Differential regulation of cumulus cell transcription during oocyte maturation in vivo and in vitro

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ABSTRACT Differences in cumulus cell gene expression after oocyte maturation in vitro (IVM) or in vivo have been described in previous studies. However, the possible impact of follicle stage on gene expression deregulation during human oocyte IVM remains unknown. Expression of selected genes of interest was compared in cumulus cell of three classes of human cumulus cell-oocyte complexes (COCs): a) COCs derived from human chorionic gonadotropin (hCG)-triggered IVM cycles, collected at the germinal vesicle (GV) stage from mid-sized follicles (4-12 mm) and matured in vitro (IVM-GV); b) COCs derived from hCG-triggered IVM cycles, collected from mid-sized follicles (4-12 mm) and matured in vivo (IVM-MII); c) COCs derived from controlled ovarian stimulation in vitro fertilization (IVF) cycles, collected from large/preovulatory follicles and matured in vivo (IVF-MII). Overall, mRNA levels of the large majority of the 20 genes of different regulative and metabolic pathways subject to analysis were altered in IVM samples compared with in vivo matured COCs. In some cases, follicle size appeared to have a role in determining transcription deregulation. For example, in comparison to the IVF-MII control, the luteinizing hormone receptor was largely overexpressed in both IVM-GV and IVM-MII COCs, therefore irrespective of IVM. However, in other circumstances follicle size and IVM had distinct and opposite impacts on gene expression, as shown by transcription of amphiregulin, which was increased in IVM-MII COCs, but decreased in COCs matured in vitro (IVM-GV) compared with the IVF-MII control. This study confirms and extends previous data on gene expression dysregulation during IVM and indicates that the size of follicles from which immature oocytes are retrieved can be an independent factor of differential transcriptional regulation.

KEY WORDS: oocyte, in vitro maturation, gene expression, mRNA, cumulus cell, follicle

Oocyte-cumulus cell interactions are realized through different signalling modalities and respond to a wide variety of demands of the maturing oocyte, including outsourcing of anabolites and catabolites (Su et al., 2007; Sugiuira et al., 2008), control of meiotic arrest and resumption (Wigglesworth et al., 2013), and regulation of organelle and cytoskeletal rearrangements (Barrett and Albertini, 2010; Coticchio et al., 2015). By revealing the mutual interdependence between oocyte and cumulus cells, oocyte in vitro maturation (IVM) studies have suggested the concept that biochemical or molecular traits expressed by cumulus cells offer important clues of the process of oocyte maturation and, therefore, can represent biomarkers of oocyte quality (Labrecque and Sirard, 2014).

In a previous study, applying a global RNA sequencing methodology and assessing comparatively the cumulus cell transcriptome before and after maturation in vivo, we identified a library of genes regulated during cumulus expansion and oocyte maturation (Yerushalmi et al., 2014). Analysis of such sequences led us to characterise new important genes, transcription factors and non-coding RNAs presumably involved in the function of the cumulus cell-oocyte complex (COC) during maturation. In the present study, we extend such findings by comparing, after maturation in vivo or

Abbreviations used in this paper: AREG, amphiregulin; COC, cumulus cell-oocyte complex; EREG, epiregulin; FSH, follicle-stimulating hormone; FSHR, follicle-stimulating hormone receptor; GV, germinal vesicle; hCG, human chorionic gonadotropin; IVF, in vitro fertilization; IVM, in vitro maturation; LHR, luteinizing hormone receptor.
in vitro, the expression in cumulus cells of selected genes of interest. Use of appropriate material ("genuine" in vitro matured COCs obtained from IVM cycles, rather than leftover immature oocytes form stimulated cycles) and suitable controls (in vivo matured COCs collected from both mid-antral and preovulatory follicles) make our findings particularly informative and probably unique. Overall, our results are indicative of significant changes occurring during maturation in cumulus cell gene expression as an effect of IVM, or indeed in specific cases follicle size, and suggest important reflections relevant to the improvement of IVM as a clinical tool.

**Results**

Overall, the transcripts of 20 genes (Table 1) were quantitatively assessed by qRT-PCR (Fig. 1). These genes were selected for their involvement in crucial cumulus cell functions (Table 2).

**Follicle-stimulating hormone receptor (FSHR) and luteinizing hormone receptor (LHR) gene expression**

Significant differences were observed in the case of the LHR gene which, in comparison to the IVF-MII control, was largely overexpressed in both IVM-GV and IVM-MII COCs.

