Cumulus Cells Gene Expression Profiling in Terms of Oocyte Maturity in Controlled Ovarian Hyperstimulation Using GnRH Agonist or GnRH Antagonist

Rok Devjak1, Klementina Fon Tacer2,3, Peter Juvan2, Irma Virant Klun1, Damjana Rozman2, Eda Vrtančnik Bokal1*

1 Division of Reproductive Medicine, Department of Obstetrics and Gynecology, University Medical Centre Ljubljana, Ljubljana, Slovenia, 2 Centre for Functional Genomics and Bio-Chips, Institute of Biochemistry, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia, 3 Institute for Hygiene and Pathology of Animal Nutrition, Veterinary Faculty, University of Ljubljana, Ljubljana, Slovenia

Abstract

In vitro fertilization (IVF) cycles controlled ovarian hyperstimulation (COH) is established by gonadotropins in combination with gonadotropin-releasing hormone (GnRH) agonists or antagonists, to prevent premature luteinizing hormone (LH) surge. The aim of our study was to improve the understanding of gene expression profile of cumulus cells (CC) in terms of ovarian stimulation protocol and oocyte maturity. We applied Affymetrix gene expression profiling in CC of oocytes at different maturation stages using either GnRH agonists or GnRH antagonists. Two analyses were performed: the first involved CC of immature metaphase I (MI) and mature metaphase II (MII) oocytes where 359 genes were differentially expressed, and the second involved the two GnRH analogues where no differentially expressed genes were observed at the entire transcriptome level. A further analysis of 359 differentially genes was performed, focusing on anti-Müllerian hormone receptor 2 (AMHR2), follicle stimulating hormone receptor (FSHR), vascular endothelial growth factor C (VEGFC) and serine protease inhibitor E2 (SERPINE2). Among other differentially expressed genes we observed a marked number of new genes connected to cell adhesion and neurotransmitters such as dopamine, glycine and γ-Aminobutyric acid (GABA). No differential expression in CC between the two GnRH analogues supports the findings of clinical studies where no significant difference in live birth rates between both GnRH analogues has been proven.

Citation: Devjak R, Fon Tacer K, Juvan P, Virant Klun I, Rozman D, et al. (2012) Cumulus Cells Gene Expression Profiling in Terms of Oocyte Maturity in Controlled Ovarian Hyperstimulation Using GnRH Agonist or GnRH Antagonist. PLoS ONE 7(10): e47106. doi:10.1371/journal.pone.0047106

Editor: Jason Glenn Knott, Michigan State University, United States of America

Received March 29, 2012; Accepted September 10, 2012; Published October 17, 2012

Copyright: © 2012 Devjak et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by Slovenian Research Agency (www.arrs.gov.si) grants P1-0104 and L3-4162. Rok Devjak is funded as young researcher by Slovenian Research Agency. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: eda.bokal@guest.arnes.si

Introduction

In vitro fertilization (IVF) has become one of the most common treatments of infertility. In an IVF cycle, ovarian stimulation is established by gonadotropins in combination with gonadotropin-releasing hormone (GnRH) analogues, i.e. GnRH agonists or GnRH antagonists. GnRH analogues are used to prevent premature luteinizing hormone (LH) surge during ovarian stimulation, which improves oocyte yield and increases pregnancy rate [1]. In the 1980s a long protocol of GnRH agonists was used starting in the midluteal phase of the preceding cycle [2]. In the 1990s, GnRH antagonists were introduced into clinical practice and proved to be safe and effective [3,4,5]. In contrast to GnRH agonists, GnRH antagonists cause immediate and rapid gonadotropin suppression without an initial period of gonadotropin hypersecretion. GnRH antagonists have several advantageous effects over GnRH agonists [6,7], of which the most important is having fewer follicles and lower estradiol level on the day of human chorionic gonadotropin (hCG) application [4] leading to a lower incidence of ovarian hyperstimulation syndrome (OHSS) [8], a serious complication of assisted reproductive therapy. Further, with a shorter period of application GnRH antagonists are friendlier to patients. Earlier studies have shown that GnRH antagonists result in lower pregnancy and delivery rates compared to GnRH agonists [6], whereas recent meta analyses show that the difference between them is not significant [9,10].

Despite great improvements in assisted reproductive technology the success of IVF still remains relatively low. Most of the oocytes retrieved after ovarian stimulation are capable of fertilization; however, only half of them develop into embryos and only a few implant. Therefore, more than one embryo is usually transferred to increase the pregnancy rate, which leads to multiple pregnancies, and increased fetal and maternal morbidity and mortality [11]. For the development of high quality embryos the maturity and quality of oocytes is fundamental. At present, oocyte competence is estimated only on the basis of morphological evaluation of the polar body, meiotic spindle, zona pellucida and cytoplasm. There is increasing evidence that morphological evaluation is not a reliable predictor of oocyte competence and embryo implantation potential. The development of functional genomics technologies has made more objective measures available such as gene expression in cumulus cells (CC) as a non-invasive prognostic indicator of oocyte fertilization competence [12,13].
Cumulus cells are essential for oocytes development. During folliculogenesis, an intense bidirectional communication exists between oocytes and surrounding CC [14], which is crucial for the development of mature and competent oocytes. Consequently, CC may reflect oocyte quality and can be used for oocyte selection. The oocyte itself also plays an active role by secreting paracrine factors that maintain the appropriate microenvironment for the acquisition of its developmental competence [15,16]. The oocyte-secreted paracrine factors influence gene expression and protein synthesis in granulosa cells (GC) and CC that in turn regulate oocyte developmental competence. Consequently, GC and CC can serve as indirect markers of oocyte quality. In IVF procedures, GC and CC are separated from oocytes and discarded, which is why they are easily accessible and also suitable for gene expression analysis of oocyte maturity [15].

