Human Cumulus Cells Molecular Signature in Relation to Oocyte Nuclear Maturity Stage

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Abstract

The bi-directional communication between the oocyte and the surrounding cumulus cells (CCs) is crucial for the acquisition of oocyte competence. We investigated the transcriptomic profile of human CCs isolated from mature and immature oocytes under stimulated cycle. We used human Genome U133 Plus 2.0 microarrays to perform an extensive analysis of the genes expressed in human CCs obtained from patients undergoing intra-cytoplasmic sperm injection. CC samples were isolated from oocyte at germinal vesicle, stage metaphase I and stage metaphase II. For microarray analysis, we used eight chips for each CC category. Significance analysis of microarray multiclass was used to analyze the microarray data. Validation was performed by RT-qPCR using an independent cohort of CC samples. We identified differentially over-expressed genes between the three CC categories. This study revealed a specific signature of gene expression in CCs issued from MII oocyte compared with germinal vesicle and metaphase I. The CC gene expression profile, which is specific of MII mature oocyte, can be useful as predictors of oocyte quality.

Introduction

The bidirectional exchanges between oocyte and contiguous CCs are important for oocyte competence acquisition, early embryonic development and CC expansion [1-3]. Oocyte maturation starts with the resumption of the first meiosis process, and is divided in nuclear and cytoplasmatic maturation. During oocyte nuclear maturation, there is progression from prophase I characterized by germinal vesicle breakdown (GVBD) to metaphase II (MII) of the second meiosis [2,4]. At the end of this process, the oocyte should be considered as mature and able to be fertilized. However, the main problem, which hinders IVF/ICSI success, is how to select oocytes competent for embryonic development and implantation. Gene expression profile of CCs has been suggested to predict embryo development and pregnancy outcome [5-12]. However, in the majority of these studies, they did not consider the possibility that CC gene expression profile might vary according to the stages of oocyte nuclear maturation and thus were focused mostly on a single specific phase of oocyte maturation, such as the MII stage [6]. In humans, it is not known whether MII oocytes are systematically surrounded by specific CC molecular signature. Hence, the objective of the present study was to investigate gene expression profiles of human CCs isolated from oocytes at the germinal vesicle (CCGV), metaphase I (CCMI) and metaphase II (CCMII) stage, under controlled ovarian stimulation (COS) cycle and to evaluate the % of MII mature oocyte surrounded by mature CCs. This study has been performed by microarray analysis in order to identify potential biomarkers related to oocyte nuclear maturity and/or oocyte quality.

Materials and Methods

Processing of cumulus cells

Normal responder patients (age<36) referred to our center for intra-cytoplasmic sperm injection (ICSI) were included in this study after written informed consent. This project was approved by the Institute Review Board. Patients were stimulated with a combination of GnRH agonist or antagonist protocols with recombinant FSH or with HP-hMG. COCs were recovered under ultrasound echo-guidance 36 h after human Chorionic Gonadotrophin (5 000 UI, hCG) administration. CCs were separated mechanically from the corresponding oocyte as previously described [8]. A total of 111 CC samples obtained from 40 patients were used in this study.

For microarray analyses, 24 individual CC samples obtained from 16 patients were issued from COC (i) at germinal vesicle stage, (ii) metaphase I stage, and (iii) metaphase II stage. The differential gene expression profile in the three CC groups was investigated. For reverse-transcription quantitative polymerase chain reaction (RT-qPCR), 24 CC samples (8 samples for each stage of nuclear maturation) obtained from 19 patients were used.

