

# The cumulus cell gene expression profile of oocytes with different nuclear maturity and potential for blastocyst formation

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## Abstract

**Purpose** Gene expression in human ART cumulus cell (CC) has been related to oocyte maturity and competence but requires further validation. Expression dynamics were investigated in CC of oocytes at different maturational stages and with different developmental competence in a standard in vivo mouse superovulation model.

**Methods** Quantitative PCR analysis of *Has2*, *Vcan*, *Sdc4*, *Alcam*, *Grem1*, *Ptgs1* and *Ptgs2* in CC collected at regular time intervals from 0 to 24 h post hCG injection.

**Results** Three expression patterns were observed each with strong regulation (4–230× differences). Immediately prior to ovulation CC of GVBD oocytes have 5× less *Sdc4* and *Ptgs1* and 5× more *Ptgs2* when compared to the CC of freshly ovulated PB oocytes. When compared to the latter, the post-ovulatory aged PB oocytes had a 2× reduced blastocyst forming capacity and their CC expressed 2× more *Sdc4* and 6× less *Alcam*.

**Conclusions** Morphologically identical cumulus oocyte complexes with different developmental competence can be differentiated by CC gene expression.

**Keywords** Cumulus cells · Gene expression · Oocyte maturation · Oocyte quality

## Introduction

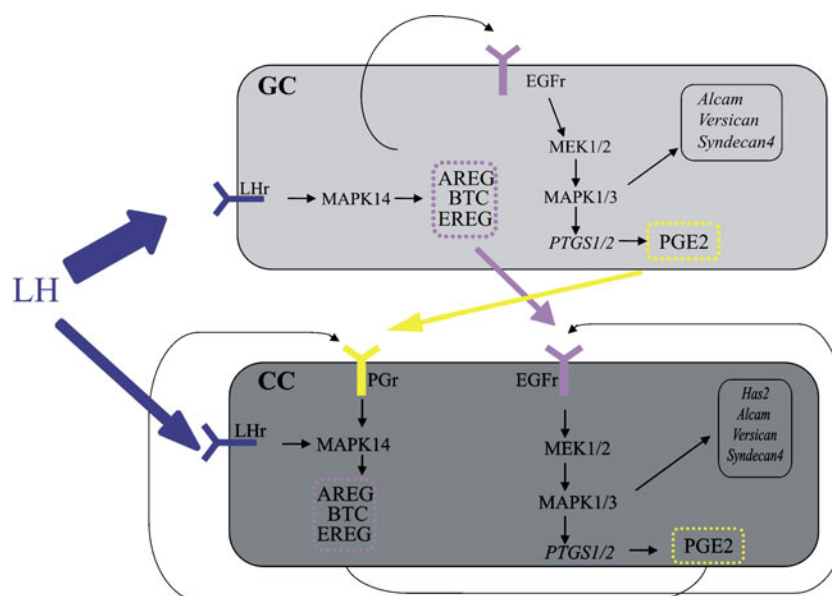
Oocyte maturation is induced by the pre-ovulatory gonadotrophin surge and requires a strictly timed coordination of events in nuclear and cytoplasmic compartments [1]. The time course of successive nuclear stages of in vivo and in vitro matured oocytes from stimulated adult mice has been well-documented [2, 3]. In stimulated cycles in adult mice, chromatin condensation, dissolution of the Germinal Vesicle (GV) and reorganization of the oocyte's cytoskeleton are first seen 2 h after stimulation and most oocytes resume maturation by 4 h post hCG. The formation of the first metaphase spindle is completed by 8 h post stimulation. Approximately 12 h after hCG, the first polar body (PB) is extruded and a MII oocyte is ovulated [2, 3]. Within 6 h after ovulation, oocytes are expected to be fertilized [4]. Outside this fertilization window, post-ovulatory oocyte ageing sets in as demonstrated in in vivo [4, 5] and in vitro maturation settings [6].

Also in the cumulus cells (CC), a timed cascade of events occurs [7–9]. Post hCG, the CC transform and start producing a specific extracellular environment for the oocyte. The presence and contact with the CC were found to be essential to obtain good blastocyst rates in several species [10–12].

