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

GIOVANNI COTICCHIO*[,1], LIBBY OPHIR[2], YUVAL YUNG[2], MICHA BAUM[2], MARIABEATRICE DAL CANTO[1], MARIO MIGNINI-RENZINI[1]; FAUSTA BRAMBILLASCA[1], RUBENS FADINI[1] and ARIEL HOURVITZ[2]

[1] Biogenesi Reproductive Medicine Centre, Istituti Clinici Zucchi, Monza, Italy and
[2]IVF Unit and Reproduction Lab, Department of Obstetrics and Gynaecology, Sheba Medical Centre, Tel Hashomer, Affiliated with the Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

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; Sugiura *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.

***Address correspondence to:** Giovanni Coticchio. Biogenesi Reproductive Medicine Centre, Istituti Clinici Zucchi, Via Zucchi, 24 Monza, Italy. Tel: +39-039-8383314; Fax: +39-039-838-3314. E-mail: coticchio.biogenesi@grupposandonato.it - http://orcid.org/0000-0003-1635-9205

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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

TABLE 1

PRIMERS USED FOR qRT-PCR OF THE mRNAs LISTED IN TABLE 2

Name	Sequence 5' to 3'	Product size	Accession
3BHSD _F	GCTGAGGAGATCAGCATCCA	130bp	M58567.1
3BHSD _R	AGCCATGGTCAACACAGGAA		
ADAMTS1_F	AAGGACAGGTGCAAGCTCAT	104bp	NM_006988
ADAMTS1_R	GAGGTGGAATCTGGGCTACA		
AREG_F	AGCCGACTATGACTACTCAG	94bp	NM_001657.3
AREG_R	CTTAACTACCTGTTCAACTCTGAC		
BMPR2_F	GTCCTGGATGGCAGCAGTAT	150bp	NM_001204.6
BMPR2_R	GTCCAGCGATTCAGTGGAGA		
CYP11A1_F	TGGGTCGCCTATCACCAGTAT	Bp82	NM_000781.2
CYP11A1_R	CCACCCGGTCTTTCTTCCA		
CYP19A1_F	TGCAAAGCACCCTAATGTTG	135bp	NM_000103
CYP19A1_R	TGGTACCGCATGCTCTCATA		
ERBB2_F	TGACCTCTCCTACATGCCCA	105bp	NM_004448.3
ERBB2_R	CCCTTGTCATCCAGGTCCAC		
EREG_F	TCCCAGGAGAGTCCAGTGAT	76bp	NM_000963.3
EREG_R	ATTGACACTTGAGCCACACG		
FSHR_F	TGCTGGCATTCCTGAGC	150bp	NM_000145.3
FSHR_R	GCTTGGTGAGGACAAACCTC		
GJA1_F (CX43)	GGAGTTCAATCACTTGGCGT	123bp	NM_000165
GJA1_R	CCAGCAGTTGAGTAGGCTTGA		
GPX3_F	AGGCCTGACGGGCCAGTACATT	90bp	NM_002084
GPX3_R	TGGTTGCAGGGAAAGCCCAGA		
GREM1_F	TGCTGGAGTCCAGCCAAGA	65bp	AF110137.2
GREM1_R	GCACCAGTCTCGCTTCAGGTA		
HAS2_F	AGCCTTCAGAGCACTGGGACGA	81bp	U54804.1
HAS2_R	ACAGATGAGGCTGGGTCAAGCA		
LHR_F	TGGAGAAGATGCACAATGGA	122bp	NM_000233.3
LHR_R	GGCAATTAGCCTCTGAATGG		
NOS2_F	TGTTGGTGGTGACCAGTACG	147bp	NM_000625.4
NOS2_R	AGAACCGAGGGTACATGCTG		
PTGS2_F	TTCCTCCTGTGCCTGATGAT	Bp120	NM_000963.3
PTGS2_R	GGGGATCAGGGATGAACTTT		
SFRP4_F	AGGACAACGACCTTTGCATC	89bp	NM_003014.3
SFRP4_R	TTTGCAGGCTTCACATACCTT		
SFRP5_F	ACCAAGATCTGCGCCCAGTGT	130bp	NM_003015
SFRP5_R	TCAGCTTCCGGTCCCCATTCT		
STAR_F	CTCAACAACCAGGAAGGCTG	112bp	NM_011485.4
STAR_R	CTACCACCACCTCCAAGCGA		
UGP2_F	AGGGGCCTTCTGTGGATTGGG	89bp	NM_006759.3
UGP2_R	AGGCAAGCCCCTGGCCTTTAT		

