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Extensive effects of in vitro oocyte maturation on rhesus monkey cumulus cell transcriptome

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Lee YS, VandeVoort CA, Gaughan JP, Midic U, Obradovic Z, Latham KE. Extensive effects of in vitro oocyte maturation on rhesus monkey cumulus cell transcriptome. *Am J Physiol Endocrinol Metab* 301: E196–E209, 2011. First published April 12, 2011; doi:10.1152/ajpendo.00686.2010.—The elaboration of a quality oocyte is integrally linked to the correct developmental progression of cumulus cell phenotype. In humans and nonhuman primates, oocyte quality is diminished with in vitro maturation. To determine the changes in gene expression in rhesus monkey cumulus cells (CC) that occur during the final day prior to oocyte maturation and how these changes differ between in vitro (IVM) and in vivo maturation (VVM), we completed a detailed comparison of transcriptomes using the Affymetrix gene array. We observed a large number of genes differing in expression when comparing IVM-CC and VVM-CC directly but a much larger number of differences when comparing the transitions from the prematuration to the post-IVM and post-VVM states. We observed a truncation or delay in the normal pattern of gene regulation but also remarkable compensatory changes in gene expression during IVM. Among the genes affected by IVM are those that contribute to productive cell-cell interactions between cumulus cell and oocyte and between cumulus cells. Numerous genes involved in lipid metabolism are incorrectly regulated during IVM, and the synthesis of sex hormones appears not to be suppressed during IVM. We identified a panel of 24 marker genes, the expression of which should provide the foundation for understanding how IVM can be improved for monitoring IVM conditions and for diagnosing oocyte quality.

oocyte quality; in vitro maturation

LIFE BEGINS IN THE OOCYTE. The oocyte accumulates a remarkable and complex macromolecular reservoir that mediates and controls the initial development of each new individual while at the same time eliminating a range of molecules that promote gametogenesis but would otherwise impede early embryogenesis (8, 36, 37, 67). Failure to complete these transitions appropriately yields an oocyte with limited developmental potential, unable to undergo fertilization or activation, or unable to sustain embryogenesis after fertilization and activation.

Because of the key role of the oocyte in early development, understanding the molecular features that confer a high oocyte developmental potential is of paramount importance in reproductive biology. Such information will reveal the underlying mechanisms and processes that drive oogenesis and early embryogenesis. Moreover, such information will provide markers with which to screen and select high-quality oocytes for assisted reproduction in humans and could enhance success in agricultural reproductive biology and species conservation applications.

The oocyte develops in close coordination with the somatic companion cells, particularly the cumulus oophorous cells, which maintain direct contact with the oocyte via transzonal processes and gap junctions until ovulation. Through its secretion of several exogenous factors, the oocyte exerts a commanding role in the overall development of the follicle and the overall differentiation of the cumulus cells (24, 27, 28, 45, 91). In return, the cumulus cells play a key supportive role by providing to the oocyte a range of extracellular and intracellular molecules that sustain oocyte growth, regulate meiotic progression, and serve as essential metabolic precursors, among other functions (21, 39, 72).

This intimate relationship between the oocyte and attached cumulus cells implies that the two cell types develop and differentiate progressively and in concert with one another and that disruption in the development of either cell component could compromise oocyte quality. Accordingly, clues to the molecular determinants of oocyte quality may be sought by examination of the cumulus cell phenotype. Meeting this objective could be advantageous as a noninvasive means of evaluating oocyte quality clinically, and moreover, it could illuminate the mechanisms of successful follicle development and oogenesis. Cumulus cell gene expression has been studied at the mRNA and protein levels recently in several species to gain insight into the relationship between cumulus cells and oocytes (1, 2, 4–7, 16, 56, 74, 76, 92). However, a clear picture of how cumulus cell phenotype relates to oocyte developmental competence has not yet emerged.

In the rhesus monkey, in vivo maturation (VVM) yields oocytes of high quality that can support efficient development, whereas in vitro maturation (IVM) consistently yields oocytes of limited developmental potential (62–65). Oocytes retrieved after 7 days of in vivo FSH stimulation and then signaled to mature in vitro are able to be fertilized and undergo cleavage and in vitro development to blastocyst stage, but long-term development is very limited (65, 66). Modified culture conditions can increase this success (19, 66) and affect gene expression in the oocyte (50), but improvements can be limited to attaining intermediate stages, and successful development to advanced stages or term after IVM remains limited (10, 62,

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66). Studies indicate that embryos derived from IVM oocytes in the monkey are deficient in the timely onset of embryonic gene transcription (66).

True IVM, wherein oocytes are given the initial stimulus to mature in an in vitro environment, has also not been very successful in the human. Only a few reports have been published for IVM of human oocytes from nonstimulated cycles in women, leading to term development (9, 17, 60, 78, 90). Oocyte viability and embryonic development after IVM are substantially lower than with VVM. Often, oocytes are retrieved from patients either after failure to mature subsequent to in vivo stimulation or after a low-level dose of human chorionic gonadotropin to initiate the process in vivo (18, 89), and it has been shown that mRNA content with IVM even after short-term human chorionic gonadotropin (hCG) stimulation is profoundly altered compared with in vivo matured oocytes (32).

It is remarkable that the difference between IVM and VVM in the rhesus monkey, yielding such a profound effect on oocyte quality (38, 62–66), is just that the last 24 h of maturation in response to hormonal stimulation occurs within cumulus-oocyte complexes (COCs) that have been removed from the follicular environment. This indicates that follicular signals during the final 24 h of maturation must normally modulate cumulus cell and/or oocyte phenotype in a manner that confers high oocyte quality.

The basis for reduced developmental competence can be sought at the level of either the oocyte or the companion cumulus cells. Recent studies indicated alterations in the transzonal processes between the cumulus cells and oocyte with rhesus monkey IVM (20). We reported recently that the transcriptomes of IVM and VVM metaphase II stage oocytes displayed a very limited degree of difference, with only 59 mRNAs showing significant alterations, most of which were mRNAs that failed to decline in abundance appropriately (38). Here, we examine the transcriptomes of the companion cumulus cells. We found that the transcriptomes of IVM and VVM cells display extensive differences related to divergent patterns of gene regulation during that final 24 h of maturation. The results highlight specific pathways and processes that are regulated aberrantly and point toward specific mechanisms that control the acquisition of high oocyte developmental potential. Moreover, we identified a panel of 24 marker genes, the expression of which was significantly related to maturation condition and which may be useful cumulus cell markers of oocyte quality.

MATERIALS AND METHODS

Collection and lysis of rhesus cumulus cells. Adult female rhesus macaques were housed at the California National Primate Research Center. All procedures employed to obtain COCs were conducted according to recommendations of the *Guide for the Care and Use of Laboratory Animals* and the Animal Welfare Act and its amendments. All procedures for maintenance and handling of the animals were reviewed and approved in advance by the Institutional Animal Use and Care Administrative Advisory Committee at the University of California at Davis. Animals were caged individually with a 0600-1800 light cycle and maintained at a temperature of 25–27°C. Animals were allowed to socialize by being housed in pairs during the day from ~0800 to 1400. Animals were fed a diet of Purina Monkey Chow and water ad libitum, and seasonal produce, seeds, and cereal were offered as supplements for environmental enrichment. Only females with a history of normal menstrual cycles were selected for study.

Females were observed daily for signs of vaginal bleeding, and the 1st day of menses was assigned cycle day 1. Beginning on cycle days 1–4, recombinant human FSH (rhFSH; Organon, West Orange, NJ) was administered (37.5 IU im) twice daily for 7 days total. For IVM experiments, COCs were collected on day 8. To obtain in vivo matured (VVM) COCs, females were given recombinant hCG (1,000 IU Ovidrel; Serono, Rockland, MA) on *treatment day 8* in addition to the rhFSH treatment outlined above. COCs were aspirated from follicles at 28–30 h following hCG by ultrasound-guided aspiration (82, 83).

All COCs were collected into Tyrode lactate (TL)-HEPES medium (37°C) containing 0.1 mg/ml polyvinyl alcohol and 5 ng/ml rhFSH (Organon). Aspirates were immediately placed in a heated isolette (37°C), where COCs were retrieved from aspirates and placed in fresh TL-HEPES medium. Immature oocytes were recovered at the germinal vesicle stage and cultured for 24 h in 70-μl drops under oil in chemically defined basal medium (CMRL) (11) containing 10% bovine calf serum (Gem Cell, Woodland, CA), rhFSH, human luteinizing hormone (0.03 IU/ml Pergonal, Ares-Serono), and 1 μg/ml androstenedione (Steraloids, Newport, RI) [also reported as CMRL medium (66)]. Cumulus cells were obtained from IVM oocytes after culture for 24 h and from prematuration (PM) or VVM oocytes only immediately after collection. The cumulus oocyte complexes were placed individually into drops of TL-HEPES medium, and cumulus cells were removed from oocytes by trituration through a small bore pipette. Only cumulus cells from oocytes that had a germinal vesicle were included in the prematuration IVM control group, and only oocytes with one polar body were utilized for the IVM and VVM groups.

RNA isolation, amplification, and array hybridization. We generated transcriptome profiles of three groups of rhesus monkey cumulus cells (PM-CC, IVM-CC, and VVM-CC) using the Affymetrix Rhesus Genome arrays (Fig. 1). Each array sample represents multiple COCs obtained from one monkey. In total, 11 array samples (4 PM-CC, 3 IVM-CC, and 4 VVM-CC) were collected from 11 different female monkeys. Total RNA was isolated from cumulus cells using the PicoPure RNA isolation kit (MDS Analytical Technologies, Sunnyvale, CA) according to the manufacturer's instructions. Fifty nanograms of total RNA from each array sample containing COC pools from each individual monkey was subjected to two rounds of cDNA synthesis and in vitro transcription labeling to achieve a linear amplification (Eukaryotic Small Sample Target Labeling Assay, Affymetrix GeneChip Expression Analysis Technical Manual) with minor modifications (initial 5-μl volume for annealing and reverse transcription for 30 min at 42°C followed by 30 min at 45°C). The biotin-labeled cRNA samples were fragmented, and 10 μg were hybridized onto Affymetrix Rhesus Genome GeneChip arrays at the University of Pennsylvania Microarray Core Facility. Posthybridization washing, staining and scanning were performed as described in the Affymetrix GeneChip Expression Analysis Technical Manual.

Microarray data analysis. Probe hybridization intensity data were imported into the Affymetrix Expression Console Software and summarized using the Robust Multichip Analysis algorithm with a global background correction and a quantile normalization (31). To minimize false detections, expression data were filtered on the basis of Present/Absent calls determined by MAS 5.0 algorithm at the default settings (detection P value <0.05 and tau = 0.015). Only those probe sets called "present" in all biological replicates of one of the two groups in comparison were selected for the analysis. To minimize the chance of false positives in subsequent analyses, probe sets with maximum raw intensity values <100 were omitted from the data set. To identify differentially expressed genes, the Significance Analysis of Microarray (81) was performed. Differentially expressed probe sets were identified at a false discovery rate $<1\%$, and Student's t-test was used to select further for the genes with statistical significance ($P < 0.01$).