**Expression of paracrine regulation genes**

Transcription of amphiregulin (AREG) was increased in IVM-MII COCs, but decreased in COCs matured in vitro (IVM-GV). Likewise, compared with the control, epiregulin (EREG) expression was 4-fold higher in IVM-MII COCs. GREM1 expression was instead 4-fold increased in COCs matured in vitro, but not in IVM-MII. Finally, BMPR2 mRNA levels were moderately increased only in IVM-GV COCs.

**Signalling cascade gene expression**

After IVM, SFRP4 was 8-fold more expressed in comparison with IVF-MII and IVF-MII COCs. The expression of NOS2 was also increased in in vitro matured COCs. Instead, relative levels of SFRP5 mRNA were 2-fold lower in COCs matured in vitro, compared with the control and the IVM-MII group.

**Steroidogenesis gene expression**

Compared with the IVF-MII control CYP11A1, involved in the conversion of cholesterol into pregnenolone, was three-fold more

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**Table 1**

| Gene Name | Accession | Product size | Sequence 5' to 3' | Name
|-----------|-----------|--------------|-------------------|-----|
| FSHR      | NM_000165 | 123 bp       | GCTGAGGATCGATCCCA | FSHR_F
|           |           |              | CTACCACGCTGACTGAT | FSHR_R
| LHR       | NM_006988 | 135 bp       | AAGGACAGGTGCAAAGCTGAT | LHR_F
|           |           |              | GGGCAGGAGTGGGTCGTGAT | LHR_R
| BMPR2     | NM_001204 | 150 bp       | GTCCTGGATGGCAGCAGTAT | BMPR2_F
|           |           |              | GCTTGGTGAGGACAAACCTT | BMPR2_R
| AREG      | NM_001657 | 94 bp        | AGCCGACTATGACTACCTGAC | AREG_F
|           |           |              | CTCCAATGCTGTCACTCTAG | AREG_R
| EREG      | NM_000963 | 150 bp       | GGTGAGGATCGATCCCA | EREG_F
|           |           |              | TCCAATGCTGTCACTCTAG | EREG_R
| STAR      | NM_001465 | 130 bp       | GCTGAGGATCGATCCCA | STAR_F
|           |           |              | TCCAATGCTGTCACTCTAG | STAR_R
| SFRP4     | NM_003015 | 89 bp        | GGTGAGGATCGATCCCA | SFRP4_F
|           |           |              | TCCAATGCTGTCACTCTAG | SFRP4_R
| SFRP5     | NM_006759 | 89 bp        | GGTGAGGATCGATCCCA | SFRP5_F
|           |           |              | TCCAATGCTGTCACTCTAG | SFRP5_R
| CYP11A1   | NM_00103  | 135 bp       | GCTGAGGATCGATCCCA | CYP11A1_F
|           |           |              | TCCAATGCTGTCACTCTAG | CYP11A1_R
| CYP19A1   | NM_000145 | 123 bp       | GCTGAGGATCGATCCCA | CYP19A1_F
|           |           |              | TCCAATGCTGTCACTCTAG | CYP19A1_R
| GPX3      | NM_006759 | 89 bp        | GGTGAGGATCGATCCCA | GPX3_F
|           |           |              | TCCAATGCTGTCACTCTAG | GPX3_R
| PTGS2     | NM_00663  | 89 bp        | GGTGAGGATCGATCCCA | PTGS2_F
|           |           |              | TCCAATGCTGTCACTCTAG | PTGS2_R
| UGP2      | NM_006759 | 89 bp        | GGTGAGGATCGATCCCA | UGP2_F
|           |           |              | TCCAATGCTGTCACTCTAG | UGP2_R

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**Fig. 1.** qRT-PCR analysis of expression levels of a panel of genes (see Table 2 for detailed description) in cumulus cells from cumulus-oocyte complexes (COCs) collected from 4-12 mm follicles and matured in vitro (IVM-GV, blue bars) or in vivo (IVM-MII, red bars). Values were normalized with respect to actin expression and plotted as fold changes with respect to expression in cumulus cells of COCs matured in vivo and collected from large/preovulatory follicles (IVF-MII), which was set to 1. Results were reported as mean ± SEM. P-values <0.05 were considered statistically significant.
expressed in COCs matured in vitro, but not in IVM-MII. mRNA levels of the aromatase gene (CYP19A1) were underexpressed in both types of COCs collected from 4-12 mm follicles and matured in vivo or in vitro.