Therefore, we used transcription profiling to perform two analyses: the first was focused on oocyte maturity and the second on the type of ovarian stimulation protocol used: recombinant gonadotropins in combination with either GnRH agonists or GnRH antagonists. The aim of this study was to improve the understanding of the CC gene expression profile in terms of ovarian stimulation protocol. To our knowledge this is the first assessment of both GnRH analogues at the molecular level in a prospective study.

Materials and Methods

Patients and IVF treatment

In this prospective, randomized study, 21 patients undergoing classical IVF cycle at the Department of Obstetrics and Gynecology, University Medical Center Ljubljana, were included. The study was approved by the national medical ethics committee and all patients have signed informed consent. Randomization was performed according to JL Fleiss [17]; the randomization list was prepared in advance. Each patient who agreed to participate in the study was assigned to either a GnRH agonist or a GnRH antagonist group, and had an equal chance to be assigned to either group. The allocation was carried out by revealing a therapy group by a third person to medical staff and the patient at the moment of entering the study. The inclusion criteria were as follows: age less than 35 years, body mass index (BMI) ranging from 18.5 to 30, female partner’s fertility factor infertility, and the partner’s spermiogram had to be normal according to WHO criteria.

Eleven randomly selected patients were administered GnRH agonist buserelin acetate (Suprefact; Hoechst AG, Frankfurt/Main, Germany) from day 22 at a daily dose of 0.6 ml (600 pg) subcutaneously. When the criteria for ovarian desensitization were fulfilled (oestradiol ≥143 pmol/L, follicles <3 mm in diameter), they were subcutaneously administered 225 IU of gonadotrophin follitropin β (Gonal F; Industria Farmaceutica Serono S.p.A, Bari, Italy). To the remaining 10 patients 225 IU of gonadotrophin follitropin β was subcutaneously administered on day 2. When the dominant follicle measured ≥14 mm in diameter, GnRH antagonist cetrorelix acetate (Cetrotide; Asta Medica AG, Frankfurt, Germany) in a dose of 0.25 mg was administered subcutaneously.

When at least three follicles were ≥17 mm and serum oestradiol was ≥0.40 nmol/L per follicle all patients were administered 10,000 IU of human chorionic gonadotrophin (hCG) (Pregnyl; N.V. Organon); 34–36 h later an ultrasound guided transvaginal oocyte retrieval was performed.

Cumulus cell collection and oocyte follow up

Oocytes were removed from the follicular fluid. Immediately after oocyte retrieval, CC of each oocyte were removed by a needle and a glass demudation pipette (Swemed, Sweden), washed in PBS, snap frozen in liquid nitrogen and stored at −80°C in vials until RNA isolation.

The oocytes were further inseminated (classical IVF) and cultured individually. After 24 hours, oocyte fertilization status was assessed. Fertilized oocytes expressed two pronuclei and two polar bodies, whereas unfertilized oocytes did not. All unfertilized cells were denuded to assess the maturation stage. Immature MI oocytes were round cells which did not extrude polar bodies and did not express germinal vesicles, whereas mature MI oocytes had a clearly visible and developed polar body. Only CC obtained from MI and unfertilized MI oocytes were considered for transcriptome analysis. Fertilized oocytes were further cultured to the blastocyst stage in the Universal IVF Medium followed by BlastAssist System (M1 and M2; Origio, Denmark) for five days. On day 5, at most two embryos at the blastocyst or morula stage were transferred into the uterus. Supernumerary blastocysts were cryopreserved. Only CC obtained from MI oocytes developed to blastocyst stage embryo were considered in transcriptome analysis.

Experimental design

The difference between the two IVF cycle stimulation protocols using GnRH analogues was studied at three different levels of oocyte quality: metaphase I oocytes (MI), unfertilized metaphase II (MII) oocytes (MII-NF) and MII oocytes developed to blastocyst stage embryo (MII-BL). We performed two analyses: firstly we assessed differences in CC gene expression according to the oocyte stage achieved (MI versus MII), and secondly we assessed the differences in CC gene expression according to the GnRH analogue used. By comparing the CC MI and MII expression profiles we sought for gene markers linked to oocyte maturity.

Figure 1 shows the number of patients (n = 21) and CC samples (n = 46) included in the study. Eleven patients were administered GnRH agonist and 10 GnRH antagonist. Altogether, 10 CC samples from MI oocytes, 15 from MII-NF oocytes and 21 from MII-BL oocytes were collected and considered in transcriptome analysis. Table 1 shows 21 patients randomized to either the GnRH agonist or GnRH antagonist group and the type and number of samples analyzed.