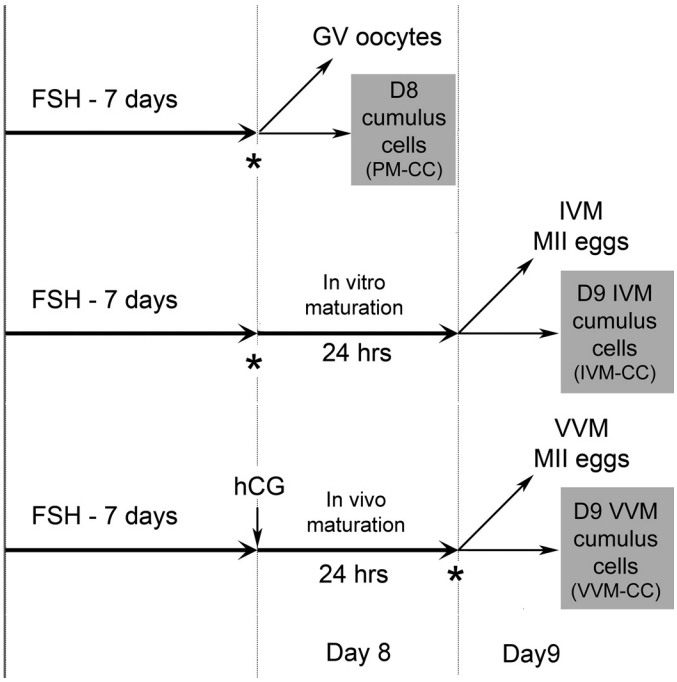


Fig. 1. Schematic summary of experimental design. Cumulus cells (CC)were collected from rhesus monkey cumulus-oocyte complexes on *day 8* (D8) after 7 days of recombinant human (rh)FSH treatment before final maturation (PM-CC), on *day 9* (D9) after in vitro maturation (IVM-CC), and on D9 after in vivo maturation following an ovulatory stimulus with hCG (VVM-CC). *Aspiration of follicles. GV, germinal vesicle; PM, prematuration; hCG, human chorionic gonadotropin; IVM, in vitro maturation; VVM, in vivo maturation.

Full data sets have been deposited in NCBI's Gene Expression Omnibus (22) and are accessible through GEO Series accession no. GSE25288 (http://www.ncbi.nlm.nih.gov/geo/) and at www.preger.org.

Pathway analysis and biological functional analysis were performed using the rhesus array annotations provided in the Ingenuity Pathway Analysis software (Ingenuity Systems, Redwood City, CA). For biological functional analysis, Fisher's exact test was performed with the *P* value threshold of 0.05 to identify molecular and cellular functional categories with statistical significance.

Real-time quantitative RT-PCR analysis. A panel of 43 oocyte quality marker candidates was chosen on the basis of a combination of fold change, array signal strength, and biological function. The mRNA expression of these 43 genes was compared between IVM-CC and VVM -CC by the quantitative real-time RT-PCR method. Each sample used for the quantitative (q)RT-PCR assay corresponds to total RNA pooled from multiple COCs obtained from one monkey. For a greater statistical power, total number of IVM-CC and VVM-CC samples was expanded for quantitative (q)RT-PCR from seven to 13 samples, including one additional IVM-CC and five additional VVM-CC samples. In total, nine samples of VVM-CC and four samples of IVM-CC were used for the qRT-PCR assay. Total RNA was isolated using the PicoPure RNA isolation kit (Molecular Devices, Sunnyvale, CA) and subjected to a whole transcriptome amplification using the QuantiTect whole transcriptome kit (Qiagen, Valencia, CA). Custom TaqMan gene expression assays were designed based on rhesus cDNA sequences using Primer Express software version 3.0 (Applied Biosystems, Foster City, CA). The primer and probe sequences of the custom-desgined TaqMan assays are listed in Supplemental Table S1 (Supplemental Material for this article is available online at the *AJP-Endocrinology and Metabolism* website). Quantitative real-time PCR was performed on ~100 ng of cDNA for each assay using ABI StepOne Plus instrument according to the manufacturer's recommendations (Applied Biosystems). The mRNA abundance of a target gene was normalized to the endogenous mitochondrial ribosomal protein S18C gene (*MRPS18C*) for sample-to-sample comparisons, and the relative expression ratio of IVM to VVM groups was obtained by the comparative C_T method (44). Statistical analysis was performed between IVM and VVM groups using the Relative Expression Software Tool for the genes assayed by qRT-PCR method (57).

Statistical analysis. Gene expression values were analyzed for a relationship to oocyte quality. Single- and multiple-gene generalized estimating equations (GEE) were used to calculate the relationship of gene expression to oocyte quality. GEE is a special case of the generalized linear model, taking into consideration the occurrence of a binary outcome (oocyte quality) from multiple events of a single experimental unit.

RESULTS

Overview of the array analysis. With array data inspected, all quality control parameters were within the acceptable ranges for all 11 array samples (Supplemental Table S2). The average detection rate for PM-CC samples was 47%, corresponding to ~25,000 probe sets. The detection rates for IVM-CC and VVM-CC were 38 and 41%, respectively. A hierarchical clustering analysis revealed that the biological replicates cluster to their corresponding groups without any apparent outliers (Supplemental Fig. S1). The three-dimensional principal component analysis also showed the clustering of all replicates to their corresponding groups (Supplemental Fig. S2).

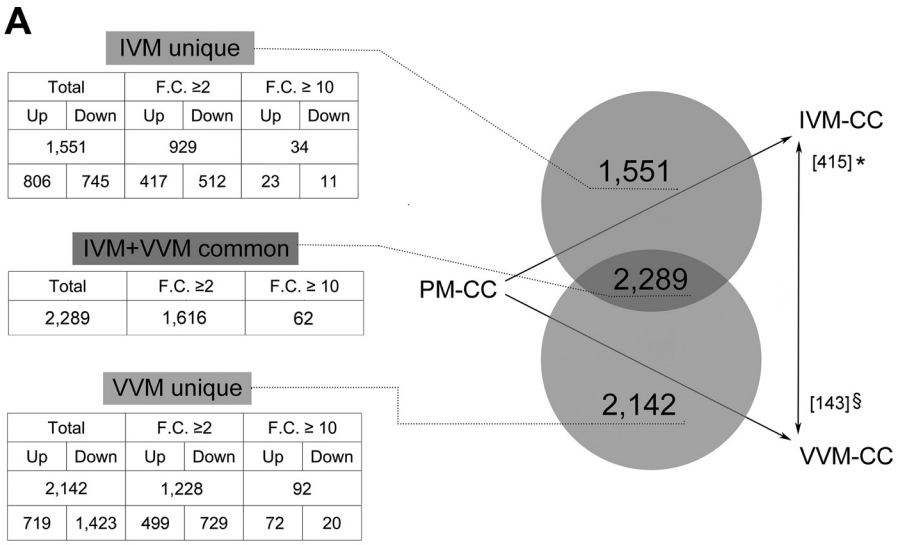
We observed that cumulus cells undergo a dramatic change in their transcriptome during the last 24 h of maturation. A total of 3,252 out of 19,076 annotated genes (17%; 4,431 probe sets) on the rhesus array were either up- or downregulated during VVM, and 14.5% (2,772 genes; 3,840 probe sets) of all genes were modulated during IVM. There were more genes downregulated than upregulated during the cumulus cell maturation process (Supplemental Table S3). Of the probe sets differentially regulated during VVM, 74% [2,459 unique genes; 3,284 probe sets (2,776 annotated)] were downregulated, whereas only 26% [793 unique genes; 1,147 probe sets (883 annotated)] were upregulated. A similar relationship was observed during IVM [1,865 genes (2,592 probe sets) downregulated compared with 907 genes (1,248 probe sets) upregulated].

Identification of differentially regulated gene sets. To assess the biological processes or functions affected by this vast amount of change, our objective was to identify sets of differentially regulated genes and then apply ingenuity pathway analysis (IPA) to understand the biological implications of these differences. Some genes displayed inconsistent results between different probes sets, and their inclusion in both up- and downregulated categories can confound pathway analysis. Moreover, with such large numbers of genes affected, IPA can fail to yield a clear picture of the major pathways affected. To simplify and focus our analysis, 485 genes (1,099 probe sets) with inconsistencies between probes sets were omitted from further consideration, and only the genes that differed in expression at the threshold of twofold or greater were employed for IPA. We began our analysis by categorizing genes as correctly or incorrectly regulated (i.e., same direction of change) and as either up- or downregulated during maturation, comparing IVM-CC and VVM-CC with PM-CC. Our objective for this was to understand the transitions that occur or do

not occur during IVM or VVM rather than just comparing the end-stage IVM-CC and VVM-CC to each other. Genes were thus classified by comparing the temporal transitions and identified as either correctly or incorrectly regulated and within these categories either up- or downregulated or oppositely regulated. Once the differences in developmental transitions were characterized, VVM-CC and IVM-CC were also compared directly to each other.

Identification of genes incorrectly regulated during IVM. Genes that changed in opposite directions between IVM-CC and VVM-CC compared with PM-CC or those changed in only one or the other condition were classified as "incorrectly regulated." The first two groups of genes incorrectly regulated during IVM included those for which mRNA expression changed (increased or decreased compared with PM-CC) during IVM but not VVM. These genes, designated as "IVM unique" (Fig. 2*A*), included 1,551 probe sets (1,216 annotated) corresponding to 1,126 unique genes. About one-half of these genes were downregulated, and the other half were upregulated (Supplemental Table S4).