For evaluating the reliability of the specific MII CC molecular signature, we tested this molecular signature on 53 CC samples
Table 1. Candidate transcripts expressed in human cumulus cells.

| Probeset | Gene symbol | Gene title | DNA chip p-value | Identified in follicular cells | Species       | References |
|----------|-------------|------------|------------------|-------------------------------|---------------|------------|
| 202052_s_at | RAI14 | retinoic acid induced 14 | 0.00195 | No | Human | Current study |
| 235542_at | TET3 | tet oncogene family member 3 | 0.00024 | No | Human | Current study |
| 201091_s_at | CBX3 | chromobox homolog 3 (HP1 gamma homolog, Drosophila) | 0.00024 | No | Human | Current study |
| 213506_at | F2RL1 | coagulation factor II (thrombin) receptor-like 1 | 0.00024 | CCs | Bovine | [18] |
| 201109_s_at | THBS1 | thrombospondin 1 | 0.00024 | CCs | Human | [20] |
| 207198_s_at | LIMS1 | LIM and senescent cell antigen-like domains 1 | 0.00073 | No | Human | Current study |
| 201995_at | EXT1 | exostoses (multiple) 1 | 0.00073 | CCs | Human | [20] |
| 219496_at | ANKRD57 | ankyrin repeat domain 57 | 0.00073 | No | Human | Current study |
| 206631_at | PTGER2 | prostaglandin E receptor 2 (subtype EP2), 53 kDa | 0.00024 | CCs | Human | [20] |
| 212265_at | QKI | quaking homolog, KH domain RNA binding (mouse) | 0.00024 | No | Human | Current study |
| 220924_s_at | SLC38A2 | solute carrier family 38, member 2 | 0.00024 | No | Human | Current study |
| 213614_x_at | EEF1A1 | eukaryotic translation elongation factor 1 alpha 1 | 0.00024 | CCs | Bovine | [18] |
| 226850_at | SUMF1 | sulfatase modifying factor 1 | 0.00073 | No | Human | Current study |
| 222430_s_at | YTHDF2 | YTH domain family, member 2 | 0.00024 | No | Human | Current study |
| 223474_at | C14orf4 | chromosome 14 open reading frame 4 | 0.00024 | GCs | Mouse | [28] |
| 201566_x_at | ID2 | inhibitor of DNA binding 2, dominant negative helix-loop-helix protein | 0.00024 | GCs | Porcine | [29] |
| 203880_at | COX17 | cytochrome c oxidase assembly homolog (S. cerevisiae) | 0.02392 | No | Human | Current study |
| 226896_at | CHCHD1 | coiled-coil-helix-coiled-helix domain containing 1 | 0.00122 | No | Human | Current study |
| 230029_x_at | UBR3 | ubiquitin protein ligase E3 component n-recognin 3 (putative) | 0.00073 | No | Human | Current study |
| 220952_s_at | PLEKHA5 | pleckstrin homology domain containing, family A member 5 | 0.00024 | No | Human | Current study |
| 207064_s_at | AOC2 | amine oxidase, copper containing 2 (retina-specific) | 0.00024 | No | Human | Current study |
isolated from mature (MII) oocytes issues from patients underwent ICSI procedure for male infertility (n = 5).

Complementary RNA preparation and microarray hybridization

Total RNA from CC samples was extracted using the RNeasy Micro Kit (Qiagen). RNA was quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). RNA integrity and quality were evaluated with an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA). RNA samples were stored at −80°C until microarray analysis. The Affymetrix 3’ IVT express protocol (ref 901229) was used to prepare cRNA (one-cycle amplification) with a starting concentration of 100 ng of total RNA. First-strand DNA was synthesized using an oligo-dT primer that incorporates a T7 promoter sequence. cDNA was then amplified in vitro transcription (IVT) with T7 RNA polymerase. During RNA amplification (aRNA) a biotinylated nucleotide analog was incorporated to be used as a label for the message. After fragmentation, the labeled anti-sense aRNA was hybridized to HG-U133 Plus 2.0 arrays (Affymetrix™) as described previously [13].

**Data processing**

Scanned GeneChip images were processed using the Affymetrix GCOS 1.4 software. Microarray data were analyzed using the Affymetrix Expression Console™ software and normalization was performed with the MAS5.0 algorithm to obtain the signal intensity and the detection call (present, marginal, or absent) for each probe set. This algorithm determines whether a gene is expressed with a defined confidence level or not (“detection call”). This “call” can either be “present” (when the perfect match probes are significantly more hybridized to the mismatch probes, FDR<0.04), “marginal” (for FDR<0.04 and ≤0.06) or “absent” (FDR>0.06). FDR, false discovery rate. The data are accessible at the Gene Expression Omnibus (GEO) through the provisional accession series number GSE31681.