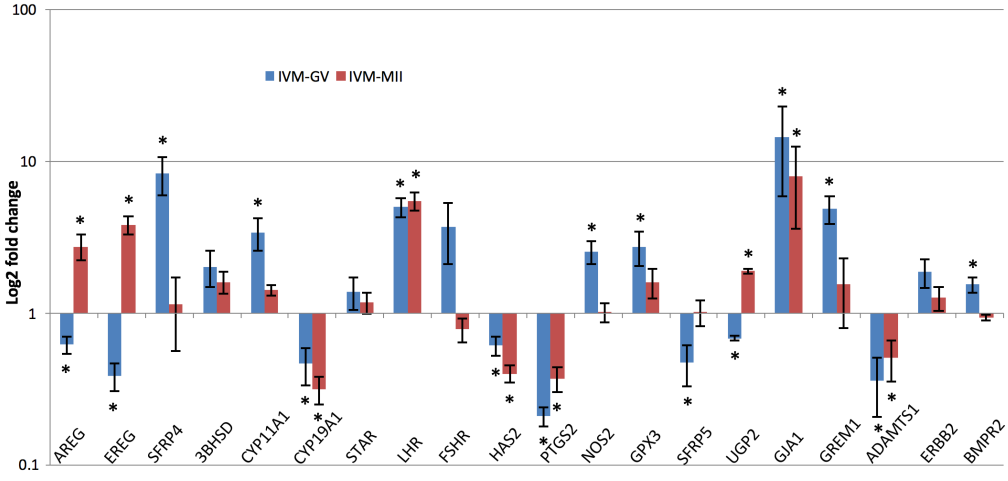

Fig. 1. qRT-PCR analysis of expression levels of a panel of genes (see Table 2 for detailed description) in cumulus cells from cumulus cell-oocyte complexes (COCs) collected from 4-12 mm follicles and matured *in vitro* (IVM-GV, blue bars) or *in vivo* (IVM-MII, red bars). *Values were normalized with 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 studies 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.

TABLE 2

ANALYZED GENES

Gene name	Description
3BHSD	3beta-hydroxysteroid dehydrogenase
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif 1
AREG	amphiregulin
BMPR2	bone morphogenetic protein receptor type II
CYP11A1	cytochrome P450 family 11 subfamily A member 1
CYP19A1	cytochrome P450 family 19 subfamily A member 1
ERBB2	erb-b2 receptor tyrosine kinase 2
EREG	epiregulin
FSHR	follicle stimulating hormone receptor
GJA1	gap junction protein alpha 1
GPX3	glutathione peroxidase 3
GREM1	gremlin 1, DAN family BMP antagonist
HAS2	hyaluronan synthase 2
LHR	luteinizing hormone/choriogonadotropin receptor
NOS2	nitric oxide synthase 2
PTGS2	prostaglandin-endoperoxide synthase 2
SFRP4	secreted frizzled related protein 4
SFRP5	secreted frizzled related protein 5
STAR	steroidogenic acute regulatory protein
UGP2	steroidogenic acute regulatory protein

source: National Center for Biotechnology Information. www.ncbi.nlm.nih.gov/gene

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

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 as 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.

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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, Buccinasco, 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% CO_2 humidified atmosphere for 30 hours. Following IVM, cumulus cells were collected only from COC associated to a MII oocyte (IVM-GV).

COCs with an expanded cumulus were cultured for 4 hours in IVM medium (Origio, Maløv, Denmark) and cumulus cells associated to a MII 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, Merck) 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 StepOnePlus 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 20 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.

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