The next two groups of incorrectly regulated genes included those that changed significantly (increased or decreased compared with PM-CC) during VVM but not IVM (VVM unique; Fig. 2*A*). A total 1,607 unique genes (2,142 probe sets, 1,760



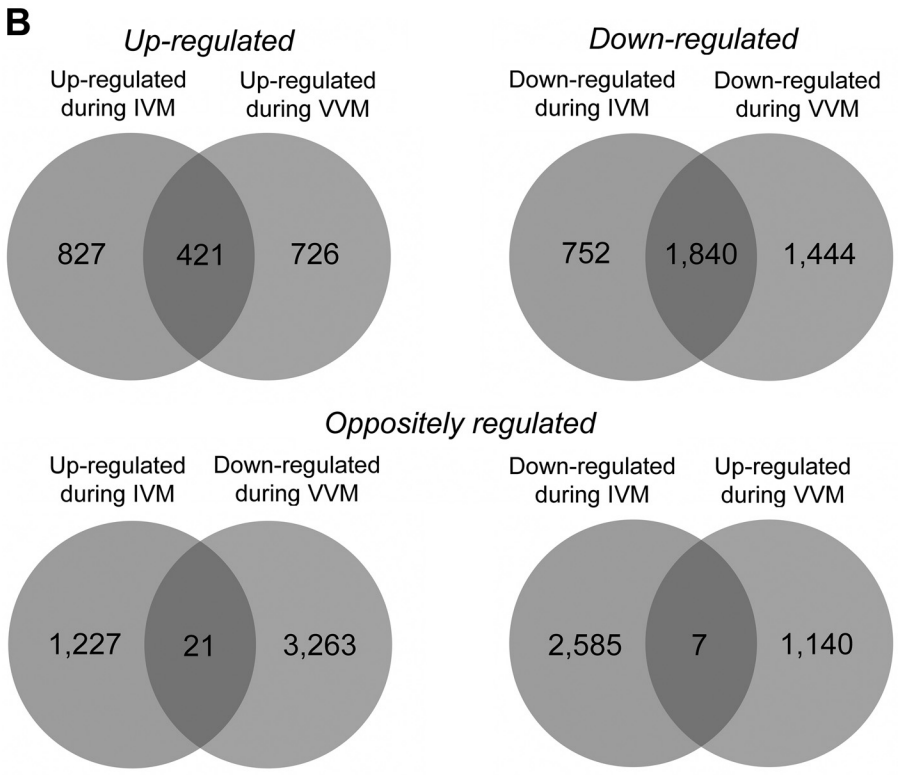


Fig. 2. *A*: Venn diagram to identify differential patterns of mRNA regulations during IVM and VVM. Probe sets for which mRNA abundance either changed only during IVM (IVM-unique) or only during VVM (VVM-unique) represent the genes that may be incorrectly regulated during IVM. Probe sets for which mRNA expression changed during both IVM and VVM may be either correctly or incorrectly regulated genes depending on the directionality of the change. IVM unique: no. of probe sets differentially regulated only during in vitro maturation. VVM unique: no. of probe sets differentially regulated only during in vivo maturation. IVM + VVM common: probe sets differentially regulated during both in vitro and in vivo maturations. FC, fold change of difference. *Probe sets more highly expressed on *day 9* IVM-CC; §probe sets more highly expressed in *day 9* VVM-CC. *B*: Venn diagrams to identify genes regulated in the opposite directions during IVM and VVM. Probe sets regulated in the same direction during IVM and VVM (421 probe sets upregulated during both IVM and VVM and 1,840 probe sets downregulated during both IVM and VVM) are considered as correctly regulated genes. Twenty-eight probe sets regulated in the opposite directions during IVM and VVM are incorrectly regulated genes.

annotated) belonged to the VVM-unique category. Compared with the IVM-unique genes, a higher proportion (1,423 probe sets, 1,112 unique genes) of the "VVM-unique" genes was downregulated during maturation than upregulated (719 probe sets, 495 unique genes; Supplemental Table S5).

A small number of genes (28 probe sets corresponding to 28 unique genes) was regulated in opposite directions (Fig. 2*B*). Twenty-one of these genes were upregulated during IVM but downregulated during VVM, and seven genes were downregulated during IVM but upregulated during VVM (Supplemental Table S6).

Identification of correctly regulated genes. Genes that changed in common and in the same direction going from PM-CC to either IVM-CC or VVM-CC were classified as "correctly regulated." The genes that were correctly regulated during IVM were identified by comparing the probe sets that showed significant changes in gene expression during IVM with those that showed significant changes during normal VVM maturation. These genes may represent a minimally required set of changes for cumulus cell accompanying oocyte maturation. About 4% of the probe sets (2,289 out of 52,865) or 8.6% of all annotated genes on the rhesus array (1,648 out of 19,076) were regulated during both IVM and VVM (IVM + VVM common; Fig. 2*A*). The majority of these [2,261 probe sets (1,824 annotated), 1,620 unique genes] were regulated in the same direction during IVM and VVM, either increased or decreased in expression during maturation (Fig. 2*B* and Supplemental Table S7). Among the relatively small pool of upregulated genes ($n = 293$), serglycin (*SRGN*, a.k.a. *PRG1* or proteoglycan 1) showed the highest upregulation in gene expression, as high as 128-fold.

Direct comparison of IVM and VVM cumulus cells and identification of more incorrectly regulated genes. The array analyses discussed above identified differences in the transition from PM-CC to IVM-CC and VVM-CC. These analyses may indicate which gene or pathway interactions are misregulated during IVM. The information from these analyses may be critical in aiding us to understand the cause of the poor quality of IVM oocytes. However, it is also necessary to identify the genes that show differential mRNA abundance at the final stage of maturation to understand the overall difference in CC phenotype that is established and to identify markers that may predict oocyte quality.

Interestingly, we found that despite the large number of genes showing incorrect modes of regulation relative to PM-CC, the final outcome of maturation was a much smaller set of differentially expressed mRNAs. There were 558 probe sets (452 unique genes) that showed differential mRNA abundance between IVM and VVM cumulus cells. Of these, 76% were more abundant in IVM cells than in VVM cells (Supplemental Table S8). The disparity between the number of VVM-CC vs. IVM-CC differences and the number of differences in the transitional comparisons with PM-CC indicates that many of the differences seen comparing the transitions are likely to reflect quantitative differences rather than qualitative differences in gene regulation.

An additional group of oppositely regulated genes (89 probe sets corresponding to 68 unique genes) was identified by comparing the transcriptomes from IVM- and VVM-CCs. (Supplemental Table S9). These genes were not identified above as "incorrectly regulated" due to either a small fold change or *P* value greater than the cutoff setting of 0.01 in the comparison with PM-CC but as significant end-stage differences between VVM-CC and IVM-CC. Among these genes, 58 were upregulated during IVM but downregulated during VVM.

Functional characteristics of genes correctly regulated during in vitro maturation. Biological functions and pathways significantly overrepresented in genes regulated either correctly or incorrectly during IVM or differing between VVM-CC and IVM-CC (Supplemental Tables S4–S9) were examined. Biofunction analysis of correctly regulated genes revealed that "cell cycle" was a biological function enriched at the highest significance among the genes downregulated during both IVM and VVM (Table 1). Genes positively associated with cell cycle progression (GO:0045786; e.g., *AKT1*, *CDC2*, *CDC20*, *CDC25A*, *CDC25C*, *CDC45L*, *CENPF*, *NUSAP1*, *PGGT1B*) were downregulated, and some genes negatively associated with cell cycle progression (*BUB3*, *ESR1*, *BCL2*, *CHMP1A*, *CDKN1B*, *ILKAP*, *BUB1*) were also downregulated during maturation. Cell cycle regulatory genes also comprised the most highly affected biological function among the genes upregulated during maturation. The mRNA abundances of *CDKN1A* and *APBB1* genes, which promote cell cycle arrest (GO:0045787), were increased during maturation.

Functional characteristics of genes incorrectly regulated during in vitro maturation. To understand the biological functions regulated in VVM- and IVM-unique genes, IPA overrepresentation analysis was performed on the genes with the fold change of difference greater than two (Table 2). For VVM-unique genes, DNA replication, recombination, and repair was the most significantly affected functional category. Among IVM-unique genes, cellular growth and proliferation genes were significantly regulated. Differences in pathway regulation were plotted for easier evaluation (Fig. 3). DNA replication, recombination, and repair was most different between IVM and VVM cumulus cells in both the confidence interval and gene number. Eighty-one genes involved in DNA replication, recombination, and repair were either up- or downregulated during VVM, whereas only nine were regulated during IVM. In VVM cumulus cells, most of the genes in this biofunctional category are involved in DNA repair or synthesis and replica-

Table 1. *Top 10 molecular and cellular functions overrepresented in genes correctly regulated during IVM (fold change of difference ≥2)*

Category	Range of *P* Values	No. of Molecules
Downregulated		
Cell cycle	4.98×10^{-16}–7.86×10^{-3}	132
Cellular assembly and organization	5.66×10^{-11}–8.07×10^{-3}	79
DNA replication, recombination, and repair	5.66×10^{-11}–7.86×10^{-3}	108
Cellular movement	3.25×10^{-10}–3.25×10^{-10}	20
Cell death	3.08×10^{-9}–7.86×10^{-3}	144
Upregulated		
Cell death	1.44×10^{-6}–1.94×10^{-2}	34
Small-molecule biochemistry	8.97×10^{-5}–1.85×10^{-2}	20
Cellular assembly and organization	1.22×10^{-4}–1.9×10^{-2}	16
Cellular movement	1.69×10^{-4}–1.9×10^{-2}	12
Cell cycle	1.8×10^{-4}–1.85×10^{-2}	19

IVM, in vitro maturation.

Table 2. *Molecular and cellular functions enriched in VVM- and IVM-unique genes (fold change of difference ≥2)*

Category	Range of *P* Values	No. of Genes
VVM-unique genes		
DNA replication, recombination, and repair	2.65×10^{-8}–6.42×10^{-3}	81
Cell cycle	2.22×10^{-6}–7.03×10^{-3}	84
Cellular growth and proliferation	1.37×10^{-5}–7.06×10^{-3}	201
Cell death	1.69×10^{-5}–6.63×10^{-3}	170
Lipid metabolism	1.04×10^{-4}–7.03×10^{-3}	38
IVM-unique genes		
Cellular growth and proliferation	1.88×10^{-6}–1.6×10^{-2}	158
Cellular assembly and organization	4.04×10^{-6}–1.6×10^{-2}	68
Cell death	7.81×10^{-6}–1.6×10^{-2}	138
Cell cycle	2.89×10^{-5}–1.63×10^{-2}	65
Cellular development	2.89×10^{-5}–1.63×10^{-2}	63

VVM, in vivo maturation.

tion. IVM cumulus cells regulated a handful of genes involved in DNA packaging or genome instability (Table 3). For the cell cycle functional category, not only was the total number of affected cell cycle genes greater in VVM cumulus cells than in IVM cells, there was also slightly higher expression of genes that delay cell cycle in VVM cumulus cells compared with IVM cumulus cells (Table 3). IVM and VVM cells differed particularly in the regulation of the genes involved in the G_1/S transition of the cell cycle (Table 4). VVM-CC showed a preference in upregulating many genes involved in the cell cycle arrest at the G_1/S transition phase, whereas IVM cumulus cells showed the opposite trend, upregulating more genes that promote the G_1/S cell cycle progression. Interestingly, although IVM-CC did not regulate the same genes as the VVM-CC, they upregulated a distinct set of G_1/S cell cycle arrest genes, such as insulin-like growth factor-binding protein-3 (*IGFBP-3*) and transforming growth factor-β 1 (*TGFβ1*). Additionally, IVM-CC successfully downregulated some genes that promote progression at the G_1/S transition. This analysis suggests that IVM cumulus cells may utilize pathways or mechanisms distinct from VVM cumulus cells as the oocyte matures. The reduced oocyte developmental competence may result from the fact that these alternate pathways used by IVM-CCs may not be as efficient as the normal pathway used by VVM-CCs for supporting oocyte development, and this may be the reason why IVM cumulus cells yield reduced quality.

The modulation of distinct sets of genes by IVM and VVM cells was also observed in other biofunctional categories. Changes in the "cell death" and "cell growth and proliferation" functions were associated with distinct sets of genes during IVM and VVM (Supplemental Tables S10 and S11), but the overall directionality seemed to be regulated similarly. For example, the percentages of proapoptotic and antiapoptotic genes in IVM-CC were almost the same as those in VVM-CC (Table 3). Genes involved in growth promotion and growth inhibition were likewise distinct but distributed in similar proportions in IVM and VVM cells. These observations suggest that IVM cumulus cells may regulate the maturation process using gene sets distinct from those used by VVM cumulus cells.