The genes studied here, hyaluronic acid synthetase-2 (*Has2*), Versican (*Vcan*), Syndecan 4 (*Sdc4*), activated leukocyte cell adhesion molecule (*Alcam*), Gremlin 1 (*Grem1*) and prostaglandin-endoperoxide synthase 1 and 2 (*Ptgs1* and 2), are involved in the signaling and the extracellular matrix formation during the ovulatory response (Fig. 1) and were found differentially expressed in human CC in relation to oocyte developmental quality and patient and treatment characteristics [13–15].

**Capsule** Gene expression patterns in cumulus cell are different when surrounding an immature, mature or postovulatory aged oocyte

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**Fig. 1** The interactions occurring in the ovarian mural granulosa cells (GC) and cumulus cells (CC) after the administration of hCG for ovulation induction. The hypothetical figure is based on earlier literature [16, 56, 57]. LH will mainly act on the mural granulosa cells where it will induce the production of EGF-like family members: Amphiregulin (Areg), Betacellulin (Btc), Epiregulin (Ereg). All can bind to the EGF receptor (Egfr) present in both GC and CC. Egfr stimulation involves the MEK1/2 and MAPK1/3 pathway and will induce *Ptgs* expression and therefore the production of Prostaglandin

E2 (Pge2). Both prostaglandin and the EGF-like family members are secreted by GC and can transduce the ovulatory response in the CC and also have an autocrine function in the CC. The ovulatory response will trigger the production of Hyaluronic acid synthetase 2 (*Has2*), Versican (*Vcan*) and activated leukocyte cell adhesion molecule (*Alcam*), genes occurring predominantly in the CC. The expression of *Ptgs1* and *Syndecan 4* (*Sdc4*) in GC or CC was not earlier documented so its involvement as represented here needs to be further documented

The ovulation cascade in CC depends on a strong autocrine regulation involving the EGF-like family members and the prostaglandins [16]. EGF-like factors bind to CC and induce *Ptgs2* [17]. *Ptgs2* is the rate limiting enzyme in Prostaglandin E production, and prostaglandin receptor *Ep2* null mice do not ovulate and have a defective COC expansion [18–20]. This indicates that *Ptgs2* expression is crucial for obtaining competent oocytes. The expression of *Ptgs1* is less well-documented and is described as constitutive, whereas the induction of *Ptgs2* seems to be rate limiting [21]. The relation between *Ptgs1* and *Ptgs2* in the ovary or CC is currently unknown.

During the first hours following the ovulatory hCG, CC start to express *Has2*, which produces hyaluronic acid (HA), the principal glycosaminoglycan attached to the CC [22]. The degree of expansion caused by HA is positively related to developmental competence [23, 24]. The CC derived matrix also contains the proteoglycans brevican (*Bcan*) and versican (*Vcan*) also known as chondroitin sulfate proteoglycan 2. Versican is a proteoglycan described in mouse mural granulosa cells after the ovulatory stimulus [25] and can bind HA via its N-terminal link protein domain, while the C-terminal domain interacts with cell surface proteins like the EGF receptor [26] and integrins [27]. Versican is cleaved by a disintegrin-like and metalloproteinase with thrombospondin type 1 motif 1 (*Adams1*),

the main proteolytic enzyme in the COC [28]. *Adams1* is also induced by the ovulatory dose of LH and cleaved Versican is detected in ovulated COCs [28]. Since deficient *Adams1* reduces fertilization [29] and since Versican binds important growth factor receptors, an altered Versican expression might also alter the functional properties of the COC matrix during oocyte maturation, ovulation and fertilization.

The next two genes, *Alcam* and *Sdc4*, code for transmembranal ECM related proteins. *Alcam* may be involved in promoting cell-cell adhesion and cell to matrix adhesion. In female reproductive tissue, *Alcam* was described in endometrial epithelium and blastocysts where it could contribute to implantation [30], and in cumulus and granulosa during the ovulatory response [7]. *Sdc4* is a heparan sulfate proteoglycan that appears to act as receptor or co-receptor [31, 32] and is involved in intracellular communication by binding anticoagulant heparan sulfate chains (aHSPG) on the cell surface and in the extracellular matrix. The aHSPG expression in the ovarian follicle is hormonally induced to culminate in preovulatory follicles and controls proteolysis and fibrin formation at ovulation, possibly protecting the oocyte [33].