RNA preparation

RNA was extracted using TRI reagent (Sigma – Aldrich, St. Louis, USA) according to slightly modified manufacturer’s instruction. Due to small sample volume, glycogen was used as a carrier to increase RNA yield. Briefly, CC from individual cumulus – oocyte complexes were homogenized in 500 µL TRI reagent supplemented with 125 µg of glycogen (Ambion, Austin, USA). After 2 min incubation at room temperature, 100 µL chloroform was added and the sample was vortexed vigorously. RNA was precipitated with isopropanol from the aqueous phase and collected after 15 min centrifugation at 12,000 × g and 4°C. RNA pellet was washed 3 times by 75% ethanol, dried and dissolved in 15 µL of RNAse free water. The integrity of the RNA samples was assessed on Agilent 2100 Bioanalyzer to assure high quality of total RNA; the RIN number was more than 7 for each sample.

Transcriptome analysis

Transcriptome analysis was performed using the GeneChip Human Gene 1.0 ST Arrays (Affymetrix, Santa Clara, USA). The
arrays were hybridized according to manufacturer’s recommendations. Briefly, 200 ng of RNA was amplified and converted to cDNA using the WT Expression Kit (Ambion, Austin, USA). The resulting cDNA was fragmented and labeled using the GeneChip WT Terminal Labeling and Controls Kit (Affymetrix, Santa Clara, USA) and hybridized to the arrays for 16 hours. The arrays were washed using GeneChip Fluidics Station 450 according to manufacturer’s recommendations, and scanned on the Affymetrix GeneChip Scanner 3000 7G. The images were analyzed and quality of data checked by Affymetrix GeneChip Expression Console software.

Data were processed and analyzed using different R/Bioconductor packages [18]. Data were normalized using the RMA algorithm from the XPS package. The raw and normalized gene expression data together with experimental information were deposited to Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo) in compliance with MIAME standards, under series accession number GSE34230.

The two-way ANOVA model was used to assess the differential expression of genes using LIMMA package by controlling the false discovery rate (FDR) [19] at significance level $\alpha = 0.05$. Considered were 4 contrasts between different oocyte stages without considering the therapy (see Figure 2) and 3 contrasts between GnRH agonist and GnRH antagonist for each oocyte stage separately.

Annotation of genes and data representation was managed using ANNAFFY and AFFYCORETOOLS packages. Simultaneously, gene set enrichment analyses were performed using Parametric Gene Set Enrichment Analysis (PGSEA) package in combination with LIMMA package by controlling FDR at significance level $\alpha = 0.05$. Gene sets related to different KEGG pathways were tested for their enrichment with respect to the contrasts of interest and sorted by their enrichment scores. Gene Ontology analysis was performed using GeneCodis [20]; a top ranked gene network among differentially expressed genes was identified by Ingenuity Pathway Analysis software (Ingenuity Systems, www.ingenuity.com).

Quantitative real time PCR (qPCR)

Quantitative real time PCR (qPCR) was used to validate 4 differentially expressed genes on a subset (30 out of 46) of samples using TaqMan Gene Expression pre-designed assays (Applied Biosystems, Foster City, USA). Genes for qPCR validation were selected considering their significance and their biological function in folliculogenesis. Cumulus cells samples are small and do not provide enough RNA to perform microarray analysis and qPCR validation in all samples. In our study 30 of the 46 samples provided enough RNA to perform both analyses. Peptidylprolyl isomerase B (PPIB) and 18s rRNA were added for normalization. Genomic DNA contamination was eliminated by DNAse treatment using DNAse I (Roche, Basel, Switzerland). cDNA for qPCR assays was prepared from 200 ng DNAse treated RNA using SuperScript RT III (Invitrogen, Carlsbad, USA) in 20 μl final volume. Following cDNA synthesis, RNAse free water was added to increase the sample volume to 30 μl. The measurements were performed using LightCycler 480 System (Roche Applied Science, Basel, Switzerland).

Table 1. A list of patients included in GnRH agonist and GnRH antagonist group with a number CC samples regarding to oocyte stage.

| Patient | Therapy group | CC MI | CC MII-NF | CC MII-BL |
|---------|--------------|-------|-----------|-----------|
| 1       | GnRH agonist | 1     | 1         | 1         |
| 2       | GnRH agonist | 1     | 1         | 1         |
| 3       | GnRH agonist | 1     | 1         | 1         |
| 4       | GnRH agonist | 1     | 1         | 1         |
| 5       | GnRH agonist | 1     | 1         | 1         |
| 6       | GnRH agonist | 1     | 1         | 1         |
| 7       | GnRH agonist | 1     | 1         | 1         |
| 8       | GnRH agonist | 1     | 1         | 1         |
| 9       | GnRH agonist | 1     | 1         | 1         |
| 10      | GnRH agonist | 1     | 1         | 1         |
| 11      | GnRH agonist | 1     | 1         | 1         |
| 12      | GnRH agonist | 1     | 1         | 1         |
| 13      | GnRH antagonist| 1      | 1         | 1         |
| 14      | GnRH antagonist| 1      | 1         | 1         |
| 15      | GnRH antagonist| 1      | 1         | 1         |
| 16      | GnRH antagonist| 1      | 1         | 1         |
| 17      | GnRH antagonist| 1      | 1         | 1         |
| 18      | GnRH antagonist| 1      | 1         | 1         |
| 19      | GnRH antagonist| 1      | 1         | 1         |
| 20      | GnRH antagonist| 1      | 1         | 1         |
| 21      | GnRH antagonist| 1      | 1         | 1         |