Next, to explore the functional relevance of genes most highly altered during IVM, a more stringent fold change criterion (≥10-fold) was applied to the IVM-unique and VVM-unique genes. This identified periostin (*POSTN*) and FBJ murine osteosarcoma viral oncogene homolog B (*FOSB*) as molecules prominently upregulated or downregulated, respectively, during IVM (Supplemental Table S12). Many of the genes upregulated at ≥10-fold during IVM encode extracellular matrix proteins (POSTN, EFEMP1, SPON2, LAMB1), membrane proteins (KCNK3, TMEM26, TTMA, THBD) or those related to extracellular matrix proteins (IGFBP-3, TNFAIP6, MMP20). IPA showed that this group of genes is overrepresented in molecular functions such as cellular growth and proliferation, cellular development, and cell-to-cell signaling. There were only four genes downregulated ≥10-fold during VVM. Three out of these four genes are transcription factors. *FOSB* and *EGR3* mRNA expression is maintained at a higher level in VVM cumulus cells compared with IVM cumulus cells (Supplemental Table S12). In addition to *FOSB* and *EGR3*, other early transcription factors *FOSL2* and *JUND* mRNAs were downregulated during IVM but maintained at a more steady level during VVM.

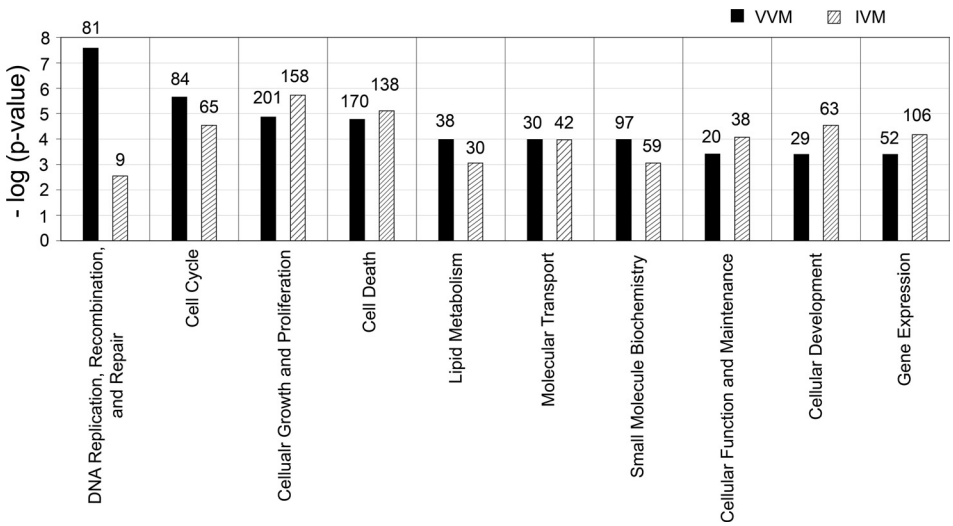


Fig. 3. Biofunctions overrepresented in VVM- and IVM-unique genes share similar categories and significance level (represented in *P* values) despite their distinct gene sets (the number on the top of each bar graph represents the number of genes in the corresponding biofunctional category).

Table 3. *No. of genes overrepresented in cell cycle, cell death, and cellular growth and proliferation*

	VVM Unique	IVM Unique
Upregulated	*Cell cycle*	
Delay in cell cycle progression	25 of 36 (69%)	7 of 26 (27%)
Increased cell cycle progression	7 of 36 (19%)	14 of 26 (54%)
Downregulated		
Delay in cell cycle progression	17 of 48 (35%)	11 of 40 (28%)
Increased cell cycle progression	16 of 48 (33%)	22 of 40 (55%)
	Cell death	
Upregulated		
Pro-survival	7 of 72 (10%)	7 of 59 (12%)
Antiapoptosis	20 of 72 (28%)	17 of 59 (29%)
Proapoptosis	25 of 72 (35%)	21 of 59 (36%)
Downregulated		
Pro-survival	11 of 98 (11%)	5 of 79 (6%)
Antiapoptosis	22 of 98 (22%)	31 of 79 (39%)
Proapoptosis	30 of 98 (31%)	29 of 79 (37%)
	Cellular growth and proliferation	
Upregulated		
Decreased growth	21 of 86 (24%)	11 of 74 (15%)
Decreased proliferation	25 of 86 (29%)	9 of 74 (12%)
Increased growth	17 of 86 (20%)	17 of 74 (23%)
Increased proliferation	21 of 86 (24%)	22 of 74 (30%)
Downregulated		
Decreased growth	16 of 115 (14%)	10 of 84 (12%)
Decreased proliferation	18 of 115 (16%)	15 of 84 (18%)
Increased growth	32 of 115 (28%)	18 of 84 (21%)
Increased proliferation	41 of 115 (36%)	26 of 84 (31%)
	DNA replication, repair, and recombination	
Upregulated		
DNA replication	7 of 30 (23%)	0 of 2 (0%)
DNA synthesis	14 of 30 (46%)	0 of 2 (0%)
DNA packaging	0 of 30 (0%)	1 of 2 (50%)
DNA repair	7 of 30 (23%)	0 of 2 (0%)
Instability of genomes	0 of 30 (0%)	1 of 2 (50%)
DNA replication	12 of 51 (24%)	0 of 7 (0%)
DNA synthesis	12 of 51 (24%)	0 of 7 (0%)
Downregulated		
DNA packaging	0 of 51 (0%)	2 of 7 (29%)
DNA repair	14 of 51 (27%)	0 of 7 (0%)
Instability of genomes	0 of 51 (0%)	5 of 7 (71%)

The characteristics of the genes differentially regulated during VVM are distinct from those regulated during IVM. For both the genes upregulated and the genes downregulated during VVM with the highest degree of changes in mRNA expression, lipid metabolism was the most highly overrepresented function (Table 5 and Supplemental Table S13). Genes upregulated during VVM by ≥10-fold were enriched in the biological functions of lipid metabolism and small molecule biochemistry. Those genes are *HSD11B1*, steroidogenic acute regulatory (*STAR*) protein, *EREG*, prostaglandin-endoperoxide syhthase 2 (*PTGS2*), *CEBPA*, *PTGER3*, *ANXA1*, *ACSL1*, *CD36*, *RORA*, *PRKCA*, *IGF1*, *ABHD5*, *UGP2*, *SLC16A10*, and *CLDN1* (Table 5 and Supplemental Table S13). They are involved in various subcategories of lipid metabolism function, such as the synthesis, metabolism and transport of lipids, β-oxidation of fatty acids, the synthesis and metabolism of cholesterol and prostaglandins, etc. It is also notable that a well-known cumulus cell maturation marker, *PTGS2* (prostaglandin-endoperoxide synthase 2), was identified as differentially upregulated during VVM but not during IVM. The genes downregulated during VVM (fc ≥ 10) are connected in a network involved in small molecule biochemistry and lipid metabolism (Supplemental Table S14 and Supplemental Fig. S3). Cytochrome P450 family 19 subfamily A polypeptide 1 (*CYP19A1*) and hydroxysteroid (11β) dehydrogenase 2 (*HSD11B2*), for example, interact with sex steroid hormones such as progesterone, β-estradiol, and prolactin (14, 41, 79, 85). Also, CYP19A1 may increase the expression of progesterone receptor (PGR) (42). This is consistent with our finding that *PGR* mRNA expression was significantly upregulated in IVM-CC, in which *CYP19A1* mRNA expression failed to get downregulated. (Supplemental Table S14).

Included in the group of oppositely regulated genes were those involved in RNA posttranscriptional modification (*POP5*, *AARS*), carbohydrate metabolism (*NUDT5*, *TKT*, *PTHLH*, *SLC37A4*), and cell-to-cell signaling interaction (*ITGAE*, *PTHLH*). Only 10 of 68 genes were downregulated during IVM but upregulated during VVM. Among these were genes involved in posttranslational modification (*SERP1*, *PTPN1*, *HERPUD1*, *NCSTN*) and cell morphology (*DLG1*, *EGR2*, *PTPN1*) (Supplemental Table S9).

Identification of oocyte quality markers by qRT-PCR and statistical analysis. The mRNA expression of 43 candidate markers of oocyte quality was examined by qRT-PCR. The rationale was that VVM oocytes are of the highest quality, whereas IVM oocytes are known to have limited developmental potential; hence, analysis of adherent cumulus cells could reveal markers predictive of ooctye quality. The qRT-PCR results confirmed differential expression for these genes between IVM-CC and VVM-CC (Fig. 4). Univariate GEE analysis showed that the expression levels of 24 of the 43 genes

Table 4. *Comparison of G_1/S cell cycle regulation genes overrepresented during VVM and IVM*

Cell Cycle	VVM Unique	IVM Unique
Upregulation		
G_1/S arrest	*PTGS2*(+212), *ANXA1*(+47), *PRKCA*(+16), *GADD45B* (+15), *IFI16*(+12), *CEBP* (+12), *GAS1*(+7), *PRKCH*(+8), *GALS3*(+7), *GALS1*(+3), *SOD2*(+3)	*IGFBP3*(+36), *PLAGL1*(+4), *PURA*(+3), *TGFB1*(+3)
G_1/S progression	*CAMK2D*(+2)	*SMAD7*(+10), *ITGA5*(+8), *FN1*(+4), *TAF10*(+3), *PLAC1*(+3), *GMNN*(+3), *CCND1*(+2)
Downregulation		
G_1/S arrest		*DDX3*(−7),*TXNIP*(−4), *LATS2*(−4), *APC*(−2)
G_1/S progression	*ESR2*(−8), *CBX2*(−4), *SMC1A*(−4), *E2F2*(−4), *CCND2*(−4), *CCND3*(−3), *CCNE1*(−3), *TP53*(−3), *POLE*(−3)	*PIM1*(−8), *KLF4*(−6), *KRAS*(−4), *PPP3CA*(−3), *SIRT1*(−3), *MYC*(−3), *FKBPIA*(−2)

Table 5. *Molecular and cellular functions overrepresented among genes up- or downregulated during VVM (fold change of difference >10)*