**Microarray data analysis**

To compare the gene expression profile of the 24 CC samples according to the oocyte maturation stage, we first filtered the samples based on the “detection call” (i.e., absent/present). Probe sets were used when they were present in at least 7 samples out of 24. A second filter that uses the variation coefficient (40%) between all the samples was also applied. To compare groups of CCs at different stages of oocyte nuclear maturation, a Significance Analysis of Microarrays-Multi-class (SAM-M) [14] was performed. This algorithm provides the score values and a false discovery rate (FDR) confidence percentage based on data permutation. SAM-M allowed the identification of genes whose expression varied significantly among the CC GV, CC MI and CC MII categories.

The SAM-M results were used to perform a supervised hierarchical clustering, based on the expression level of the probe sets (multiclass gene set), and the cluster was visualized using the Tree View software [15].

**Reverse-Transcription quantitative Polymerase Chain Reaction (RT-qPCR)**

We performed RT-qPCR to validate the expression of the candidate genes using the Superscript First Strand Synthesis System (Invitrogen) according to the manufacturer’s recommendation. An independent cohort of CC samples was used for the validation. Strand cDNA was generated starting from 300 ng of total RNA from each sample and used (dilution 1:10) to assess gene expression by qPCR in 384-wells plates on a Light Cycler 480 (Roche) as described in [16]. Details of the primers used are reported in Table S1. Normalization was performed using the Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) housekeeping gene.

**Embryo outcome in relation to MII CC gene expression profile**

The embryo outcome on day 5 or day 6 of fertilized oocytes has been performed in relation to their gene expression profile of CCs.

**Statistical analysis**

The data obtained by RT-qPCR was analyzed with the GraphPad Instat software (http://www.graphpad.com/instat/instat.htm) using the Kruskal-Wallis non-parametric test. The differences among groups were considered significant when the p-value was <0.05.

**Results**

Identification of sets of genes over-expressed in CCs according to each stage of oocyte nuclear maturity

Using SAM-M, we identified a total of 25 genes (multiclass gene set) with a FDR≤3.30 that significantly distinguished the three groups. These 25 genes were differentially over-expressed according to the stage of nuclear maturity of the associated oocyte. Totally, 10, 4 and 11 genes were specifically over-expressed in the CC GV, CC MI and CC MII categories respectively (Table 1). The number of genes that are specific for a given category of CCs indicates that there is a significant variation across

| Probeset | Gene symbol | Gene title | DNA chip p-value | Identified in follicular cells | Species | References |
|----------|-------------|------------|-----------------|------------------------------|---------|------------|
| 226720_at | PWWP2A | PWWP domain containing 2A | 0.00195 | No | Human | Current study |
| 210367_s_at | PTGES | prostaglandin E synthase | 0.00073 | GCs, TCs | Monkey | [32] |
| 228805_at | C5orf25 | chromosome 5 open reading frame 25 | 0.00024 | No | Human | Current study |
| 203825_at | BRD3 | bromodomain containing 3 | 0.00024 | CCs | Bovine | [18] |

This table shows the genes differentially expressed in human. These data indicates that for each stage of oocyte nuclear maturity, the molecular signature is different in CCs. GCs, granulosa cells; GLCs, granulosa luteal cells; TCs, thecal cells; CCs, cumulus cells.

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Table 1. Cont.
the three categories of CCs as demonstrated also by the supervised hierarchical clustering which shows a clear segregation of the CC samples based on this list of 25 genes (Fig. 1). For complete name of these 25 genes, see table 1.

The 25 genes were then screened by RT-qPCR using an independent cohort of CC samples to strongly validate the microarray results. Fifteen genes were statistically validated as being differentially expressed in the three categories (Fig. 2).