CC MI: cumulus cells of metaphase I oocytes; CC MII-NF: cumulus cells of unfertilized metaphase II oocytes; CC MII-BL: cumulus cells of metaphase II oocytes developed to blastocyst stage embryo.

doi:10.1371/journal.pone.0047106.t001

Figure 1. Experimental design. Number of patients included in the study with respect to the two GnRH analogue treatments and the number of collected CC samples; CC MI: cumulus cells of metaphase I oocytes; CC MII-NF: cumulus cells of unfertilized metaphase II oocytes; CC MII-BL: cumulus cells of metaphase II oocytes developed to the blastocyst stage.

doi:10.1371/journal.pone.0047106.g001
Results

Comparison with microarray data.

Resulting values were log2 transformed (log2 fold change) for rRNA values and were expressed in arbitrary units [21]. The expression by normalization factor calculated from PPIB and 18s dividing the averaged, efficiency corrected values for mRNA Penzberg, Germany). Normalized mRNA levels were obtained by dividing the averaged, efficiency corrected values for mRNA expression by normalization factor calculated from PPIB and 18s rRNA values and were expressed in arbitrary units [21]. The resulting values were log2 transformed (log2 fold change) for comparison with microarray data.

Microarray data validation by qPCR

Four of the 359 differentially expressed genes were validated by qPCR. Figure 4 shows the expression (log2 fold change) of anti-Mullerian hormone receptor 2 (AMHR2), follicle stimulating hormone receptor (FSHR), vascular endothelial growth factor C (VEGFC) and serine protease inhibitor E2 (SERPINE2) as assessed by microarray analysis and qPCR. All genes matched the direction of expression changes using either of the measurement method. Correlation factor (r) between log2 fold change of both methods for all the 4 genes was 0.98 (p = 0.02).

Discussion

To our knowledge, this is the first prospective study comparing the effects of two different GnRH analogues and maturity stage of the oocyte at the level of gene expression in CC. Considering oocyte maturity we observed 359 differentially expressed genes between CC MI and CC MII. Using either GnRH agonist or GnRH antagonists, we have not observed differentially expressed genes. Moreover, we have not observed differentially expressed genes at any level of maturity stage of the oocyte (MI, MII-NF and MII-BL) between the two GnRH analogues used. Since only MII oocytes are capable of fertilization, our results support and supplement the clinical studies on GnRH analogues [9,22] by showing that no significant differences between CC of MII oocytes exist at the...
### Table 2. Patients baseline characteristics.

|                        | GnRH agonists       | GnRH antagonists     | p-value |
|------------------------|---------------------|----------------------|---------|
| Age (years)            | 30.7 ± 3.88         | 30.5 ± 3.03          | 0.88    |
| BMI (kg/m²)            | 23.1 ± 2.68         | 22.7 ± 2.80          | 0.72    |
| Oestradiol level on day of hCG (nmol/L) | 5.3 ± 2.3 | 4.6 ± 2.4 | 0.48    |
| Retained oocytes (n)   | 11.7 ± 5.24         | 8.0 ± 1.89           | 0.08    |
| Degenerated (ratio)    | 0.02 ± 0.05         | 0.04 ± 0.11          | 0.46    |
| MI (proportion)        | 0.07 ± 0.08         | 0.14 ± 0.15          | 0.19    |
| MII (proportion)       | 0.91 ± 0.08         | 0.82 ± 0.21          | 0.18    |
| Fertilized (proportion)| 0.65 ± 0.14         | 0.50 ± 0.19          | 0.06    |
| MI-NF (proportion)     | 0.68 ± 0.08         | 0.62 ± 0.22          | 0.36    |
| MI-BL (proportion)     | 0.23 ± 0.08         | 0.20 ± 0.07          | 0.31    |
| Endometrial lining thickness (mm) | 9.91 ± 1.58 | 9.8 ± 0.63 | 0.84    |
| Pregnancy rate per cycle (proportion) | 0.55 ± 0.16 | 0.60 ± 0.16 | 0.81    |
| Delivery rate per cycle (proportion) | 0.55 ± 0.16 | 0.40 ± 0.16 | 0.53    |
| Frozen embryos remained (number) | 0.70 ± 0.95 | 0.40 ± 0.70 | 0.43    |

Age, body mass index (BMI), serum oestradiol level on day of hCG, number of retrieved oocytes; proportions of degenerated oocytes, metaphase I oocytes (MI), metaphase II oocytes (MII), fertilized oocytes, unfertilized metaphase II oocytes (MII-NF) and metaphase II oocytes developed to the blastocyst stage (MII-BL).