Growth differentiation factor 9 (*Gdf9*), i.e. an oocyte-secreted factor required for normal ovarian follicle development and female fertility, acts using transforming growth

factor-beta signaling as a paracrine factor on granulosa cell [34]. *Gdf9* is a key mediator of cumulus expansion and induces the expression of *Has2* and *Ptgs2* in the presence of FSH [35]. Gremlin is secreted and favors the GDF9 pathway (and mucification) in cumulus/granulosa over the BMP pathway [36, 37].

Studying the expression dynamics in a mouse model can provide insight into a) how these genes are regulated and what the magnitude is of the changes observed during the ovulatory response, b) whether up or down regulation can be related to oocyte maturity, c) how stable the expression is in CC of PB oocytes (the target population in human ART-cycles), and d) whether a known population of COC with identical morphological characteristics, but different oocyte quality, could be distinguished (as a proof of concept).

## Materials and methods

### Mouse cumulus cells

Mice used for retrieval of COCs were 7-to-9-week old F1 hybrids (C57Bl/6J x CBA/ca, Charles River, Brussels, Belgium), housed and bred according to the national legislation on animal care and with the consent of the “Commissie voor Ethisch Proefdiergebruik VUB” (project n° 05-395-2). The adult anoestrous mice were primed for 48 h with a single injection of 5IU eCG (Folligon, Intervet, Mechelen, Belgium), and CC were collected before (0 h) and 2, 4, 8, 12, 16 and 24 h after administration of the ovulatory stimulus of 5IU hCG (Chorulon, Intervet, Mechelen, Belgium). Collection of the *in vivo* grown COCs was repeated 3 times with two mice for each of the 7 time points. The CC of individual COCs were analyzed and per time point the CC of at least 2 COCs from each biological replicate were used in the current study. Because of the low detection levels for *Ptgs1* and *Ptgs2*, the number of samples was tripled for these 2 genes.

The pre ovulatory COCs were punctured from the large antral follicles in the ovary. The post-ovulatory COCs (from 12 h post hCG onwards) were flushed from the ampulla using L15 Leibovitz-GlutaMAXI medium supplemented with 10% heat inactivated Fetal Bovine Serum (HIA FBS), 100 IU/ml penicillin and 100 µg/ml streptomycin (all from Life Technologies, Merelbeke, Belgium). At 0 and 2 h, the collected oocytes were in germinal vesicle (GV) stage; at 4 and 8 h, all oocytes were undergoing germinal vesicle breakdown (GVBD), and from 12 h onwards all oocytes extruded a polar body (PB). All manipulations were performed under a stereomicroscope SZX9 (Olympus, Aartselaar, Belgium) on heated stages. COCs with 2–5 cumulus cell (CC) layers were washed and transferred using the mouth pipette to an individual 10 µl droplet containing 40 IU/ml of Hyaluronidase (Sigma-Aldrich, Bornem, Belgium) where oocytes were freed. The CC from individual COCs were snap-frozen in liquid nitrogen and stored at –80°C for further use.

### RNA extraction & RT reaction

Total-RNA of CC of individual COCs was extracted using the RNeasy Micro kit (Qiagen, Westburg, Venlo, The Netherlands) and reverse transcribed using the Taqman Reverse Transcription Reagents (Applied Biosystems, Lennik, Belgium) as described earlier [38]. Negative controls were generated by omitting the RNA or the reverse transcription enzyme and proved to be blank. All resulting cDNA was frozen at –80°C and used for PCR within 2 weeks.

### Quantitative PCR (QPCR)

PCR primer design was performed using the Universal Probe Library (UPL) software (<https://www.roche-applied-science.com/sis/rtqpcr/upl/index.jsp>). Primers were selected to be complementary to the sequence of *Has2*, *Ptgs1*, *Ptgs2*, *Sdc4*, *Alcam*, *Grem1*, *Vcan* (Table 1). Ribosomal *Rn18s* was used as endogenous control (primer sequences were kindly