**Metaphase II mature oocytes present distinct expression patterns in their surrounding CCs and embryo outcome**

The gene expression profile of 53 CGs isolated from fertilized oocytes has been established. 50% of fertilized oocytes present a CC molecular signature corresponding to CCs at VG or MI stages. The blastulation rate on day 5 or day 6 was more higher in fertilized oocytes surrounded by mature CCs compared with fertilized oocytes surrounded by immature CC gene expression profile (62 vs. 17%, P<0.05 respectively).

**Discussion**

Cross-talk between CCs and oocyte plays a pivotal role during oocyte maturation. In this study, we identified several genes that are differentially expressed in CGs associated with an oocyte at the GV, MI, or MII stage. These molecular signatures showed that mature oocytes could be surrounded with CCs presenting distinct gene expression profiles.

**Gene expression profile of CCs according to oocyte nuclear maturation stages**

There are only few genes differentially expressed in human CCs according to oocyte nuclear maturation stages. This finding is not really surprising for several reasons. First, the current study focuses on a fine biologic question in the same type of cells. Secondly, several studies reported differences in CC gene expression profile according to patients and treatments characteristics [12,17], probably limiting the observed differences. On the other hand, this is the first study on a large cohort of human CCs comparing differential gene expression profile of individual CCs at each stage of oocyte nuclear maturation under COS and using a transcriptomic global approach. All reported papers in this topic were often restricted to one or two oocyte nuclear maturation stages, and targeted, for the majority, some known genes [18,19,20–32]. Indeed, CC genes previously described to be related to oocyte nuclear maturation [7], oocyte developmental potential [6,8–11] or embryo development [5] were expressed, but not differentially expressed between our three CC categories.

**Matute oocytes can be surrounded by distinct CC gene expression profiles**

Concomitantly with oocyte nuclear maturation, we observed that CCs undergo a molecular maturation process. Our findings demonstrate that mature MII oocyte can be surrounded by either CGs corresponding to CC_MG, CC_MI or CC_MII stage respectively. Although the notion of synchronized maturation during folliculogenesis between oocyte and CCs is well documented in mammalian models, it was not yet clearly demonstrated in humans [19]. In the present study, we observed in an independent CC cohort that less than 50% of mature oocytes were surrounded by CGs displaying CC_MII signature.

**Oocyte quality associated with mature CCs**

We observed a high blastulation rate issues from MII oocyte surrounded by CCs over-expressing CC_MII. Inversely, mature oocytes over-expressing CC_GV or CC_MI signature were related to poor blastocyst formation rate. To test the CC status (mature or immature) is thus of a major importance in case of IVF/ICSI failure. In practical value, the quantitative mRNA expression of our CC signatures must rapidly be performed by RT-qPCR (<4 hours) and can help to select the best oocyte quality. Another best way to rapidly (<1 hour) develop and produce assays with high specificity and sensitivity consists in the determination of the quantity of proteins encoded by said genes using a particularly relevant novel approach that combines the use analytical chromatography with a new highly selective mass spectrometry technique called MRM (MRM, Multiple Reaction Monitoring; Applied Biosystems, SCIEX QTRAP® 5500). This technology is compatible in terms of reproducibility and robustness with a clinical application.

In summary, this study highlights the distinct gene signature of individual CC samples isolated from oocytes at GV, MI and MII stages. Assessing the expression of such signatures is a necessary first step to qualify the CC status as competent or incompetent. CGs screening at the mature oocyte stage is likely to be an accurate tool for detecting competent CCs and may permit the identification of oocyte competence during IVF/ICSI cycles. In addition, these molecular signatures are relevant to elucidate the
embryo disorders and IVF failure independently to morphology aspects.

Supporting Information

Table S1 Sequences of the primers used for RT-qPCR quantification.

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Author Contributions

Conceived and designed the experiments: ZGO SA SH. Performed the experiments: ZGO. Analyzed the data: ZGO DH SA SH. Contributed reagents/materials/analysis tools: ZGO DH SA JDV HD IJK SH. Wrote the paper: ZGO DH SA SH. Final approval: SH.