Significance of differences between the two treatment groups was assessed using Student’s t-test; two-tailed p-values are shown.

doi:10.1371/journal.pone.0047106.t002

### Table 3. Top down and up regulated genes between CC MII and CC MI.

| Symbol | Description                                                                 | p-value | Fold Change |
|--------|-----------------------------------------------------------------------------|---------|-------------|
| SFRP4  | secreted frizzled-related protein 4                                         | <0.01   | −5.0        |
| ITGB3  | integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)                 | <0.01   | −3.3        |
| MGP    | matrix Gla protein                                                          | 0.01    | −3.1        |
| CRHBP  | corticotropin releasing hormone binding protein                             | 0.04    | −3.0        |
| BUB1   | budding uninhibited by benzimidazoles 1 homolog (yeast)                     | 0.01    | −2.7        |
| ANK2   | ankyrin 2, neuronal                                                         | <0.01   | −2.5        |
| TSPAN7 | tetraspanin 7                                                              | 0.02    | −2.4        |
| TNFSF4 | tumor necrosis factor (ligand) superfamily, member 4                        | <0.01   | −2.4        |
| PALLD  | palladin, cytoskeletal associated protein                                    | <0.01   | −2.2        |
| DSE    | dermatan sulfate epimerase                                                  | <0.01   | −2.2        |
| CCDC99 | coiled-coil domain containing 99                                            | <0.01   | −2.2        |
| GPR63  | G protein-coupled receptor 63                                               | <0.01   | −2.2        |
| GLRA2  | glycine receptor, alpha 2                                                   | <0.01   | −2.1        |
| BMP3   | bone morphogenetic protein 3                                                | 0.01    | −2.1        |
| CDH3   | cadherin 3, type 1, P-cadherin (placental)                                  | <0.01   | −2.0        |
| FRMD4B | FERM domain containing 4B                                                   | 0.01    | −2.0        |
| ID3    | inhibitor of DNA binding 3, dominant negative helix-loop-helix protein      | 0.01    | −2.0        |
| NDP    | Norrie disease (pseudoglioma)                                               | <0.01   | −2.0        |
| GABRA5 | gamma-aminobutyric acid (GABA) A receptor, alpha 5                         | 0.04    | −2.0        |
| MAOB   | monoamine oxidase B                                                        | 0.01    | −2.0        |
| HSD11B1| hydroxysteroid (11-beta) dehydrogenase 1                                   | 0.02    | 1.7         |
| PTGES  | prostaglandin E synthase                                                    | 0.04    | 1.9         |
| SPDOCK2| sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 2    | 0.02    | 2.0         |
| C10orf10| chromosome 10 open reading frame 10                                        | <0.01   | 2.3         |
| NIKAIN1| Na+/K+ transporting ATPase interacting 1                                    | <0.01   | 3.1         |

differentially expressed genes which expressed the highest down and up according to log2 fold change and p-value<0.05. CC MI: cumulus cells of metaphase I oocytes, CC MII: cumulus cells of metaphase II oocytes.

doi:10.1371/journal.pone.0047106.t003
transcriptome level. However, the GnRH antagonist protocol is more patient friendly because of shorter stimulation time, and is also considered safer because of a lower incidence of OHSS.

The analysis of baseline characteristics of the GnRH agonist and GnRH antagonist-treated patients showed that experimental groups were comparable, excluding sample-dependent biases in data. In line with previous findings [10], we have observed a higher number of retrieved oocytes and higher delivery rate in the GnRH agonist group although the differences were not significant. FDR corrected data analysis of CC transcriptome have shown that the two GnRH analogues do not differ significantly. A non detectable difference between GnRH agonists and GnRH antagonists at the CC transcriptional level is in accordance with clinical data considering pregnancy and delivery rates, showing slight and insignificant variances between the two GnRH analogues [9,10,22].

Figure 3. Subgroup of genes connected to follicle stimulating hormone (FSH) and luteinizing hormone (LH) from a top ranked gene network. It is associated with Post-Translational Modification, Cellular Development, Cellular Growth and Proliferation as identified by Ingenuity Pathways Analysis. Top ranked gene network was obtained from comparisons of differential expression between CC MII and CC MI. Genes are represented as nodes, and the biological relationship between two nodes is represented as edge (line): 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’. The green colour intensity of the nodes indicates the degree of down-regulation in the CC MII group. CC MI: cumulus cells of metaphase I oocytes. CC MII: cumulus cells of metaphase II oocytes.

doi:10.1371/journal.pone.0047106.g003

Figure 4. Expression (log2 fold change) of \textit{VEGFC, SERPINE2, AMHR2} and \textit{FSHR} from microarrays and qPCR showing contrast between CC MII and CC MI. Bars represent expression of CC MII vs. CC MI as log2 fold change with standard deviation. Blue represents microarray data and red qPCR data. Correlation coefficient \((r = 0.98)\) and \(p = 0.02\) indicate strong and significant correlation between microarray and qPCR data.

doi:10.1371/journal.pone.0047106.g004
The analyses of gene expression in CC for each stage of oocyte maturity have shown that the most significant change at the transcriptional level occurs during oocyte transition from the MI to the MII stage. This is in accordance with previous studies [23,24] reporting massive transcriptional changes in CC accompanied by a substantial transcript degradation in oocytes during the process of maturation [23]. We have found that the genes involved in the pathways of cell division, multicellular organismal development, signal transduction and cell adhesion play a significant role in the process of meiosis.

