray commercial arrays. While the whole genome OneArray microarray was expected to yield more gene candidates, our custom-made library should reveal more specific candidates (Fig. 2); and thus the two platforms are thought to be complementary. In fact, the

OneArray candidates represented respectively 73.4% (191/260) and 87.4% of the overexpressed and the underexpressed candidates with more than 2-fold change that were kept in our microarray data analysis. We believe that these *in vivo* candidates expressed just prior to ICSI could reflect the normal physiological and genomic contexts needed for good oocyte production and successful pregnancy. The proximity of the oocyte would confer CCs a high potential to notify its developmental potential both in ICSI programs or IVF cycles [27, 49]. Due to the complexity of the signalling pathways in CCs, the study of both over- and underexpressed genes seems to be important in order to understand the molecular events in this cell compartment and identify potential pathways that might yield insights about the oocyte competence acquisition process.

The global analysis of both up- and down-regulated candidates in CCs of ZGP versus ZGNP by the IPA software revealed the main molecular and cellular functions triggered by each gene expression pattern (Table 2). Just prior to ovulation, CCs are in full expansion which may explain that around 18% of the overexpressed genes are

Fig. 7 A custom-made IPA network made of selected list of microarray differentially expressed genes of human CCs between the ZGP and ZGNP groups. Overexpressed genes (*red*) are CALM1 (calmodulin 1), AR (androgen receptor), NRP1 (neuroligin 1), PKN2 (protein kinase N2), CALU (calumenin), and PLCB2 (phospholipase C, beta 2). Underexpressed genes (*green*) are TOM1 (target of myb1 (chicken)), GJA1 (gap junction protein, alpha 1, 43 kDa), PTX3 (pentraxin-related gene, rapidly induced by IL-1 beta), PGRMC1 (progesterone receptor membrane component 1), IL8 (interleukin 8), CASP9 (caspase 9, apoptosis-related cysteine peptidase), CAV1

(caveolin 1, caveolae protein, 22 kDa) and HSPD1 (heat shock 60 kDa protein 1 (chaperonin)). Other genes: hCG (human chorionic gonadotropin), FSH (follicle stimulating hormone), LH (luteinizing hormone), Akt (protein kinase B), AP1(JUN, enhancer-binding protein AP1), PDGF family (platelet-derived growth factor), VEGF family (vascular endothelial growth factor), PKC (protein kinase C), ERK1/2 and p38 MAPK (mitogen-activated protein kinase family), PI3K family (phosphoinositide-3-kinase), and HSP70 & 90 (heat shock protein 70 and 90 kDa family)

involved in cellular assembly and organization. At this stage, CCs were reported to be less homogenous and therefore of various shapes [44, 50] which may explain the down-regulation of cell morphology genes.

The underexpression of main functions such as cell development and protein folding and/or post-translational modifications in CCs (Table 2) is consistent with an apoptotic phenotype shown by 40% of the overexpression

Table 2 The main molecular and cellular functions governed by human CCs harvested before ICSI. These functions are ranked by *p*-value according to the IPA software analysis

Underexpressed genes			Overexpressed genes		
Function	<i>p</i> -value	% of genes	Function	<i>p</i> -value	% of genes
Cell morphology	1,24E-04–3,45E-02	23,6	Cell death	3,13E-04–3,21E-02	40,2
Cellular development	1,32E-04–3,45E-02	11,3	Cellular growth & proliferation	3,46E-04–3,21E-02	21,4
Cell death	1,85E-04–3,45E-02	28,1	Cellular assembly & organization	6,88E-04–3,21E-02	17,9
Post-translational modification	2,73E-04–2,61E-02	30,3	Gene expression	6,88E-04–3,21E-02	12
Protein folding	2,73E-04–2,73E-04	6,7	Cell cycle	1,14E-03–3,21E-02	8,5