References

1. Salustri A, Yanagishita M, Hascall VC (1989) Synthesis and accumulation of hyaluronic acid and proteoglycans in the mouse cumulus cell-oocyte complex during follicle-stimulating hormone-induced mucification. J Biol Chem 264: 13840–13847.

2. Cha KY, Chian RC (1998) Maturation in vitro of immature human oocytes for clinical use. Hum Reprod Update 4: 103–120.

3. Goubr P, Gourd AP, Qian C, Laverge H, Van der Elst J, et al. (1998) In-vitro maturation of human germinal vesicle stage oocytes: role of cumulus cells and epidermal growth factor in the culture medium. Hum Reprod 13: 1638–1644.

4. Marteil G, Richard-Parpaillon L, Kubiak JZ (2009) Role of oocyte quality in meiotic maturation and embryonic development. Reprod Biol 9: 203–224.

5. McKenzie LJ, Pangas SA, Carson SA, Kovanci E, Casneros P, et al. (2004) Human cumulus granulosa cell gene expression: a predictor of fertilization and embryo selection in women undergoing IVF. Hum Reprod 19: 2679–2674.

6. Zhang X, Jafari N, Barnes RB, Confino E, Mlad M, et al. (2005) Studies of gene expression in human cumulus cells indicate pentraxin 3 as a possible marker for oocyte quality. Fertil Steril 85: 1169–1179.

7. Feuerstein P, Cadorret Y, Dables-Traun R, Guerif F, Bidault R, et al. (2007) Gene expression in human cumulus cells: one approach to oocyte competence. Hum Reprod 22: 3069–3077.

8. Assou S, Haouzi D, Mahmoud K, Aouacheria A, Guillemyn Y, et al. (2008) A non-invasive test for assessing embryo potential by gene expression profiles of human cumulus cells: a proof of concept study. Mol Hum Reprod 14: 711–719.

9. Hanel M, Dufort J, Robert C, Gravel G, Leveille MC, et al. (2000) Identification of differentially expressed markers in human follicular cells associated with competent oocytes. Hum Reprod 3: 1118–1127.

10. Van Montfoort AP, Geraerts JP, Dumoulin JC, Stassen AP, Evers JL, et al. (2008) Differential gene expression in cumulus cells as a prognostic indicator of embryo viability: a microarray analysis. Mol Hum Reprod 14: 157–168.

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

12. Adriasenas T, Wathlet S, Segers I, Verheyen G, De Vos A, et al. (2010) Cumulus cell gene expression is associated with oocyte developmental quality and influenced by patient and treatment characteristics. Hum Reprod 25: 1259–1270.

13. Haouzi D, Assou S, Mahmoud K, Hedon B, De Vos J, et al. (2009) LH/bCGR gene expression in human cumulus cells is linked to the expression of the extracellular matrix modifying gene TFFAP1 and to serum estradiol levels on day of hCG administration. Hum Reprod 24: 2868–2870.

Figure 2. Quantitative RT-PCR confirmation of the microarray data. This figure shows the mRNA relative abundance of genes that were differentially expressed in CCs issued from oocytes at different stages of nuclear maturation. The signal intensity for each gene is shown on the y axis in arbitrary units determined by RT-qPCR analysis. *Indicates a significant difference of gene expression between CCs categories (*p<0.01, *p<0.05). Results were presented as the mean ± SEM.