Among top down and up regulated genes comparing CC MI and CC MII there were previously described genes which are members of the Wnt signaling pathway (SERP4 and CDH5) [25], tumor growth factor beta (TGFβ) pathway (BMP3) [26], cell cycle (BUB1) [24] and prostaglandin formation (PTGES) [24,27]. But the most numerous was a group of genes connected to cell adhesion, cytoskeleton and extracellular matrix formation [28] which included previously described genes (ITGB3, ADAMTS1) [29,30] and many previously not described genes (TSPAN7, SPOCK2), and group of genes connected to signal transduction (GLRA2, MAOB, GBR45, GPR63). These pathways have been previously recognised to have a key role in folliculogenesis and oocyte maturation [28,31].

In the group of genes for extracellular matrix formation there were TSPAN7, which works through ITGB3 [29,32], MGP, which is a BMP2 binding protein [33]; they were among the top regulated genes between CC MI and CC MII. Further, a group of serpine peptidase inhibitors (SERPINE2, SERPINF1, HTRA1, SERINC5) with tissue plasminogen activator (PLAT) were also among downregulated genes. On the contrary, SPOCK2, NID2 and ADAMTS1 showed upregulation between CC MI and CC MII oocytes. These genes are responsible for extracellular matrix binding. Extracellular matrix of CC has been found crucial for ovulation, oviduct passage and fertilization, especially through TNFp protein [34]. The genes of CC extracellular matrix have been proved to be hCG-dependant [35]. ANK2, ANK3 and PALLD are genes which function in cytoskeleton formation. All these genes were much less expressed in CC MII oocytes compared to CC MI oocytes. ANK2 and ANK3 both contain ANKRD57 protein, where the ANKRDS7 gene has already been recognized to be influenced by oocyte maturation in CC [36].

Among signal transduction genes other than Wnt signaling and TGFβ there were also top down and up regulated genes connected to neurotransmitters. Receptors for two inhibitory neurotransmitters glycine and γ-Aminobutyric acid GABA (GLRA2, GABRA5) and dopamine degrading enzyme gene (MAOB) were down regulated in CC MII compared to CC MI oocytes. So far little is known on the function of glycine and GABA in the folliculogenesis and oocyte maturation. A comparative analysis of amino acids in the follicular fluid of preovulatory follicles and in serum showed the amount of glycine differed the most, and was higher in the follicular fluid [37]. On the other hand more is known on the dopamine function in folliculogenesis. Dopamine derives from ovarian neurons in follicles [38] and during oocyte maturation its concentration rises [39]. From the follicular fluid dopamine is transported to the oocyte where it is degraded to noradrenaline [30]. Another role of dopamine agonists is a blockade of VEGF vascular permeability in prevention of OHSS without compromising the result of IVF [40]. Considering the results of our study as well as of previous studies [37,38,40,41] we suppose that neurotransmitters have an important role in folliculogenesis and oocyte maturation. To our knowledge this is the first implication that the genes responsive to dopamine, glycine and GABA might serve as biomarkers of oocyte maturation in CC.

The Ingenuity Pathway Analysis identified AMHR2, FSHR, SERPINE2 and VEGFC to be in the top gene network among differentially expressed genes between CC MI and CC MII, and are connected to FSH and LH. The expression of AMHR2 and FSHR in CC MI oocytes was significantly higher compared to CC MII oocytes. The expression of FSHR and AMHR2 in CC has been reported to be strongly related to the expression level of anti-Müllerian hormone (AMH) and androgen receptor (AR) [42,43,44]. In the ovary, AMH regulates primordial follicle recruitment and FSH sensitivity of growing follicles in an inhibitor manner. AMH expression in GC and CC has been found to be the highest in pre-antral and small antral follicles [45,46], and gradually diminishes during folliculogenesis [46,47] which is in accordance with AR, FSHR and AMHR2 mRNA expression [42]. FSHR is highly expressed in CC and is critical for oocyte maturation [48]. Its expression decreases along with the progression of maturation of bovine oocytes in vitro [49] and in vivo after hCG administration [49]. Cattaneo-Jonard et al. have found that FSHR together with AMH, AMHR2, and AR are overexpressed in GC from stimulated follicles of PCOS women indicating an oocyte maturation defect [50].

In addition, SERPINE2 is significantly altered by oocyte stage. SERPINE2 expression is increased by FSH [51] and is decreased after LH surge in GC of growing dominant bovine follicles [52,53]. This goes in line with the results of our study where CC MII shows a lower expression of SERPINE2 than CC MI. Finally, the expression difference of VEGFC has been observed between CC MI and CC MII level, where the latter have a significantly higher expression. Vascular endothelial growth factor (VEGF) is an angiogenic substance synthesized in theca cells and GC [54]. Structurally related to VEGF are also VEGFB and VEGFC [55] and they are all expressed in human GC [56]. It is known that follicular VEGF concentrations are higher in preovulatory follicles compared to early antral follicles and that oocyte quality is related to the intrafollicular influence of VEGF [58]. VEGF concentrations in follicular aspirates containing MII oocytes that have fertilized are higher than of those containing MII oocytes that are not fertilized [58].