**Cumulus expansion and prostaglandin biosynthesis gene expression**

The expression of the HAS2 gene, involved in cumulus expansion, was reduced in COCs collected from hCG-primed IVM cycles, particularly in the IVM-MII group. mRNA levels of PTGS2, having a role in prostaglandin biosynthesis, were also consistently reduced in both IVM-GV and IVM-MII COCs.

**Hydrogen peroxide detoxification and glycosaminoglycan metabolism gene expression**

GPX3 and UGP2 were also dysregulated following in vitro maturation. The former was more than 2-fold more expressed in IVM-GV, but not IVM-MII, samples. mRNA of the latter gene was instead underexpressed in IVM-GV COCs and overexpressed in IVM-MII samples.

**Cell coupling and intercellular remodelling gene expression**

GJA1, a gene playing a role in gap junction communication, was 14- and 8-fold more expressed in IVM-GV and IVM-MII COCs, respectively, while the expression pattern of ADAMTS1, whose action is required for tissue remodelling during folliculogenesis, had an opposite profile.

**Discussion**

Collective experience suggests that our ability to reproduce efficiently in vitro the complex and delicate process of oocyte maturation remains limited. This has reflections in the developmental competence of oocytes matured in vitro and, consequently, the clinical outcome of IVM treatments. Oocyte maturation after IVM can be studied through a variety approaches many of which are however invasive (Coticchio et al., 2016). Transcription analysis of cumulus cells represents an excellent alternative, being totally non-invasive and significantly informative of oocyte quality and attendant maturation processes (Uyar et al., 2013). In such a context, we trust that the present work offers valuable information, especially taking into account the two levels of control included in the study design. In fact, not only was cumulus cell gene expression after IVM compared with a condition in which oocyte maturation occurred in vivo, but also it was assessed relative to follicle stage, which per se can represent a source of variability.

Overall, our results describe a situation in which after IVM cumulus cell gene expression is largely dysregulated. In general, although not in all cases, after IVM mRNA levels were found altered compared with in vivo matured COCs collected from both mid-sized and large/preovulatory follicles, leading to the conclusion that dysregulation was not a consequence of the different follicle origin but rather an effect of IVM conditions. This is an important inference derived from our data, because previous studies did not address the specific point of the implications of IVM relative to follicle size.

** Gonadotropin receptor genes**

Expression of the LHR gene increases as follicles expand (Maman et al., 2012; Yung et al., 2014), but in mural granulosa cells it undergoes major repression after exposure to ovulatory doses of hCG (Jeppesen et al., 2012). We did not observed underexpression of this gene in in vitro matured and IVM-MII COCs. This suggests that, after oocyte maturation, repression may be hindered by an immature mid-antral stage follicular environment. Information regarding the expression of this receptor gene has practical implications for IVM technology, as LH is one of the major biomolecules adopted to support IVM.

**Paracrine regulation genes**

Following IVM, the mRNAs of the AREG and EREG genes were less abundant compared with a situation in which maturation occurred in vivo in large antral follicles. In addition, the fact that in IVM-MII COCs levels of the AREG and EREG transcripts were not reduced, but in fact increased, rules out that the follicle stage (mid-antral, in both IVM-GV and IVM-MII COCs) plays a role in determining a reduced transcription of the AREG and EREG genes in in vitro matured COCs. Instead, levels of ERBB2 were not reduced following IVM. This is suggestive of a possible deficiency in the production of amphiregulin and epiregulin during in vitro maturation, while sensitivity assured by their receptor probably remains unaltered.

**Steroidogenesis genes**

The expression of genes having a role in the conversion of cholesterol to pregnenolone was not affected by IVM, with the exception of CYP11A1 whose mRNA was more abundant in IVM-GV COCs. Conversely, mRNA levels of the aromatase gene (CYP19A1), crucial for the production of estrogens, were lower in both IVM-GV and IVM-MII, thus suggesting that a smaller follicle size, rather than IVM, may be the cause of a reduced expression. This finding is of particular relevance because estrogens synthesis is a distinct sign of healthy communication between oocyte and cumulus cells especially during oocyte maturation (Kidder and