**Table 1** Overview of the primersequences 5'→3'

Gene symbol	Accession n°	Forward primer	Reversed primer	Amplicon length
<i>Alcam</i>	NM_009655.1	CTGATTGTGGGAATTGTCGTT	TCCTTGTTACATGTTTIGATGC	110bp
<i>Grem1</i>	NM_011824.3	GAGGACCCACGGAAGTGA	CCTCAGCTGTTGGCAGTAGG	92bp
<i>Has2</i>	NM_008216.2	TGAGCAGGAGCTGAACAAGA	GCCAACAATATAAGCAGCTGTG	118bp
<i>Ptgs1</i>	NM_008969.2	CCTCTTCCAGGAGCTCACA	TCGATGTCACCGTACAGCTC	70bp
<i>Ptgs2</i>	NM_011198.3	GGGAGTCTGGAACATTGTGAA	GCACATTGTAAGTAGGTGGACTGT	110bp
<i>Sdc4</i>	NM_011521.1	CCCTTCCCTGAAGTGATTGA	AGTTCCTTGGGCTCTGAGG	98bp
<i>Vcan</i>	NM_001081249.1	CTGTGTGGATGGTTTTAATACATTTAG	CAGCCATAGTCACATGTCTCAGT	96bp
<i>Rn18s</i>	NR_003278.1	TCAAGAACGAAAGTCGGAGG	GGACATCTAAGGGCATCACA	489bp

provided by Prof. Dr. R. Einspanier, HU Berlin, Germany). Quantitative PCR was performed on the LightCycler 480 (Roche Diagnostics, Vilvoorde, Belgium) with the LC480 SYBR Green I Master (Roche Diagnostics, Vilvoorde, Belgium) containing 2  $\mu$ l of cDNA and 0.6  $\mu$ M of primers in a total volume of 15  $\mu$ l. After 10 min activation at 95°C, cycling conditions were 10 s at 95°C, 30 s at 60°C for 45 cycles. A log<sub>6</sub> dilution series of a synthetic oligonucleotide, corresponding to the amplicon, and no template controls were simultaneously run. The specificity of the PCR products was confirmed by melting curve analysis performed at the end of the amplification and by gel electrophoresis. Each sample was tested in triplicate and the standard curve was used to calculate the absolute quantity, taking into account the actual PCR efficiency prior to the normalization to his *Rn18S*. Results are depicted in graphs with relative ratios, using the median of the condition with the lowest expression level different from 0 as the calibrator.

### In vitro fertilization

Mucified COCs of F1 mice stimulated as described above were collected at 12, 16 and 24 h post hCG for in vitro fertilization and embryo culture. Sperm was collected from two adult F1 mice and sperm capacitation and IVF were performed in M16 (Sigma-Aldrich, Bornem, Belgium) supplemented with 2.6% bovine serum albumin fraction V (BSA; Calbiochem, Merck, Leuven, Belgium) and 10  $\mu$ l/ml non-essential amino acids (100 $\times$  NEAA; Life Technologies, Merelbeke, Belgium). Embryos were cultured in M16 supplemented with NEAA (10  $\mu$ l/ml) and essential amino acids (20  $\mu$ l/ml; 50 $\times$  EAA; Life Technologies, Merelbeke, Belgium). Embryo and blastocyst evaluation was performed as described earlier [39]. Three biological replicates were performed, each time with 2 female mice /time point, and each time point was considered an experimental unit and contained on average 34 $\pm$ 13 COCs.

### Statistical analysis

To ensure Gaussian distribution, the expression ratios were log transformed and the fertilization and embryo culture percentages were arcsine transformed. All data were analyzed using the two-tailed ANOVA and Tukey post test using Graphpad Prism V4.0 (Graphpad software, CA, USA).

## Results

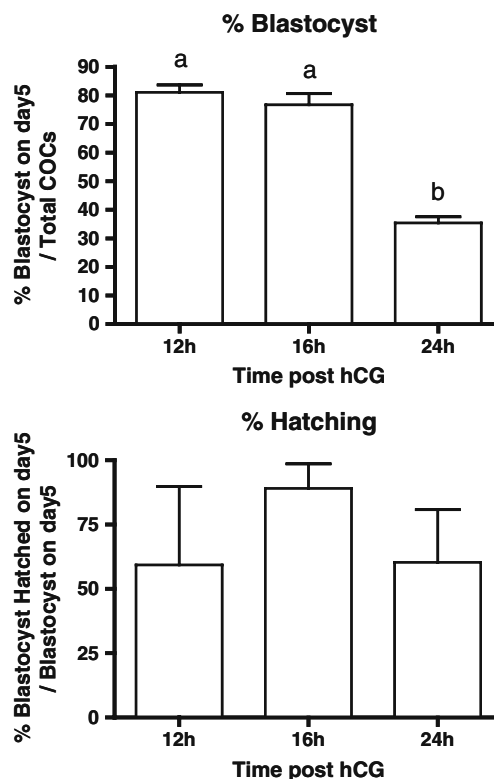
### Developmental competence of the ovulated COCs