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14. Tusher VG, Tibshirani R, Chu G (2001) Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci U S A 98: 5116–21.
15. Eisen MB, Spellman PT, Brown PO, Botstein D (1998) Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci U S A 95: 14863–14868.
16. Haouzi D, Dechaud H, Assou S, Monzo C, de Vos J, et al. (2011) Transcriptome analysis reveals dialogues between human trophoblast and endometrial cells during the implantation period. Hum Reprod 26: 3446–9.
17. Adriaenssens T, Mazoyer C, Segers I, Wathlet S, Smits J (2009) Differences in collagen expression in cumulus cells after exposure to highly purified menotropin or recombinant follicle-stimulating hormone in a mouse follicle culture model. Biol Reprod 80: 1017–23.
18. Regassa A, Rings F, Hoelker M, Cinar U, Tholen E, et al. (2011) Transcriptome dynamics and molecular cross-talk between bovine oocyte and its companion cumulus cells. BMC Genomics 12: 57.
19. Adriaenssens T, Segers I, Wathlet S, Smits J (2011) The cumulus cell gene expression profile of oocytes with different nuclear maturity and potential for blastocyst formation. J Assist Reprod Genet 28: 31–40.
20. Koks S, Velthut A, Sarapik A, Almæ S, Reinmaa E, et al. (2010) The differential transcriptome and ontology profiles of floating and cumulus granulosa cells in stimulated human antral follicles. Mol Hum Reprod 16: 229–240.
21. Higuchi T, Fujiwara H, Yamada S, Tatsumi K, Kataoka N, et al. (1999) Co-expression of integrin-associated protein (IAP/CD47) and its ligand thrombospondin-1 on human granulosa and large luteal cells. Mol Hum Reprod 5: 920–6.
22. Koenigberg S, Bentov Y, Chalifa-Caspi V, Potoshnik G, Ofr R, et al. (2009) Gene expression microarray profiles of cumulus cells in lean and overweight obese polycystic ovary syndrome patients. Mol Hum Reprod 15: 89–103.
23. Assou S, Anahory T, Pantesco V, Le Carrou T, Pellentor F, et al. (2006) The human cumulus-oocyte complex gene-expression profile. Hum Reprod 21: 1705–19.
24. Tamba S, Yodoi R, Morimoto K, Inazumi T, Suheno M, et al. (2010) Expression profiling of cumulus cells reveals functional changes during ovulation and central roles of prostaglandin EP2 receptor in cAMP signaling. Biochimie 92: 663–675.
25. Hizaki H, Segi E, Sugimoto Y, Hirose M, Saji T, et al. (1999) Abortive expansion of the cumulus and impaired fertility in mice lacking the prostaglandin E receptor subtype EP2. Proc Natl Acad Sci U S A 96: 10501–6.
26. Markosyan N, Dozier BL, Lattanzio FA, Duffy DM (2006) Primate granulosa cell response via prostaglandin E2 receptors increases late in the periovulatory interval. Biol Reprod 75: 769–786.
27. Segi E, Haraguchi K, Sugimoto Y, Tsuji M, Tsunekawa H, et al. (2003) Expression of messenger RNA for prostaglandin E receptor subtypes EP4/EP2 and cyclooxygenase isozymes in mouse periovulatory follicles and oviducts during superovulation. Biol Reprod 68: 804–11.
28. McRae RS, Johnston HM, Mihm O, O'Shaughnessy PJ (2005) Changes in mouse granulosa cell gene expression during early luteinization. Endocrinology 146: 309–17.
29. Verbraak EJ, van ’t Veld EM, Groot Koerkamp M, Roelen BA, van Haerdten T, et al. (2011) Identification of genes targeted by FSH and oocytes in porcine granulosa cells. Theriogenology 75: 362–76.
30. Johnson AL, Haugen MJ, Woods DC (2008) Role for inhibitor of differentiation/deoxyribonucleic acid-binding (Id) proteins in granulosa cell differentiation. Endocrinology 149: 3187–93.
31. Hogg K, Etherington SL, Young JM, McNally AS, Duncan WC (2010) Inhibitor of differentiation (Id) genes are expressed in the steroidogenic cells of the ovine ovary and are differentially regulated by members of the transforming growth factor-beta family. Endocrinology 151: 1247–56.
32. Duffy DM, Seachord CL, Dozier BL (2005) Microsomal prostaglandin E synthase-1 (mPGES-1) is the primary form of PGES expressed by the primate periovulatory follicle. Hum Reprod 20: 1485–92.