genes that are related to the cell death pathways and confirmed by previous studies [51]. It is expected that CCs show signs of apoptosis at the final maturation stage. Some authors have even suggested to use these apoptotic genes during folliculogenesis as potential markers of oocyte development potential [52]. Surprisingly and in parallel to these apoptotic events, the expression of some cell death genes in CCs was markedly reduced. Furthermore, many genes associated with cell cycle, cell growth and proliferation, as well as gene expression were activated (Table 2). These gene patterns show a proliferative phenotype that may reduce or delay the impact of the apoptotic events discussed earlier. This CCs genomic behaviour supports a fine gene regulation within CCs to ensure their functions (reviewed by [8]) and highlight their roles as an interface between the oocyte and its environment. It was also established in most mammals that in addition to environmental stimulations, the CCs are governed by the oocyte at many molecular levels including steroidogenesis, gene expression, apoptosis prevention, metabolic activity and inflammatory-like response production [43, 45, 46, 53–55]. The presence of several genes associated with most of these reported molecular functions in our overexpressed gene list is further confirmation of our findings. CCs are therefore a suitable target to look for efficient biomarkers associated with oocyte developmental potential [56]. Among the IPA provided pathways, two networks will be discussed later.

Based on microarray data, 17 gene candidates (14 overexpressed and 3 underexpressed) were chosen for further validation by real-time PCR. This validation was achieved on the 14 individual CCs samples (8CCs for ZGP versus 6 for ZGNP). Following normalization and statistical analysis, 6 competence biomarkers among the 14 overexpressed genes ($\approx 43\%$) analyzed were found to be statistically significant ($P \leq 0.05$). On the other hand, one of the three incompetence markers assessed was highly significant (TOM1 ($p=0.0126$)). It should be noted that the number of significant markers could have been higher without the technical limitation due to the reduced number of available patients and therefore of the CCs samples used during the study.

To our knowledge, the idea of looking for incompetence markers using high-throughput tools such as microarray is still poorly explored. We believe that CCs are the site of a two-way dialog between the oocyte and the follicular environment, and could therefore respond by the expression of several useful markers [27, 49, 57]. Biomarkers of oocyte incompetence could therefore constitute additional and important indicators of oocyte quality. Taken together, our preliminary positive and negative markers, once validated on a large number of patients, could be efficient tools to discriminate the competent oocytes to select for IVF / ICSI procedures among a set of high-grade

morphological oocytes. The confirmed biomarkers are useful tools that strengthen the morphological selection procedures already used.

In order to increase the chances of pregnancy in our study, two embryos with high morphological grade and ZP birefringence were transferred to each one of the 8 patients. Among them, three women were pregnant with singleton. PCA analysis was the suitable tool to overcome the challenge of determining which embryo from the two transferred induced the pregnancy. This mathematical tool was previously used in similar context to successfully identify the embryo that led to pregnancy among the two transferred [39]. Gene expression variation of normalized real-time PCR data of the three patients was used to identify the true positive versus the false positive embryo. The 2D representation of the six CCs samples allowed the identification of the positive embryo among the two embryos transferred for each patient (Fig. 4). This intra-patient analysis was very helpful to establish a preliminary mathematical model that predicts the oocyte's pregnancy probability based on the significant biomarkers reported herein. While previous models were based on morphological and subjective criteria [58, 59], our preliminary pregnancy model might be more efficient since it relies on quantitative and significant biomarkers associated with successful pregnancy. These markers were measured under similar and satisfactory morphological criteria, and are therefore more discriminative. Keeping in mind the dichotomous criteria of our explained variable (pregnancy=0 or 1), binary logistic regression was the appropriate model to predict the pregnancy success probability. The simplified model described before (Fig. 5) was established following many iterative steps to keep the lowest number of gene markers that accurately predict the pregnancy outcome. Odds ratio (OR) analysis was also achieved to make this preliminary model more useful to predict the pregnancy chances of an oocyte once the levels of expression of HIST1H4C and NRP1 of its CCs are known. As it is statistically recommended, the relative OR analysis of each gene in the model was measured by fixing the other candidate to its average normalized value from our data set. An increase of one log-expression unit of HIST1H4C or NRP1 provides an increase in pregnancy chances of 59% or 39% respectively. Given the experimental limitations in our study due to the reduced number of samples, this preliminary model needs an additional validation step with a large number of samples and patients. Once this validation is achieved, it will be a valuable and quantitative selection tool that could be added to the morphological criteria already in use in clinics. A large-scale study including at least 100 patients with SET will be undertaken to validate the gene biomarkers list and to strengthen the pregnancy prediction model suggested herein. Additional genomic predictors could be added to the present list. The cognate protein levels