Ovulated COCs collected at 12–24 h post hCG are all morphologically identical with expanded CC and oocytes at

the PB stage. The developmental competence of the oocytes collected at 12, 16 and 24 h was analyzed. The 3 groups had a comparable fertilization capacity with 2cell rates of (Mean $\pm$ SD) 97% $\pm$ 6, 82% $\pm$ 11, and 81% $\pm$ 10 for respectively 12, 16 and 24 h, but 24 h COCs had a 2 $\times$  reduced blastocyst forming capacity ( $p$ <0.001) (Fig. 2). The capacity to hatch was comparable in the 3 conditions (Fig. 2).

### The different COC classes

At 0 h the CC have not yet been exposed to hCG and are referred to as “GV CC”. At 2 h no morphologic distinction with 0 h COCs could be found, but expression changes are already initiated in the CC=“Activated GV CC”. At 4 and 8 h oocytes undergo the germinal vesicle breakdown=“GVBD CC”. Based on the differences in developmental capacity described above, polar body oocyte CC were subdivided into



**Fig. 2** Blastocyst forming and hatching capacity of ageing PB oocytes. Cumulus oocyte complexes (COCs) of eCG primed mice collected at 12, 16 and 24 h post hCG injection were in vitro fertilized and grown to blastocyst. The % (Mean $\pm$ SEM) of blastocysts formed and blastocysts hatched of three biological replicates are depicted. For blastocyst forming capacity the time points marked with different letters were statistically different after two-tailed ANOVA and Tukey post test ( $P$ <0.0001). Hatching capacity was comparable in the three conditions. Based on the differences in developmental capacity the 12 and 16 h CC samples are further referred to as “Optimal PB CC” and the 24 h CC samples are further called “Aged PB CC”

12 and 16 h CC samples = “Optimal PB CC” and 24 h CC samples = “Aged PB CC”. (Figure 3).

Expression in pre and post hCG cumulus cells

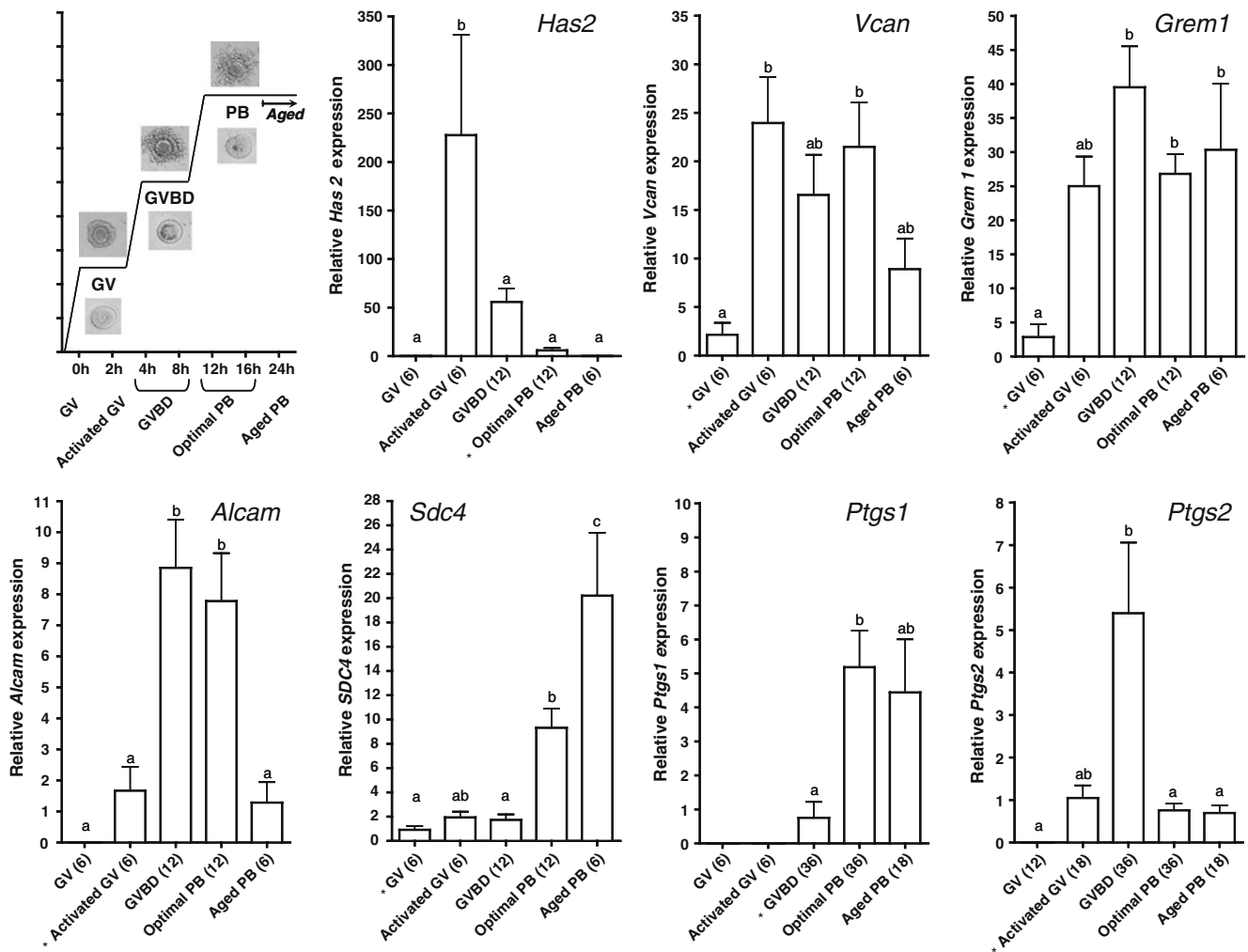
Prior to the induction of final oocyte maturation, mRNA for *Has2*, *Alcam*, *Ptgs1* and 2 was undetectable. For *Vcan*, *Grem1* and *Sdc4*, at least 50% of the samples analyzed contained quantifiable amounts of mRNA prior to hCG. Post hCG, all genes demonstrated distinct patterns (Fig. 3). In the activated GV CC, the first time point after hCG administration, mRNA was detectable in all samples for all investigated genes except for *Ptgs1*; with a significant induction for *Has2* and *Vcan* (Fig. 3).