Category	Range of *P* Values	Genes
Upregulated		
DNA replication, recombination, and repair	2.65×10^{-8}–6.42×10^{-3}	*RGS2*, *GADD45B*, *EPB41L3*, *IFI16*, *CD36*, *RARRES1*, *TFPI2*, *F11R*, *IGF1*, *ANXA1*, *CEBPA*, *ERRFI1*, *PTGS2*, *CXADR*, *EREG*, *C3AR1*, *HSD11B1*, *ACPP*, *PRKCA*
Cell cycle	2.22×10^{-6}–7.03×10^{-3}	*RGS2*, *ABHD5*, *IGF1*, *RORA*, *ANXA1*, *CEBPA*, *CD36*, *PTGS2*, *HSD11B1*, *ACSL1*, *STAR*
Cellular growth and proliferation	1.37×10^{-5}–7.06×10^{-3}	*SLC16A10*, *ABHD5*, *RGS2*, *PTGER3*, *CD36*, *STAR*, *IGF1*, *UGP2*, *CLDN1*, *RORA*, *ANXA1*, *CEBPA*, *PTGS2*, *HSD11B1*, *ACSL1*, *PRKCA*
Cell death	1.69×10^{-5}–6.63×10^{-3}	*SLC16A10*, *ABHD5*, *RGS2*, *PTGER3*, *CD36*, *STAR*, *IGF1*, *UGP2*, *RORA*, *ANXA1*, *CEBPA*, *PTGS2*, *ACSL1*, *HSD11B1*, *PRKCA*
Lipid metabolism	1.04×10^{-4}–7.03×10^{-3}	*CD36*, *RARRES1*, *SERPINA3*, *TFPI2*, *SHC4*, *F11R*, *IGF1*, *ANXA1*, *ERRFI1*, *PTGS2*, *EREG*, *FUT4*, *PRKCA*
Downregulated		
Cell morphology	7.34×10^{-4}–2.61×10^{-2}	*CACNA2D2*, *CYP19A1*, *RYR2*, *IHH*
Cellular development	7.34×10^{-4}–1.39×10^{-2}	*CYP19A1*, *IHH*
Cellular function and maintenance	7.34×10^{-4}–6.59×10^{-3}	
Cellular growth and proliferation	7.34×10^{-4}–4.77×10^{-2}	*SFRP4*, *CACNA2D2*, *NDP*, *CYP19A1*, *RYR2*, *IHH*, *CDCA7*, *SLC29A1*
Lipid metabolism	7.34×10^{-4}–3.61×10^{-2}	*CYP19A1*

tested were significantly related to oocyte quality (IVM vs. VVM) (genes with asterisks in Fig. 4, Supplemental Table S15). Thus any of these genes may be an informative marker of oocyte quality. Changes in gene expression are thus associated with increases or decreases in predicted oocyte quality as specified by the odds ratios given. Moreover, multiple genes from this large panel can be employed together to further strengthen the predictive power. Three genes (*NEK6*, *AQP11*, and *IGF1*) were identified by stepwise multiple GEE to be predictive in combination (Supplemental Table S16). The following equation may be used to predict the probability of having a high-quality oocyte: probability (high-quality oocyte) $= e^{5.608 + 0.645[NEK6] + 0.100[AQP11] - 2.17[IGF1]}/1 + e^{5.608 + 0.645[NEK6] + 0.100[AQP11] - 2.17[IGF1]}$.

DISCUSSION

Our transcriptome comparisons of PM-CC, IVM-CC, and VVM-CC yielded a number of surprising results that have significant implications for understanding the biology of oocyte maturation and its relationship to attendant oocyte quality. First, we found that a large number of genes (558 probe sets, 452 genes, 4.5% of total genes detected) differ in expression when IVM-CC and VVM-CC are compared directly. A much larger number of differences are seen when the two developmental transitions, PM-CC to IVM-CC vs. PM-CC to VVM-CC, are compared, indicating that many more genes display quantitative differences in regulation than is revealed just by comparing IVM-CC and VVM-CC. Overall, a total of 2,830 genes were incorrectly regulated (1,607 VVM-unique + 1,127 IVM-unique + 96 oppositely regulated), which is more than 20% of the genes detected on the array. This result highlights the need to consider developmental transitions rather than just end points when evaluating divergent processes and also emphasizes a striking disruption in cumulus cell development during the terminal 24-h maturation period when this is undertaken in vitro. The extensive degree of difference observed between IVM-CC and VVM-CC contrasts with the minor degree of difference reported for the oocytes (38). This suggests that reduced oocyte quality may not be reflected highly at the level of oocyte mRNA expression. This may be because key changes in the oocyte reside at the posttranslational level, whereas changes in cumulus cell phenotype that affect oocyte quality are manifested at the mRNA level.

Second, several aspects of the array data indicate truncation or delay in the normal pattern of gene regulation. A higher proportion of VVM-unique genes is normally downregulated during VVM but not downregulated during IVM, indicating that IVM-CC are delayed or defective in downmodulating gene expression. The group of VVM- and IVM-unique genes might have resulted in part from a failure of IVM cumulus cells in timely gene regulation or mRNA degradation. We observed deficient expression of some transcription factors. The expression of these early transcription factors is required for maturation and terminal differentiation of mural granulosa cells (30, 59, 69). Thus, IVM-CC may be deficient in factors necessary to complete the maturation process. Among the genes with the greatest expression differences, the cumulus cell differentiation markers *FOSB*, *FOSL2*, *JUND*, *PTGS2*, and *EGR2* failed to be regulated correctly during IVM. This suggests that IVM-CC are not attaining a phenotype identical to VVM-CC.

An increase in *PTGS2*, *STAR*, *HAS2*, and *GREM1* gene expression is associated with the cumulus cell expansion process in the mouse, and this increase is thought to be regulated by GDF9 secretion from the oocyte (23, 33, 52, 68). Although *HAS2* and *GREM1* mRNAs are expressed here, there does not appear to be a high degree of upregulation associated with either IVM or VVM. In contrast, our rhesus cumulus cell microarray results show that *PTGS2* and *STAR* are two of the genes with the highest relative increase in mRNA levels in VVM-CC compared with the PM-CC. These genes are not increased in IVM-CC, yet cumulus expansion does occur in IVM COCs (51). The association between cumulus expansion and genes that are regulated by GDF9 in the mouse does not appear to be as direct in the primate. However, higher *PTGS2*, *HAS2*, and *GREM1* mRNA levels have been associated with better-quality oocytes and embryos in humans (46).

Third, we find evidence of remarkable compensatory changes in gene expression during IVM. Although many individual genes differ in regulation between IVM-CC and VVM-CC, this is offset by up- or downregulation of other genes

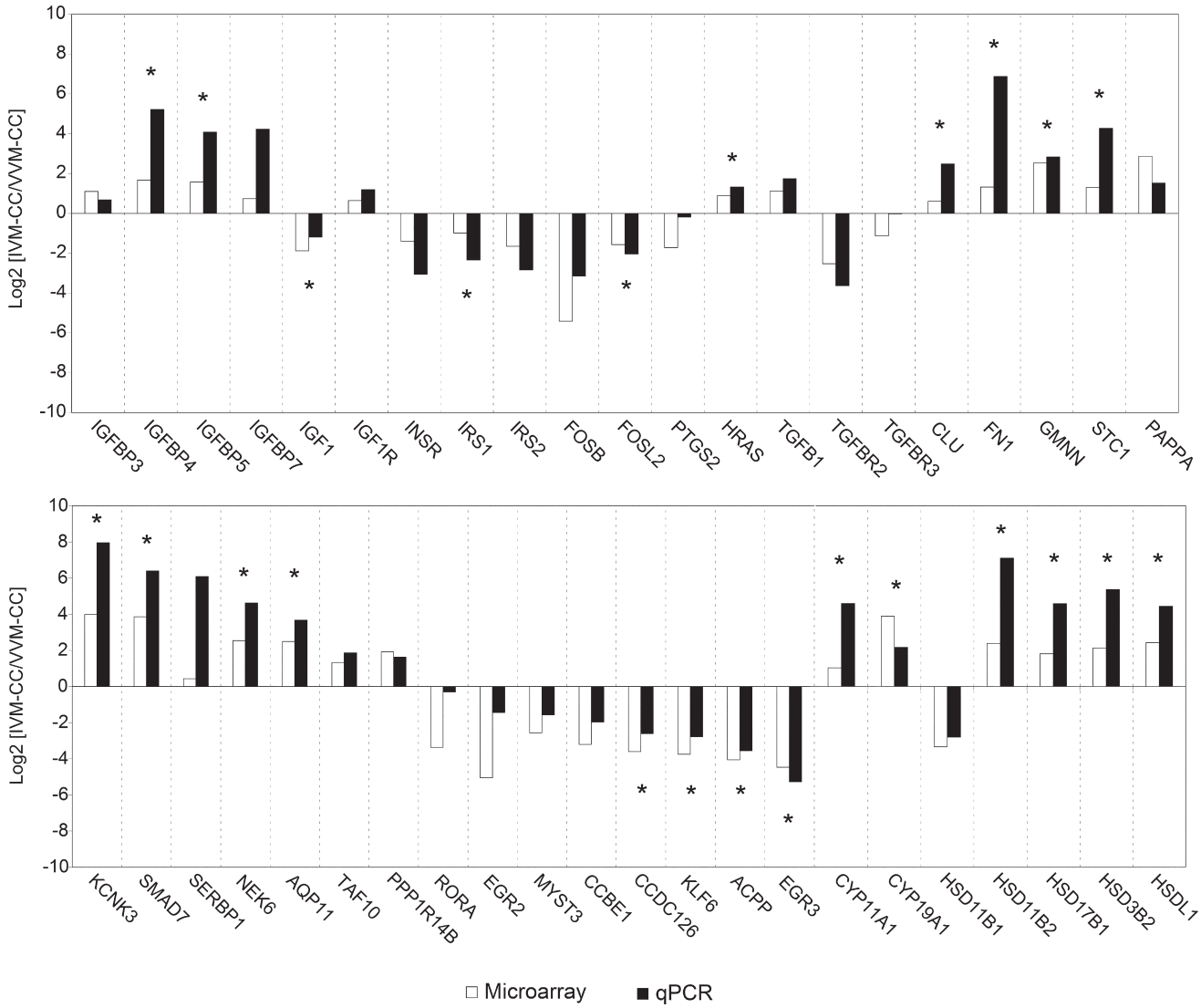


Fig. 4. Quantitative analysis of selected mRNAs by quantitative RT-PCR. *The 24 genes found by univariate analysis to be highly predictive of oocyte quality (see Supplemental Table S15). Gene symbols are defined in Supplemental Table S1.

related to specific cell functions. We observed this particularly for cell cycle arrest at the G_1/S phase transition, cell death, and cell growth and proliferation categories. This indicates that IVM-CC and VVM-CC may arrive at similar physiological states but that these similar states likely differ significantly at the level of individual regulatory gene expression. The consequences of such an altered, albeit similar, cumulus cell physiological state for the oocyte remain to be determined.

Many of the genes associated with cell cycle progression have been studied in cell types other than those in the ovarian follicle, which could partially explain some of the apparent differences found in this study. It is known that cell cycle progression decreases at ~24 h post-hCG in vivo (26), the time in which the VVM samples were obtained for this study. Furthermore, it has been demonstrated that cumulus cell morphology is different during IVM and VVM (20), which may also be reflected in the gene expression patterns of these two groups of cumulus cells.

Fourth, we found that among the genes affected in cumulus cells by IVM are those that likely contribute to productive cell-cell interactions between cumulus cell and oocyte. Our analysis of the IVM oocyte transcriptome likewise pointed to likely changes in cell-cell interactions (38). Here, we observe changes in the expression of growth factors and other ligands, their receptors, and other interactive cell surface molecules. For example, the *DLG1* gene (discs, large homolog 1) is responsible for the structural integrity of adherens junctions and tight junctions. The fact that IVM cumulus cells failed to upregulate this gene confirms again the compromised cell-to-cell interaction observed in IVM oocytes and cumulus cells. Early growth response 2 (*EGR2*) is another early response gene that is regulated downstream of MEK-ERK pathway (12, 34). The mRNA expression of *EGR2* in VVM cumulus cells tends to be maintained high, but in IVM cumulus cells the mRNA abundance for the *EGR2* gene was downregulated 18-fold (Supplemental Table S9).