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**TABLE 2**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>3BHSD</td>
<td>3beta-hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>ADAMTS1</td>
<td>ADAM metallopeptidase with thrombospondin type 1 motif 1</td>
</tr>
<tr>
<td>AREG</td>
<td>amphiregulin</td>
</tr>
<tr>
<td>BMP2</td>
<td>bone morphogenetic protein receptor type II</td>
</tr>
<tr>
<td>CYP11A1</td>
<td>cytochrome P450 family 11 subfamily A member 1</td>
</tr>
<tr>
<td>CYP19A1</td>
<td>cytochrome P450 family 19 subfamily A member 1</td>
</tr>
<tr>
<td>ERBB2</td>
<td>erb-b2 receptor tyrosine kinase 2</td>
</tr>
<tr>
<td>EREG</td>
<td>epiregulin</td>
</tr>
<tr>
<td>FSHR</td>
<td>follicle stimulating hormone receptor</td>
</tr>
<tr>
<td>GJA1</td>
<td>gap junction protein alpha 1</td>
</tr>
<tr>
<td>GPX3</td>
<td>glutathione peroxidase 3</td>
</tr>
<tr>
<td>GREM1</td>
<td>gremlin 1, DAN family BMP antagonist</td>
</tr>
<tr>
<td>HAS2</td>
<td>hyaluronan synthase 2</td>
</tr>
<tr>
<td>LHR</td>
<td>luteinizing hormone/choriogonadotropin receptor</td>
</tr>
<tr>
<td>NOS2</td>
<td>nitric oxide synthase 2</td>
</tr>
<tr>
<td>PTGS2</td>
<td>prostaglandin-endoperoxide synthase 2</td>
</tr>
<tr>
<td>SFRP4</td>
<td>secreted frizzled related protein 4</td>
</tr>
<tr>
<td>SFRP5</td>
<td>secreted frizzled related protein 5</td>
</tr>
<tr>
<td>STAR</td>
<td>steroidogenic acute regulatory protein</td>
</tr>
<tr>
<td>UGP2</td>
<td>steroidogenic acute regulatory protein</td>
</tr>
</tbody>
</table>

Vanderhyden, 2010). In addition, high levels of estradiol contribute to enhance the synthesis of Natriuretic Peptide Type C (NPPC) and its Natriuretic Peptide Receptor 2 (NPR2), which are essential element that control meiotic arrest and resumption in mouse oocytes (Lee et al., 2013).

**Cumulus expansion and prostaglandin biosynthesis genes**

HAS2 mRNA was more expressed in IVF-MII COCs. The fact that it tended to be equally less represented in IVM-GV and IVM-MII indicates that IVM does not have a specific effect on the expression of the HAS2 gene. Rather, COCs of mid-antral follicles seem to have a limited transcriptional competence, irrespective of whether oocyte maturation occurs *in vitro* or *in vivo*. This is consistent with the finding that human COCs matured *in vitro* display very little expansion, if any (Coticchio et al., 2012). A similar situation was observed for the PTGS2 mRNA, whose levels where lower in both IVM-GV and IVM-MII. Therefore, follicle stage, but not IVM, appear to affect also the expression of the cyclooxygenase-2 gene.

**Cell coupling and intercellular remodelling genes**

The GJA1 gene, encoding the connexin43 protein, is known to be expressed at low levels after maturation *in vivo* in large antral and preovulatory follicles. Our data point towards a loss of such a regulation. In fact, the GJA1 gene was much more expressed in both types of COCs obtained from hCG-primed IVM cycles, especially in IVM-GV samples. This suggest that IVM and follicle size may be independently and probably additively responsible for the deregulation of the GJA1 gene.

An opposite profile of regulation was observed in the case of the ADAMTS1 gene whose expression was lower in both IVM-GV and IVM-MII COCs compared with the IVF-MII control. This may have significant implications for oocyte quality, in the light of our previous study in which we observed a direct correlation between levels of ADAMTS1 mRNA and the oocyte ability to support fertilization (Yung et al., 2010).

Other genes analysed in this study, involved in hydrogen peroxide detoxification, glycosaminoglycan metabolism and signalling cascade were also largely deregulated.

**Implications and conclusions**

Overall, our results suggest that although oocyte meiotic maturation may be achieved apparently successfully *in vitro*, it is nevertheless accompanied by extensive transcription deregulation, confirming data of previous reports (Guzman et al., 2013). Importantly, our analysis adds another important layer of information to the question of IVM and gene expression in cumulus cells. This was made possible by the adoption of two types of control of *in vivo* matured COCs, i.e. IVF-MII and IVM-MII, in which oocyte maturation occurred in large/preovulatory and mid-sized follicles, respectively. Therefore, we were able to discriminate whether deregulation occurred exclusively as a consequence of IVM and/or a collateral effect due to follicle size. The extensive gene expression differences found in this study between *in vivo* and *in vitro* matured COCs may be therefore interpreted as a sign that human oocyte IVM is still largely suboptimal, in agreement with previous clinical evidence (Coticchio et al., 2012; Dal Canto et al., 2012).