of the confirmed candidates should be thereafter checked as protein measurements may be seen as an easier way to assess candidate levels in a clinical setting. This validation will offer in the few next years a valuable set of efficient biomarkers that may be helpful to increase both the pregnancy outcome and the SET efficiency.

The use of genomic approaches to study gene expression patterns in CCs should contribute to shed light on the molecular pathways leading to oocyte competence. This is why two IPA networks were selected for analysis. The first network (Fig. 6) included most of the 17 candidates selected for real-time PCR validation. AR is one of the main genes involved in this pathway. It is a steroid hormone receptor that, once activated, acts as an intracellular transducer signal and/or a transcription factor that interacts with transcription regulators such as TMF1. AR is also involved in CCs steroidogenesis which is under both the oocyte and gonadotropins control [45]. AR also interacts with the Ca^{2+} pathway via CALU, a calcium-binding protein localized in the endoplasmic reticulum, and CALM1. The calcium pathway in CCs was recently reported to interact with progesterone, PKA and PI3K to induce a chemical attraction of the spermatozoa to the oocyte surface during human fertilization [60]. The presence of PI3K, Ca, PLCB2 and p38 MAPK in both the first and especially the second network 1 downstream of the gonadotropins (LH/hCG) [61] (Fig. 7) supports these findings.

CCs also exhibited a differentiation status with a fine regulation of some genes associated with the catabolic process, including the ubiquitination machinery (UBQLN1), caspases (CASP9) and the proteasome activity (TOM1). In fact, selective proteolysis was shown to be required for ovulation [62, 63]. Moreover, this proteolytic activity was shown to involve a specific ubiquitin-proteasome pathway during porcine oocyte maturation, CCs expansion and fertilization [36, 64]. This selective proteolysis appears to occur simultaneously with an increase in gene expression and translational activities in CCs (RPL9, HIST1H4C) at the preovulatory stage.

We reported also in Fig. 6 the expression of some neuronal-like factors (NRP1, CHGB, and SYT11) involved in vesicle guidance and transport following E2 induction. SYT11 is a Calcium sensor that triggered the membranes' fusion in the intracellular vesicle trafficking, cell exocytosis and the neuronal synaptic vesicle secretion in eukaryotes [65–67]. For CHGB, it is an ubiquitous component of the secretory vesicles in eukaryote cells. This protein was also shown to be co-released with the hormone or the neurotransmitter following the appropriate stimuli. NRP 1, which is the most studied candidate, is a tyrosine kinase coreceptor that can bind to the membrane and is involved in angiogenic and neuronal traffic through the cytoskeleton [68]. Its expression was also reported in granulosa cells of

rat and bovine species [69, 70]. Keeping in mind the fact that synaptic vesicles transport was also achieved via microfilament and microtubule networks [71] and that similar cytoskeleton structures were reported in the transzonal cytoplasmic projections between CCs and the oocyte [72–75], one can assume the involvement of neuronal genes and molecules/vesicles exchange in CCs-oocyte dialogue.

PTX3 is another gene candidate that is involved in CCs expansion and extracellular matrix formation. Although it was previously suggested as a positive oocyte competence marker [76], our microarray data shows its underexpression in the CCs of oocytes that led to pregnancy (Fig. 7). Other similar reports were unable to find positive correlation between this candidate gene and the oocyte competency [40, 77]. Experimental conditions related to ovarian stimulation, sample collection and processing could be factors that may explain this discrepancy.