The *SRGN* mRNA is less highly upregulated with IVM. The SRGN (PRG1) protein is best known for its role in formation of mast cell secretory granules and a granule-mediated apoptosis via the association with granzyme B and perforin (47, 58).

SRGN also binds to hyaluronic acid as well as its receptor CD44 and can inhibit the adhesion of cells to extracellular matrix proteins (25, 77). Serglycins influence many aspects of cellular processes such as matrix formation, cell-cell and cell-matrix adhesion, and cell proliferation and migration, and they are also implicated in ovarian development, ovulation, and fertilization (61). The fact that proteoglycan synthesis is regulated by gonadotropins suggests the involvement of these molecules in folliculogenesis (48). Also, it has been suggested that serglycins bind circulating lipoproteins to regulate their cellular uptake and concentration in follicular fluid (86). Previous studies showed that higher LDL concentrations in follicular fluid are associated with decreased oocyte fertilization rate (84). However, the direct association or mechanisms of serglycins in steroidogenic activity of cumulus cells have not yet been fully understood.

Additional evidence of defective cumulus cell-oocyte interaction was noted when comparing the actin and tubulin labeling of the transzonal processes (TZP) of cumulus cells from rhesus monkey COCs at various times following hCG in vivo or in vitro (20). The IVM COCs exhibited a remodeling of the tubulin component of the TZPs and retained the TZPs longer, yet the timing of nuclear maturation was significantly advanced in IVM oocytes. In contrast, the VVM COCs did not exhibit TZP remodeling, and the number of TZPs decreased within 3 h of hCG administration. These microarray results provide insight into the underlying basis for the aberrant responses of the IVM cumulus cells.

A fifth major finding is that many of the genes that are misregulated to the greatest degree during IVM are involved in lipid metabolism. Lipid metabolism is implicated as a key regulation pathway in cumulus cell maturation. Steroidogenesis and prostaglandin synthesis are the two main mechanisms that must be carefully regulated for COCs to achieve complete maturation. Cytochrome P450 genes are key genes involved in the maturation of mural cells. Among the various cytochrome P450 genes, *CYP19A1* in particular was incorrectly regulated during IVM. The *CYP19A1* mRNA abundance was significantly downregulated during VVM but not during IVM. The *CYP19A1* gene is implicated in steroidogenesis, particularly the synthesis of estrogen. It is well known that estrogens play a critical role in regulating homeostatic pathways and influence fertility, lipid metabolism, and other key biological pathways (70). During follicular development, the transcription of *CYP19A1* in ovarian mural granulosa cells is upregulated by FSH and prostaglandin E2 (15, 53), but later, with the surge of LH/hCG, *CYP19A1* gene expression must be rapidly downregulated. The downregulation of *CYP19A1* gene expression in mural cells is a characteristic of ovarian follicular differentiation (3, 49). The failure of IVM-CC to downregulate *CYP19A1* expression further indicates that IVM cumulus cells are likely compromised in their terminal differentiation.

Numerous genes involved in lipid metabolism are incorrectly regulated during IVM (Fig. 5). For example, *LDLR* and *STAR* genes are responsible for regulating the transfer of cholesterol. LDL receptor (*LDLR*) is a cell surface protein that takes up cholesterol-carrying LDL molecules (13), and *STAR* mediates the transfer of cholesterol to the inner mitochondrial membrane from outer membrane (71). The expression of these two mRNAs was upregulated during VVM but not IVM. Additional enzymes involved in various steps in lipid metabolism were misregulated during IVM (Fig. 5). The *CYP11A1* and *HSD3B2* mRNAs, encoding products involved in the breakdown of cholesterol to progesterone, are overexpressed during IVM, as was the mRNA for PGR. Progesterone plays a critical role in granulosa cell differentiation via the interaction with specific receptors (PGRs). The reduction of PGR gene expression is associated with better embryo quality in humans (29); therefore, the overexpression of PGR and the enzymes involved in the progesterone production in IVM-CC may decrease the developmental competence of IVM oocytes and embryos. The association of a higher level of *PGR* mRNA with COCs that will yield less competent embryos is consistent with the observation that higher-quality human embryos came from COCs with reduced levels of PGR (29). This classic nuclear PGR does not appear in mouse cumulus cells, although it is present in the granulosa cells of preovulatory follicles after the hCG stimulus (75), and similar transient expression in response to LH has also been demonstrated in the rat (54, 55). The rhesus cumulus cell microarray results appear to delineate another significant difference between primates and rodents.

Additionally, our data suggest that the synthesis of sex hormones is not suppressed during IVM. The enzymes that convert androstenedione into testosterone or estradiol, *HSD17B1* and *CYP19A1*, were downregulated during VVM but not during IVM. The IVM procedure seems to allow the continued production of androgens that is shut down during VVM. The abnormal regulation of lipid metabolism in IVM cumulus cells is also evidenced in the glucocorticoid and mineralocorticoid pathways. Glucocorticoids are steroid hormones that play an important role in the maintenance of physiological processes. Glucocorticoids mediate their functions by interacting with their receptors. Cortisol (or hydrocortisone) is the active form of glucocorticoids that can bind and activate glucocorticoid receptors, whereas corticosterone is the inactive form. HSD11β1 predominately generates active cortisol from inactive cortisone, whereas HSD11β2 converts active cortisol to inactive cortisone (Fig. 5). Glucocorticoids activated by HSD11β1 can upregulate the expression of PTGS2; both *HSD11B1* and *PTGS2* mRNAs were upregulated during VVM but not IVM.

While this article was in preparation, another study reported on changes in mRNA expression detected in whole follicles during oocyte maturation and ovulation (88). That study observed many of the changes in gene expression that we report here for cumulus cells. Our data thus pinpoint changes specifically associated with the cumulus cell. Moreover, our study particularly discloses the difference between in vitro and in vivo maturation. By comparing the developmental transitions from PM-CC to either IVM-CC or VVM-CC, our study highlights striking differences in gene regulation, such as delays in some gene expression changes and compensatory changes seen in other changes. Additionally, our study highlights the key interactions of cumulus cells with the follicular environment during the last 24 h of maturation, which are not apparent in analysis of whole follicles.

The identification of a panel of 24 genes, the expression of which correlates highly with protocols yielding high- or low-quality oocytes, may provide the foundation for understanding how IVM can be improved and for monitoring IVM conditions to recognize such improvements. Additionally, the availability

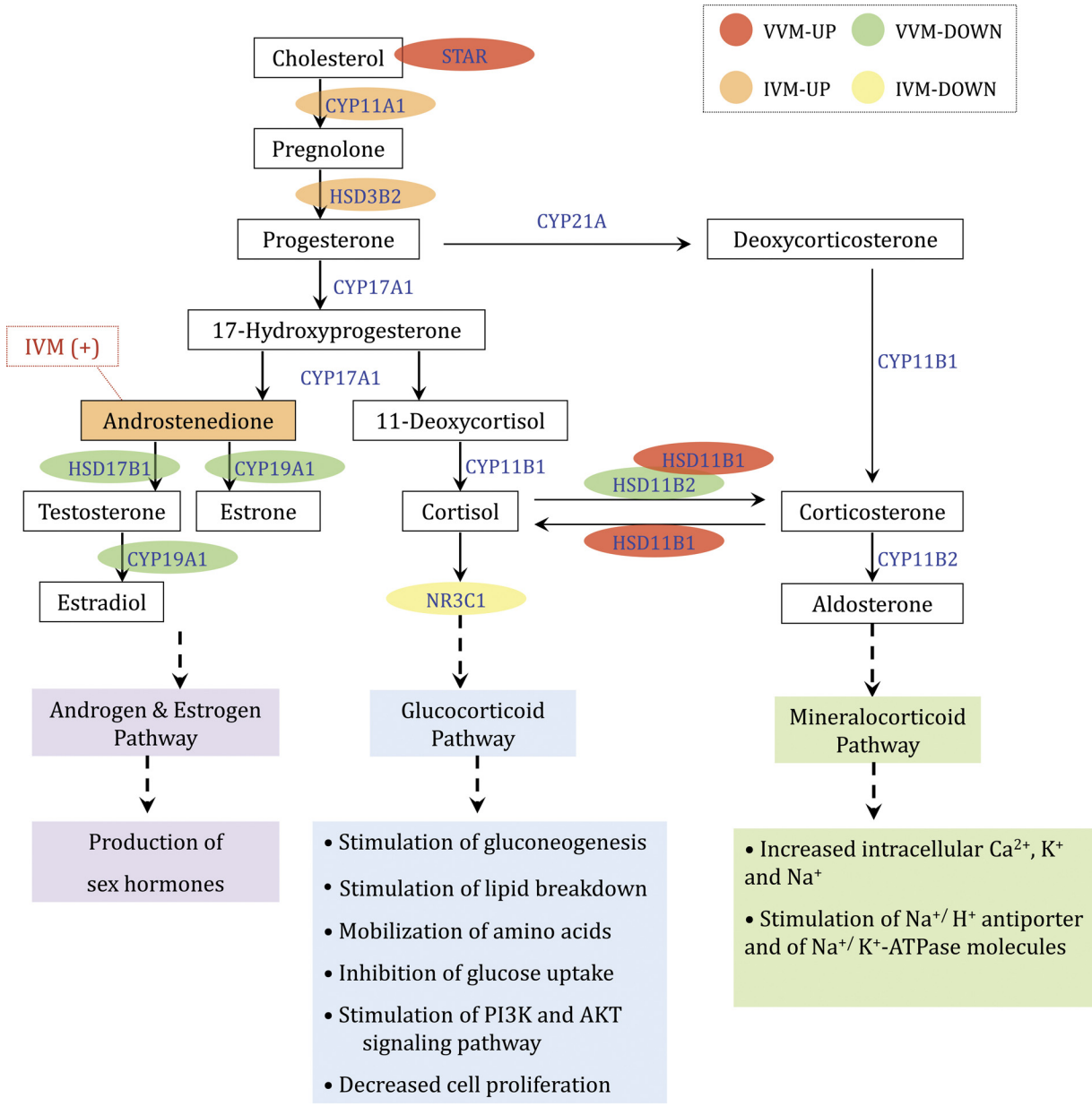


Fig. 5. Misregulation of genes involved in steroidogenesis during IVM. Numerous genes involved in steroidogenesis are incorrectly regulated during IVM. The misregulation of these genes may, in turn, compromise downstream pathways such as the production of androgen and estrogen, glucocorticoid, and mineralocorticoid pathways. PI3K, phosphatidylinositol 3-kinase.