Finally, we hypothesise that information derived from our investigation may offer cues for improving the culture conditions under which human IVM is achieved. For example, our data suggest that during IVM the AREG and EREG genes are downregulated and therefore the end products of those genes should be provided exogenously.

In conclusion, this study confirms and extends previous data on gene expression dysregulation during IVM and indicates that in specific cases follicle size/stage during antral development can be an independent source of differential transcriptional regulation.

**Material and Methods**

**Source of oocytes**

This study was approved by the ethical committee of the participating clinics. Written informed consent was obtained from each patient who provided samples.

**In vitro maturation protocol**

Patients candidates for IVM treatment were selected and treated as previously described (Fadini et al., 2009). Treatment with 150 IU/day rFSH was carried out for 3 days form day 3 of cycle. On Day 6, 10,000 IU hCG (Pregnyl; MSD, Rome, Italy) were administered when the endometrial thickness was ≥5 mm and the leading follicle was 11-12 mm. Oocyte retrieval was carried out 36 hours later under transvaginal ultrasound guidance.

Follicular aspirates from mid-sized follicles (4-12 mm) were filtered through a 70 μm cell strainer (Becton-Dickinson, Buccinaco, Italy) and washed twice. COCs were detected under a stereomicroscope and thoroughly washed. COCs were classified according to cumulus oophorus morphology and stage of oocyte maturation after culture:

- Compact COCs, formed from multiple layers of compact cumulus cells enclosing a GV-stage oocyte, were transferred to IVM Medium (Origio, Maløv, Denmark) supplemented with recombinant FSH 0.075 IU/ml (Merck, Rome, Italy) and hCG 0.10 IU/ml (Merck, Rome, Italy). IVM was carried out at 37°C in a 6% CO₂ humidified atmosphere for 30 hours. Following IVM, cumulus cells were collected only from COC associated to a MI oocyte (IVM-GV).
- COCs with an expanded culture were cultured for 4 hours in IVM medium (Origio, Malov, Denmark) and cumulus cells associated to a MI oocyte (IVM-MII) were isolated for further processing. Oocyte maturation requires 24-30 hours. Therefore, in such COC oocyte maturation occurred *in vivo*.

**In vitro fertilization protocol**

Normo-ovulatory young women (< 37 years of age) undergoing IVF because of male factor infertility or pre-implantation genetic diagnosis were selected for this study. Ovarian stimulation was carried out as previously described through a “long agonist” or a “short antagonist” protocol (Maman et al., 2011). Ovarian stimulation was achieved with personalised doses of r-FSH (Gonal-F, Merk) and *in vivo* matured oocytes (IVF-MII) were collected from preovulatory follicles after administration of 250 μg of hCG (Merck).

**Cumulus cell processing**

Collected cumulus cells of the three sample types were washed, centrifuged at 200 g and stored in liquid nitrogen. Cumulus cells of two to three patients were pooled for each sample type, to compensate for patient heterogeneity and low RNA amount retrieved from the cumulus of single COCs.

**RNA processing and analysis**

Total RNA was extracted from cumulus cells by a mini RNA Isolation I Kit (Zymo Research Corp., CA, USA). Expression levels of transcripts of interest were measured by qRT-PCR with a high-capacity cDNA RT kit (Applied Biosystems, Foster City, CA, USA). Using appropriate primers (Table 1), amplification and detection were performed using the StepOne-Plus real-time PCR system (Applied Biosystems). All samples were done in
duplicates. Actin beta (ACTB) mRNA was chosen as a housekeeping gene. mRNA quantifications were obtained by the comparative Ct method, using DDCt. Results were expressed as fold change with respect to the IVF-MII group, i.e. COCs matured in vivo and collected from large/preovulatory follicles, that was set to 1. Student’s t-test with a two-tailed distribution and two samples equal variance testing was used to compare samples for the normalization of data. For all statistical analysis of qRT-PCR, SPSS software (IBM, Armonk, NY, USA) was used. Difference in measurable gene expression 2-fold was considered biologically significant. P-values <0.05 were considered statistically significant.

References


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