When considering the physiologically most relevant timespan (0–16 h), 3 expression patterns could be distin-

guished 1) the expression is significantly induced prior to ovulation (12 h) and fades away at ovulation, the “early response genes” (*Has2*, *Ptgs2*; Fig. 3), or 2) the expression is significantly induced prior to ovulation and is equally expressed in the optimal COC after ovulation, the “early remaining genes” (*Vcan*, *Grem1* and *Alcam*; Fig. 3) or 3) the expression is only induced post ovulation, the “late response genes” (*Sdc4* and *Ptgs1*; Fig. 3).

*Has2* was the gene with the strongest induction (230-fold in activated GVs); it then gradually decreased until absent in all post-ovulatory aged PB CC samples. *Ptgs2* reached a maximal induction (5×) in GVBD CC and decreased to a baseline expression level in PB CC.

*Vcan* expression showed a strong induction immediately after hCG exposure (23×), which was maintained until the optimal PB CC stage. *Grem1* was rapidly upregulated (25×)



**Fig. 3** The relative expression observed in cumulus cells from eCG primed mice at different time points post hCG injection. The first graph depicts the source of the material and the type of oocyte it surrounded. Magnification of the pictures is 300× for cumulus oocyte complexes and 400× for the oocytes. The succeeding graphs depicts

the relative expression level (Mean±SEM) of each of the genes with the condition used as calibrator marked with an \*. Conditions marked with different letters are statistically different after two-tailed ANOVA and the Tukey post test ( $P < 0.05$ )

by the ovulatory dose of hCG, reaching a maximal response (40×) in GVBD CC, after which a steady state was maintained in the PB oocyte CC. *Alcam* mRNA was most highly expressed (5-fold) around the time of ovulation in CC of GVBD and optimal PB.

Finally, for the two late response genes, expression levels did not vary much until the PB stage. *Sdc4* expression was 10 and 20 times induced in competent and post-ovulatory aged PB CC respectively, and *Ptgs1* was mainly detected in the CC of ovulated COCs. The patterns for *Ptgs1* and 2 are complementary. It should however be noted that *Ptgs1* expression was not present in all COCs, but was only detectable in 8, 67 and 44% of the COCs of respectively GVBD CC, optimal PB CC and post-ovulatory aged PB CC. An overview of the % positive samples per gene and condition is shown in Table 2.