of this panel of markers may be applicable for screening and identifying high- or low-quality oocytes. All or a subset of these markers could be adopted into a readily applicable PCR- or protein expression-based assay that could be applied to small clumps of cumulus cells isolated from individual COCs. This could allow clinical selection of the highest-quality oocytes in situations that require fertilized embryos to be transferred (35) and may provide an invaluable means of evaluating oocyte quality after in vitro oocyte culture and IVM, for example, in the context of oncofertility programs (87), or efforts to manipulate germinal vesicle stage oocytes in emerging assisted reproduction methods involving microsurgery (40, 43, 73, 80). These markers may also provide a means of optimizing reproductive biology approaches to enhance production of valuable agricultural species or enhance species conservation approaches. Evolutionary conservation of most of these genes (Supplemental Table S17) indicates the feasibility of application across species.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES

1. **Adriaenssens T, Segers I, Wathlet S, Smitz J.** The cumulus cell gene expression profile of oocytes with different nuclear maturity and potential for blastocyst formation. *J Assist Reprod Genet* 28: 31–40, 2011.
2. **Anderson RA, Sciorio R, Kinnell H, Bayne RA, Thong KJ, de Sousa PA, Pickering S.** Cumulus gene expression as a predictor of human oocyte fertilisation, embryo development and competence to establish a pregnancy. *Reproduction* 138: 629–637, 2009.
3. **Andric N, Thomas M, Ascoli M.** Transactivation of the epidermal growth factor receptor is involved in the lutropin receptor-mediated down-regulation of ovarian aromatase expression in vivo. *Mol Endocrinol* 24: 552–560, 2010.
4. **Assidi M, Dieleman SJ, Sirard MA.** Cumulus cell gene expression following the LH surge in bovine preovulatory follicles: potential early markers of oocyte competence. *Reproduction* 140: 835–852, 2010.
5. **Assidi M, Montag M, Van Der Ven K, Sirard MA.** Biomarkers of human oocyte developmental competence expressed in cumulus cells before ICSI: a preliminary study. *J Assist Reprod Genet* 28: 173–188, 2011.
6. **Assou S, Haouzi D, De Vos J, Hamamah S.** Human cumulus cells as biomarkers for embryo and pregnancy outcomes. *Mol Hum Reprod* 16: 531–538, 2010.
7. **Assou S, Haouzi D, Mahmoud K, Aouacheria A, Guillemin Y, Pantesco V, Reme T, Dechaud H, De Vos J, Hamamah S.** A non-invasive test for assessing embryo potential by gene expression profiles of human cumulus cells: a proof of concept study. *Mol Hum Reprod* 14: 711–719, 2008.
8. **Bachvarova RF.** A maternal tail of poly(A): the long and the short of it. *Cell* 69: 895–897, 1992.
9. **Barnes FL, Crombie A, Gardner DK, Kausche A, Lacham-Kaplan O, Suikkari AM, Tiglias J, Wood C, Trounson AO.** Blastocyst development and birth after in-vitro maturation of human primary oocytes, intracytoplasmic sperm injection and assisted hatching. *Hum Reprod* 10: 3243–3247, 1995.
10. **Bavister BD, Boatman DE, Collins K, Dierschke DJ, Eisele SG.** Birth of rhesus monkey infant after in vitro fertilization and nonsurgical embryo transfer. *Proc Natl Acad Sci USA* 81: 2218–2222, 1984.
11. **Boatman DE, Bavister BD.** Stimulation of rhesus monkey sperm capacitation by cyclic nucleotide mediators. *J Reprod Fertil* 71: 357–366, 1984.
12. **Bradley EW, Ruan MM, Oursler MJ.** Novel pro-survival functions of the Kruppel-like transcription factor Egr2 in promotion of macrophage colony-stimulating factor-mediated osteoclast survival downstream of the MEK/ERK pathway. *J Biol Chem* 283: 8055–8064, 2008.
13. **Brown MS, Goldstein JL.** A receptor-mediated pathway for cholesterol homeostasis. *Science* 232: 34–47, 1986.
14. **Burton PJ, Krozowski ZS, Waddell BJ.** Immunolocalization of 11beta-hydroxysteroid dehydrogenase types 1 and 2 in rat uterus: variation across the estrous cycle and regulation by estrogen and progesterone. *Endocrinology* 139: 376–382, 1998.
15. **Cai Z, Kwintkiewicz J, Young ME, Stocco C.** Prostaglandin E2 increases cyp19 expression in rat granulosa cells: implication of GATA-4. *Mol Cell Endocrinol* 263: 181–189, 2007.
16. **Caixeta ES, Ripamonte P, Franco MM, Junior JB, Dode MA.** Effect of follicle size on mRNA expression in cumulus cells and oocytes of Bos indicus: an approach to identify marker genes for developmental competence. *Reprod Fertil Dev* 21: 655–664, 2009.
17. **Cha KY, Koo JJ, Ko JJ, Choi DH, Han SY, Yoon TK.** Pregnancy after in vitro fertilization of human follicular oocytes collected from nonstimulated cycles, their culture in vitro and their transfer in a donor oocyte program. *Fertil Steril* 55: 109–113, 1991.
18. **Chian RC, Gilbert L, Huang JY, Demirtas E, Holzer H, Benjamin A, Buckett WM, Tulandi T, Tan SL.** Live birth after vitrification of in vitro matured human oocytes. *Fertil Steril* 91: 372–376, 2009.
19. **Curnow EC, Ryan JP, Saunders DM, Hayes ES.** In vitro developmental potential of macaque oocytes, derived from unstimulated ovaries, following maturation in the presence of glutathione ethyl ester. *Hum Reprod* 25: 2465–2474, 2010.
20. **de Prada JK, Hill DL, Chaffin CL, VandeVoort CA.** Nuclear maturation and structural components of nonhuman primate cumulus-oocyte complexes during in vivo and in vitro maturation. *Fertil Steril* 91: 2043–2050, 2009.
21. **Donahue RP, Stern S.** Follicular cell support of oocyte maturation: production of pyruvate in vitro. *J Reprod Fertil* 17: 395–398, 1968.
22. **Edgar R, Domrachev M, Lash AE.** Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res* 30: 207–210, 2002.
23. **Elvin JA, Clark AT, Wang P, Wolfman NM, Matzuk MM.** Paracrine actions of growth differentiation factor-9 in the mammalian ovary. *Mol Endocrinol* 13: 1035–1048, 1999.
24. **Eppig JJ, Wigglesworth K, Pendola FL.** The mammalian oocyte orchestrates the rate of ovarian follicular development. *Proc Natl Acad Sci USA* 99: 2890–2894, 2002.
25. **Ernst H, Zanin MK, Everman D, Hoffman S.** Receptor-mediated adhesive and anti-adhesive functions of chondroitin sulfate proteoglycan preparations from embryonic chicken brain. *J Cell Sci* 108: 3807–3816, 1995.
26. **Fru KN, Cherian-Shaw M, Puttabyatappa M, VandeVoort CA, Chaffin CL.** Regulation of granulosa cell proliferation and EGF-like ligands during the periovulatory interval in monkeys. *Hum Reprod* 22: 1247–1252, 2007.
27. **Gilchrist RB, Lane M, Thompson JG.** Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. *Hum Reprod Update* 14: 159–177, 2008.
28. **Gougeon A.** Regulation of ovarian follicular development in primates: facts and hypotheses. *Endocr Rev* 17: 121–155, 1996.
29. **Hasegawa J, Yanaihara A, Iwasaki S, Otsuka Y, Negishi M, Akahane T, Okai T.** Reduction of progesterone receptor expression in human cumulus cells at the time of oocyte collection during IVF is associated with good embryo quality. *Hum Reprod* 20: 2194–2200, 2005.
30. **Hattori M, Takesue K, Nishida N, Kato Y, Fujihara N.** Inhibitory effect of retinoic acid on the development of immature porcine granulosa cells to mature cells. *J Mol Endocrinol* 25: 53–61, 2000.
31. **Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP.** Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res* 31: e15, 2003.
32. **Jones GM, Cram DS, Song B, Magli MC, Gianaroli L, Lacham-Kaplan O, Findlay JK, Jenkin G, Trounson AO.** Gene expression profiling of human oocytes following in vivo or in vitro maturation. *Hum Reprod* 23: 1138–1144, 2008.
33. **Joyce IM, Pendola FL, O'Brien M, Eppig JJ.** Regulation of prostaglandin-endoperoxide synthase 2 messenger ribonucleic acid expression in mouse granulosa cells during ovulation. *Endocrinology* 142: 3187–3197, 2001.
34. **Keeton AB, Bortoff KD, Bennett WL, Franklin JL, Venable DY, Messina JL.** Insulin-regulated expression of Egr-1 and Krox20: dependence on ERK1/2 and interaction with p38 and PI3-kinase pathways. *Endocrinology* 144: 5402–5410, 2003.
35. **La Sala GB, Nicoli A, Capodanno F, Rondini I, Villani MT, Iannotti F.** The effect of the 2004 Italian law on outcomes of assisted reproduction technology in severe male factor infertility. *Reprod Biomed Online* 20: 2–10, 2010.
36. **Latham KE.** Epigenetic modification and imprinting of the mammalian genome during development. *Curr Top Dev Biol* 43: 1–49, 1999.
37. **Latham KE.** Mechanisms and control of embryonic genome activation in mammalian embryos. *Int Rev Cytol* 193: 71–124, 1999.
38. **Lee YS, Latham KE, Vandevoort CA.** Effects of in vitro maturation on gene expression in rhesus monkey oocytes. *Physiol Genomics* 35: 145–158, 2008.
39. **Leese HJ, Barton AM.** Production of pyruvate by isolated mouse cumulus cells. *J Exp Zool* 234: 231–236, 1985.
40. **Li GP, Chen DY, Lian L, Sun QY, Wang MK, Liu JL, Li JS, Han ZM.** Viable rabbits derived from reconstructed oocytes by germinal vesicle transfer after intracytoplasmic sperm injection (ICSI). *Mol Reprod Dev* 58: 180–185, 2001.
41. **Li X, Nokkala E, Yan W, Streng T, Saarinen N, Warri A, Huhtaniemi I, Santti R, Makela S, Poutanen M.** Altered structure and function of reproductive organs in transgenic male mice overexpressing human aromatase. *Endocrinology* 142: 2435–2442, 2001.
42. **Li X, Warri A, Makela S, Ahonen T, Streng T, Santti R, Poutanen M.** Mammary gland development in transgenic male mice expressing human P450 aromatase. *Endocrinology* 143: 4074–4083, 2002.