In this study the differences in gene expression have been observed between CC MI and CC MII. This is not only in accordance with previous studies, but also provides some new aspects of oocyte maturation at the molecular level. According to our CC gene expression profiling we may conclude that both aspects of oocyte maturation at the molecular level. According to our CC gene expression profiling we may conclude that both aspects of oocyte maturation at the molecular level. According to our CC gene expression profiling we may conclude that both aspects of oocyte maturation at the molecular level. According to our CC gene expression profiling we may conclude that both aspects of oocyte maturation at the molecular level. According to our CC gene expression profiling we may conclude that both aspects of oocyte maturation at the molecular level.
with an arrowhead indicates ‘acts on’. The green colour intensity of the nodes indicates the degree of down-regulation while the red colour intensity of the nodes indicates the degree of up-regulation in the CC MII group. CC MI: cumulus cells of metaphase I oocytes; CC MII: cumulus cells of metaphase II oocytes.

Table S1 359 differentially expressed genes, p value, and their expression (log2 fold change) between CC MII and CC MI. CC MI: cumulus cells of metaphase I oocyte; CC MII: cumulus cells of metaphase II oocyte.

Table S2 Top enriched KEGG pathways by PGSEA and their expression (log2 fold change) between CC MII and CC MI. Significant (p<0.05) expression changes are shown in bold.

Table S3 Gene Ontology (GO) analysis performed by GeneCodis upon 359 differentially expressed genes.

References
1. Hayden C (2008) GnRH analogues: applications in assisted reproductive techniques. Eur J Endocrinol 159 Suppl 1: S17–25.
2. Schmutzler RK, Reichert C, Diedrich K, Wild I, Diedrich C, et al. (1998) Combined GnRH-agonist/gonadotropin stimulation in in-vitro fertilization. Hum Reprod 3 Suppl 2: 29–33.
3. Diedrich K, Diedrich C, Santos E, Zoll C, al-Hasani S, et al. (1994) Suppression of the endogenous luteinizing hormone surge by the gonadotrophin-releasing hormone antagonist Cetrorelix during ovarian stimulation. Hum Reprod 9: 788–791.
4. Albano C, Felberbaum RE, Smitz J, Riehmuller-Winzen H, Engel J, et al. (2000) Ovarian stimulation with HMG: results of a prospective randomized phase III European study comparing the luteinizing hormone-releasing hormone (LHRH)-antagonist cetrorelix and the LHRH-agonist buserelin. European Cetrorelix Study Group. Hum Reprod 15: 526–531.
5. Felberbaum R, Reissmann T, Kupker W, Al-Hasani S, Bauer O, et al. (1996) Hormone profiles under ovarian stimulation with human menopausal gonadotropin (hMG) and concomitant administration of the gonadotropin releasing hormone (GnRH)-antagonist Cetrorelix at different dosages. J Assist Reprod Genet 13: 216–222.
6. Ludwig M, Katalinic A, Diedrich K (2001) Use of GnRH antagonists in ovarian stimulation for assisted reproduction technologies compared to the long protocol. Meta-analysis. Arch Gynecol Obstet 265: 175–182.
7. Diedrich K, Ludwig M, Felberbaum RE (2001) The role of gonadotropin-releasing hormone antagonists in in vitro fertilization. Semin Reprod Med 19: 213–220.
8. Ludwig M, Felberbaum RE, Devroye P, Albano C, Riehmuller-Winzen H, et al. (2000) Significant reduction of the incidence of ovarian hyperstimulation syndrome (OHSS) by using the LHRH antagonist Cetrorelix (Cetrotide) in controlled ovarian stimulation for assisted reproduction. Arch Gynecol Obstet 264: 29–32.
9. Kohlbianakis EM, Collins J, Tarlatzis BC, Devroye P, Diedrich K, et al. (2006) Among patients treated for IVF with gonadotrophins and GnRH analogues, is the probability of live birth dependent on the type of analogue used? A systematic review and meta-analysis. Hum Reprod Update 12: 651–671.
10. Al-Inany HG, Abu-Setta AM, Aboughar M (2009) Gonadotropin-releasing hormone antagonists for assisted conception (Review). The Cochrane Library.
11. Keith L, Breborowicz G (2002) Triplet pregnancies and their aftermaths. Part I: Basic considerations. Int J Fertil Womens Med 47: 254–264.
12. Assou S, Haouzi D, De Vos J, Hannamah S (2010) Human cumulus cells as biomarkers for embryo and pregnancy outcomes. Mol Hum Reprod 16: 531–535.
13. Li Q, McKenzie LJ, Matzuk MM (2008) Revisiting oocyte-somatic cell interactions: in search of novel intracellular predictors and regulators of oocyte developmental competence. Mol Hum Reprod 14: 673–678.
14. Matzuk MM, Burns KH, Viveiros MM, Eppig JJ (2002) Intercellular communication in the mammalian ovary: oocytes carry the conversation. Science 296: 2178–2180.
15. Matzuk MM, Lamb DJ (2002) Genetic dissection of mammalian fertility pathways. Nat Cell Biol 4 Suppl: s14–49.
16. McClory SL, Byrne JA, Chavez SL, Behr B, Huajer AJ, et al. (2010) Parturogenesis/blastocysts derived from cumulus-free in vitro matured human oocytes. PLoS ONE 5: e10979.
17. Fleiss JL (1999) Design and Analysis of Clinical Experiments. John Wiley & Sons.