GJA1 (Cx43) is one of the proteins involved in cumulus-oocyte gap junctions (GJC) which play a pivotal role in this intercellular communication [78]. Its expression is regulated by gonadotropins (LH/hCG) through PKC and PI3K (Fig. 7) [79]. These connexin-43-rich GJC were ruptured or obstructed after 6 h of culture in the bovine (GVBD), and substituted by GJB1 [80]. Consistent with our results which showed a significant decrease of its expression, the GJA1 underexpression was suggested as a good marker of oocyte quality and successful pregnancy [57]. Interestingly, the CCs transzonal projections (TZP) structures based on actin filaments were recently shown to be intact in mouse CCs *in vivo* [73]. Based on these findings, selective regulation of cumulus-oocyte exchanges looks to occur through membrane cytoplasmic extensions mainly the transzonal projections (TZP) until ovulation. These communications are prerequisite for oocyte developmental competence acquisition.

The Calcium pathway is also markedly overrepresented in our data including Ca^{2+} , CALU (calcium-binding protein in the ER), Calmodulin, PLCB2, CALM1, and PI3K (Fig. 7). This pathway is involved in several signalling pathways and secretory vesicle trafficking (reviewed in [81]). It is established that the LH/hCG stimulation prior to ovulation triggers a very quick rise of intracellular Calcium in CCs that moves to the oocyte within few minutes especially when the TZP are not disrupted [82, 83]. In addition to its established roles in oocyte maturation and meiosis resumption, the calcium pathways is essential to the spermatozoa capacitation, acrosome reaction and subsequent fertilization [84, 85]

TOM1 is a gene candidate with still poorly known functions. It was reported to bind to endosomes where it regulated the ubiquitinated protein sorting and the intracellular vesicles trafficking [86, 87]. Moreover, it inhibits some immune-like factors pathways including interleukins and the tumor necrosis factor-induced proteins. Other

transcription factors like AP1 were also downregulated by TOM1[88]. This transcription factor (AP1) is involved in the LH pathway of TNFAIP6 expression in the preovulatory bovine granulosa cells [89]. Taken together, TOM1 looks to prevent some molecular events associated to the ovulation process. As a negative biomarker of oocyte competence, its inhibition in the CCs at this stage prior to ICSI becomes therefore understandable.

We should highlight that the networks studied herein may be only the tip of the iceberg of the whole molecular pathway of oocyte developmental competence acquisition. The protein kinases' interactions (PI3K, AKT, PKA, MAPK, ERK, PKC, PKN2, PLCB2) are very common pathways and involve several cross-talks which emphasizes the complexity of the molecular events in CCs prior to ovulation. Unfortunately, the whole molecular network of these candidates is still poorly understood despite recognized studies conducted in rodents and mammals including mice, porcine, bovine and human species, ([36, 56, 73, 90] and reviewed by [91]). Further studies are required to improve our understanding of these pathways (disruption in animal models) and to facilitate oocyte developmental potential prediction.

Conclusion

We reported herein a list of preliminary *in vivo* markers that may reflect the normal physiological and genomic context needed for optimal oocyte maturation and successful pregnancy. The oocyte, through its proximity impact, confers to CCs a high capacity to notify its developmental potential both in ICSI programs and IVF cycles. These CCs genomic biomarkers once validated will represent a valuable tool in the clinical setting not only in the selection of good quality oocytes that lead to successful pregnancy and healthy embryo, but also to optimize the ovulation induction protocols reported to influence the oocyte quality [92]. Consequently, the elective SET outcome could be improved, and thus reducing the risks associated with multiple pregnancies [16, 17]. These biomarkers could also serve to optimize the culture media used in IVM protocols.

Our study has the advantage of taking into account most of the COC morphological criteria, the ZP birefringence, some of the early embryo morphology parameters related to successful fertilization at zygote stage (second polar body extrusion; presence of centrally located and equal size pronuclei (2PN)), and the CCs gene expression patterns associated to later pregnancy outcome. It therefore provides a preliminary list of 7 non-invasive biomarkers with high discriminative potential of the oocyte developmental competence that could increase the pregnancy outcome, reduce multiple pregnancy incidences and improve the efficiency of the elective SET procedure.

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