43. **Liu H, Wang CW, Grifo JA, Krey LC, Zhang J.** Reconstruction of mouse oocytes by germinal vesicle transfer: maturity of host oocyte cytoplasm determines meiosis. *Hum Reprod* 14: 2357–2361, 1999.
44. **Livak KJ, Schmittgen TD.** Analysis of relative gene expression data using real-time quantitative PCR and the 2[−Delta Delta C(T)] Method. *Methods* 25: 402–408, 2001.
45. **Matzuk MM, Burns KH, Viveiros MM, Eppig JJ.** Intercellular communication in the mammalian ovary: oocytes carry the conversation. *Science* 296: 2178–2180, 2002.
46. **McKenzie LJ, Pangas SA, Carson SA, Kovanci E, Cisneros P, Buster JE, Amato P, Matzuk MM.** Human cumulus granulosa cell gene expression: a predictor of fertilization and embryo selection in women undergoing IVF. *Hum Reprod* 19: 2869–2874, 2004.
47. **Metkar SS, Wang B, Aguilar-Santelises M, Raja SM, Uhlin-Hansen L, Podack E, Trapani JA, Froelich CJ.** Cytotoxic cell granule-mediated apoptosis: perforin delivers granzyme B-serglycin complexes into target cells without plasma membrane pore formation. *Immunity* 16: 417–428, 2002.
48. **Mueller PL, Schreiber JR, Lucky AW, Schulman JD, Rodbard D, Ross GT.** Follicle-stimulating hormone stimulates ovarian synthesis of proteoglycans in the estrogen-stimulated hypophysectomized immature female rat. *Endocrinology* 102: 824–831, 1978.
49. **Ndiaye K, Fayad T, Silversides DW, Sirois J, Lussier JG.** Identification of downregulated messenger RNAs in bovine granulosa cells of dominant follicles following stimulation with human chorionic gonadotropin. *Biol Reprod* 73: 324–333, 2005.
50. **Nyholt de Prada JK, Kellam LD, Patel BG, Latham KE, Vandevoort CA.** Growth hormone and gene expression of in vitro-matured rhesus macaque oocytes. *Mol Reprod Dev* 77: 353–362, 2010.
51. **Nyholt de Prada JK, Lee YS, Latham KE, Chaffin CL, VandeVoort CA.** Role for cumulus cell-produced EGF-like ligands during primate oocyte maturation in vitro. *Am J Physiol Endocrinol Metab* 296: E1049–E1058, 2009.
52. **Pangas SA, Jorgez CJ, Matzuk MM.** Growth differentiation factor 9 regulates expression of the bone morphogenetic protein antagonist gremlin. *J Biol Chem* 279: 32281–32286, 2004.
53. **Parakh TN, Hernandez JA, Grammer JC, Weck J, Hunzicker-Dunn M, Zeleznik AJ, Nilson JH.** Follicle-stimulating hormone/cAMP regulation of aromatase gene expression requires beta-catenin. *Proc Natl Acad Sci USA* 103: 12435–12440, 2006.
54. **Park-Sarge OK, Mayo KE.** Regulation of the progesterone receptor gene by gonadotropins and cyclic adenosine 3′,5′-monophosphate in rat granulosa cells. *Endocrinology* 134: 709–718, 1994.
55. **Park OK, Mayo KE.** Transient expression of progesterone receptor messenger RNA in ovarian granulosa cells after the preovulatory luteinizing hormone surge. *Mol Endocrinol* 5: 967–978, 1991.
56. **Peddinti D, Memili E, Burgess SC.** Proteomics-based systems biology modeling of bovine germinal vesicle stage oocyte and cumulus cell interaction. *PLoS One* 5: e11240, 2010.
57. **Pfaffl MW, Horgan GW, Dempfle L.** Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res* 30: e36, 2002.
58. **Raja SM, Wang B, Dantuluri M, Desai UR, Demeler B, Spiegel K, Metkar SS, Froelich CJ.** Cytotoxic cell granule-mediated apoptosis. Characterization of the macromolecular complex of granzyme B with serglycin. *J Biol Chem* 277: 49523–49530, 2002.
59. **Russell DL, Doyle KM, Gonzales-Robayna I, Pipaon C, Richards JS.** Egr-1 induction in rat granulosa cells by follicle-stimulating hormone and luteinizing hormone: combinatorial regulation by transcription factors cyclic adenosine 3′,5′-monophosphate regulatory element binding protein, serum response factor, sp1, and early growth response factor-1. *Mol Endocrinol* 17: 520–533, 2003.
60. **Russell JB, Knezevich KM, Fabian KF, Dickson JA.** Unstimulated immature oocyte retrieval: early versus midfollicular endometrial priming. *Fertil Steril* 67: 616–620, 1997.
61. **Salustri A, Camaioni A, Di Giacomo M, Fulop C, Hascall VC.** Hyaluronan and proteoglycans in ovarian follicles. *Hum Reprod Update* 5: 293–301, 1999.
62. **Schramm RD, Bavister BD.** Development of in-vitro-fertilized primate embryos into blastocysts in a chemically defined, protein-free culture medium. *Hum Reprod* 11: 1690–1697, 1996.
63. **Schramm RD, Bavister BD.** Effects of granulosa cells and gonadotrophins on meiotic and developmental competence of oocytes in vitro in non-stimulated rhesus monkeys. *Hum Reprod* 10: 887–895, 1995.
64. **Schramm RD, Bavister BD.** Follicle-stimulating hormone priming of rhesus monkeys enhances meiotic and developmental competence of oocytes matured in vitro. *Biol Reprod* 51: 904–912, 1994.
65. **Schramm RD, Bavister BD.** A macaque model for studying mechanisms controlling oocyte development and maturation in human and non-human primates. *Hum Reprod* 14: 2544–2555, 1999.
66. **Schramm RD, Paprocki AM, VandeVoort CA.** Causes of developmental failure of in-vitro matured rhesus monkey oocytes: impairments in embryonic genome activation. *Hum Reprod* 18: 826–833, 2003.
67. **Schultz RM.** The molecular foundations of the maternal to zygotic transition in the preimplantation embryo. *Hum Reprod Update* 8: 323–331, 2002.
68. **Segi E, Haraguchi K, Sugimoto Y, Tsuji M, Tsunekawa H, Tamba S, Tsuboi K, Tanaka S, Ichikawa A.** 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: 804–811, 2003.
69. **Sharma SC, Richards JS.** Regulation of AP1 (Jun/Fos) factor expression and activation in ovarian granulosa cells. Relation of JunD and Fra2 to terminal differentiation. *J Biol Chem* 275: 33718–33728, 2000.
70. **Simpson ER, Misso M, Hewitt KN, Hill RA, Boon WC, Jones ME, Kovacic A, Zhou J, Clyne CD.** Estrogen—the good, the bad, and the unexpected. *Endocr Rev* 26: 322–330, 2005.
71. **Strauss JF 3rd, Kallen CB, Christenson LK, Watari H, Devoto L, Arakane F, Kiriakidou M, Sugawara T.** The steroidogenic acute regulatory protein (StAR): a window into the complexities of intracellular cholesterol trafficking. *Recent Prog Horm Res* 54: 369–394; discussion 394–395, 1999.
72. **Su YQ, Sugiura K, Eppig JJ.** Mouse oocyte control of granulosa cell development and function: paracrine regulation of cumulus cell metabolism. *Semin Reprod Med* 27: 32–42, 2009.
73. **Takeuchi T, Rosenwaks Z, Palermo GD.** A successful model to assess embryo development after transplantation of prophase nuclei. *Hum Reprod* 19: 975–981, 2004.
74. **Tamba S, Yodoi R, Morimoto K, Inazumi T, Sukeno M, Segi-Nishida E, Okuno Y, Tsujimoto G, Narumiya S, Sugimoto Y.** Expression profiling of cumulus cells reveals functional changes during ovulation and central roles of prostaglandin EP2 receptor in cAMP signaling. *Biochimie* 92: 665–675, 2010.
75. **Teilmann SC, Clement CA, Thorup J, Byskov AG, Christensen ST.** Expression and localization of the progesterone receptor in mouse and human reproductive organs. *J Endocrinol* 191: 525–535, 2006.
76. **Tesfaye D, Ghanem N, Carter F, Fair T, Sirard MA, Hoelker M, Schellander K, Lonergan P.** Gene expression profile of cumulus cells derived from cumulus-oocyte complexes matured either in vivo or in vitro. *Reprod Fertil Dev* 21: 451–461, 2009.
77. **Toyama-Sorimachi N, Sorimachi H, Tobita Y, Kitamura F, Yagita H, Suzuki K, Miyasaka M.** A novel ligand for CD44 is serglycin, a hematopoietic cell lineage-specific proteoglycan. Possible involvement in lymphoid cell adherence and activation. *J Biol Chem* 270: 7437–7444, 1995.
78. **Trounson A, Wood C, Kausche A.** In vitro maturation and the fertilization and developmental competence of oocytes recovered from untreated polycystic ovarian patients. *Fertil Steril* 62: 353–362, 1994.
79. **Tseng L, Mazella J.** Endometrial cell specific gene activation during implantation and early pregnancy. *Front Biosci* 7: d1566–d1574, 2002.
80. **Tucker MJ, Morton PC, Wright G, Sweitzer CL, Massey JB.** Clinical application of human egg cryopreservation. *Hum Reprod* 13: 3156–3159, 1998.
81. **Tusher VG, Tibshirani R, Chu G.** Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci USA* 98: 5116–5121, 2001.
82. **VandeVoort CA, Tarantal AF.** The macaque model for in vitro fertilization: superovulation techniques and ultrasound-guided follicular aspiration. *J Med Primatol* 20: 110–116, 1991.
83. **VandeVoort CA, Tarantal AF.** Recombinant human gonadotropins for macaque superovulation: repeated stimulations and post-treatment pregnancies. *J Med Primatol* 30: 304–307, 2001.
84. **Volpe A, Coukos G, Uccelli E, Droghini F, Adamo R, Artini PG.** Follicular fluid lipoproteins in preovulatory period and their relationship with follicular maturation and progesterone production by human granulosa-luteal cells in vivo and in vitro. *J Endocrinol Invest* 14: 737–742, 1991.

85. **Wang H, Li R, Hu Y.** The alternative noncoding exons 1 of aromatase (Cyp19) gene modulate gene expression in a posttranscriptional manner. *Endocrinology* 150: 3301–3307, 2009.
86. **Williams KJ, Fuki IV.** Cell-surface heparan sulfate proteoglycans: dynamic molecules mediating ligand catabolism. *Curr Opin Lipidol* 8: 253–262, 1997.
87. **Woodruff TK.** The Oncofertility Consortium—addressing fertility in young people with cancer. *Nat Rev Clin Oncol* 7: 466–475, 2010.
88. **Xu F, Stouffer RL, Müller J, Hennebold JD, Wright JW, Bahar A, Leder G, Peters M, Thorne M, Sims M, Wintermantel T, Lindenthal B.** Dynamics of the transcriptome in the primate ovulatory follicle. *Mol Hum Reprod* 17: 152–165, 2011.
89. **Yang SH, Qin SL, Xu Y, Yoon SH, Chian RC, Lim JH.** Healthy live birth from vitrified blastocysts produced from natural cycle IVF/IVM. *Reprod Biomed Online* 20: 656–659, 2010.
90. **Yoon HG, Yoon SH, Son WY, Lee SW, Park SP, Im KS, Lim JH.** Pregnancies resulting from in vitro matured oocytes collected from women with regular menstrual cycle. *J Assist Reprod Genet* 18: 325–329, 2001.
91. **Zhang M, Su YQ, Sugiura K, Xia G, Eppig JJ.** Granulosa cell ligand NPPC and its receptor NPR2 maintain meiotic arrest in mouse oocytes. *Science* 330: 366–369, 2010.
92. **Zhang X, Jafari N, Barnes RB, Confino E, Milad M, Kazer RR.** Studies of gene expression in human cumulus cells indicate pentraxin 3 as a possible marker for oocyte quality. *Fertil Steril* 83, *Suppl* 1: 1169–1179, 2005.