Items: GO group; Items_Details: description of GO group; Hyp_c: corrected value of hypergeometrical test.

Acknowledgments
We thank all the personnel at the Division of Reproductive Medicine, Department of Obstetrics and Gynecology, University Medical Center Ljubljana, for contributing their share to this study. Especially, we would like to thank the IVF laboratory staff Msc. Jerneja Krnicel, Lali Bačer Kermavner, Brigita Valentinčič Gruden and Jožica Mivleš for their skillful and careful work with biological material. Finally, we would like to thank Dr Tanja Burišnik Papler for performing qPCR analysis and Ms Mojca Pirc for reviewing the English text.

Author Contributions
Conceived and designed the experiments: RD KFT PJ IVK DR EVB. Performed the experiments: RD KFT PJ IVK DR EVB. Analyzed the data: RD KFT PJ IVK DR EVB. Contributed reagents/materials/analysis tools: RD KFT PJ IVK DR EVB. Wrote the paper: RD KFT PJ IVK DR EVB.
36. Ouandaogo ZG, Haouzi D, Assou S, Dechaud H, Kadoc H, et al. (2011) Human cumulus cells molecular signature in relation to oocyte nuclear maturity stage. PLoS ONE 6: e27179.

37. Jozwik M, Teng C, Battaglia FC (2006) Amino acid, ammonia and urea concentrations in human pre-ovulatory ovarian follicular fluid. Hum Reprod 21: 2776–2782.

38. Mayerhofer A, Smith GD, Danilchuk M, Levine JE, Wolf DP, et al. (1998) Oocytes are a source of catecholamines in the primate ovary: evidence for a cell-cell regulatory loop. Proc Natl Acad Sci U S A 95: 10990–10995.

39. Bodis J, Torok A, Timmerberg HR, Hanf V, Hamori M, et al. (1992) Influence of noradrenaline, dopamine and serotonin contents in follicular fluid of human graafian follicles after superovulation treatment. Gynecol Obstet Invest 33: 165–167.

40. Gomez R, Gonzalez-Izquierdo M, Zimmermann RC, Novella-Maestre E, Alonso-Muriel I, et al. (2006) Low-dose dopamine agonist administration blocks vascular endothelial growth factor (VEGF)-mediated vascular hyperpermeability without altering VEGF receptor 2-dependent luteal angiogenesis in a rat ovarian hyperstimulation model. Endocrinology 147: 5400–5411.

41. Bodis J, Torok A, Timeberg HR, Hanf V, Hansori M, et al. (1992) Influence of serotonin on progesterone and estradiol secretion of cultured human granulosa cells. Fertil Steril 57: 1006–1011.

42. Grondahl ML, Nielsen ME, Dal Canto MB, Fadini R, Rasmussen IA, et al. (2011) Anti-Mullerian hormone remains highly expressed in human cumulus cells during the final stages of folliculogenesis. Reprod Biomed Online 22: 389–396.

43. Nielsen ME, Rasmussen IA, Kristensen SG, Christensen ST, Mollgard K, et al. (2010) In human granulosa cells from small antral follicles, androgen receptor mRNA and androgen levels in follicular fluid correlate with FSH receptor mRNA. Mol Hum Reprod 17: 63–70.

44. Slomczynska M, Duda M, Slazak K (2001) The expression of androgen receptor, anti-Mullerian hormone and its receptor, FSH receptor, and androgen receptor genes are overexpressed by granulosa cells from stimulated follicles in women with polycystic ovary syndrome. J Clin Endocrinol Metab 93: 4456–4461.

45. Kawashima I, Okazaki T, Noma N, Nishihori M, Yamashita Y, et al. (2008) Sequential exposure of porcine cumulus cells to FSH and/or LH is critical for appropriate expression of steroidogenic and ovulation-related genes that impact oocyte maturation in vivo and in vitro. Reproduction 136: 9–21.

46. Salhab M, Tosca L, Cabau C, Papillier P, Perreau C, et al. (2010) Kinetics of gene expression and signaling in bovine cumulus cells throughout IVM in different mediums in relation to oocyte developmental competence, cumulus apoptosis and progesterone secretion. Theriogenology 75: 90–104.

47. Visser JA, de Jong FH, Laven JS, Themmen AP (2006) Anti-Mullerian hormone expression pattern in the human ovary: potential implications for initial and cyclic follicle recruitment. Mol Hum Reprod 10: 167–175.

48. Bokal EV, Vrtovec HM, Virant Klun I, Verdenik I (2005) Prolonged GnRH Analogues on Cumulus Cells Gene Expression