The ratio of *Ptgs2/1* expression

*Ptgs1* and 2 were both found to be expressed in CC, but seemed to be important at different moments of maturation. Since an absolute quantification to the copy number using the standard curve was performed prior to the relative quantification, a ratio of mean copy numbers could also be made. In COCs expressing both *Ptgs1* and *Ptgs2* (from 12 h post hCG onwards), the *Ptgs2/Ptgs1* ratio was more than 700. This indicated that the amount of *Ptgs2* mRNA copies is much greater in the CC.

CC expression differences in oocytes with different nuclear maturity

When comparing the expression in CC of GV (0 h), GVBD and optimal PB, the three patterns distinguished earlier correspond to: 1) the expression is only higher in the CC of the GVBD oocytes (*Ptgs2*), 2) expression is upregulated from the GVBD stage and is unchanged in the CC of competent PB oocytes (*Vcan*, *Grem1* and *Alcam*), 3) expression is only increased in the CC of PB oocytes (*Sdc4* and *Ptgs1*). It is remarkable that expression differences in each pattern are at least five-fold and can rise to 14-fold. As such, the ovulated competent PB oocytes were

surrounded with CC with, on average, a 9× decreased level of *Has2* ( $P>0.05$ ), a 5× increased *Sdc4* ( $P<0.05$ ), a 5× increased *Ptgs1* ( $P<0.05$ ) and a 5× decreased *Ptgs2* ( $P<0.05$ ) when compared to the CC of not ovulated GVBD oocytes.

Comparing CC of competent or post-ovulatory aged oocytes

Post-ovulatory aged COCs collected 24 h after ovulation induction had a morphology undistinguishable from the COCs collected at 12 or 16 h, but on average, had 2× more *Sdc4* ( $P<0.05$ ), and respectively 2 and 6× less *Vcan* (ANOVA  $P>0.05$ , t-test  $P<0.05$ ) and *Alcam* ( $P<0.05$ ) in their CC. CC of post-ovulatory aged PB oocytes did not contain *Has2*, whereas it was still detectable in 8/12 CC samples of competent PB oocytes.

## Discussion

The corona-cumulus cells were examined for their expression of factors related to extracellular matrix formation and signaling triggered by an ovulatory dose of hCG in in vivo grown and matured follicles. As expected, all genes were expressed at one or more time points during the ovulatory response. The fact that 30–50% of the in vivo grown COCs from superovulated mice expressed *Vcan*, *Sdc4* or *Grem1* prior to hCG administration might seem unexpected. However, experiments by others have indicated the presence of limited amounts of *Grem1*, *Sdc4* mRNA and *Vcan* mRNA and protein in granulosa cells of growing follicles [25, 37, 40].

The relation between the complex processes leading to cumulus expansion downstream of the ovulatory response and normal fertilization and development [41] generated expectations that some of the tested genes could prospectively be used as prognostic markers. These genes were found differentially expressed in human CC in relation to oocyte developmental quality and patient and treatment characteristics [13]. Studying the dynamics of gene expression in an animal model may provide useful information for

**Table 2** Fraction of samples containing the specific gene of the total number of samples analyzed (n), when analyzing the cumulus cells of single cumulus oocyte complexes

	<i>18S</i>	<i>Has2</i>	<i>Sdc4</i>	<i>Alcam</i>	<i>Vcan</i>	<i>Grem1</i>	<i>Ptgs1</i>	<i>Ptgs2</i>
GV	100% (12)	0% (6)	67% (6)	0% (6)	50% (6)	50% (6)	0% (6)	0% (12)
Activated GV	100% (18)	100% (6)	100% (6)	100% (6)	100% (6)	100% (6)	0% (6)	72% (18)
GVBD	100% (36)	100% (12)	67% (12)	100% (12)	100% (12)	100% (12)	8% (36)	100% (36)
Optimal PB	100% (36)	67% (12)	100% (12)	100% (12)	100% (12)	100% (12)	67% (36)	100% (36)
Aged PB	100% (18)	0% (6)	100% (6)	67% (6)	100% (6)	100% (6)	44% (18)	100% (18)

evaluating the predictive potential of this approach in human ART.

Which expression changes occur in an in vivo ovulation response?

Three categories of genes could be distinguished: early (*Has2* and *Ptgs2*), early and persistent (*Vcan*, *Grem1* and *Alcam*) and late response genes (*Sdc4* and *Ptgs1*).

The early response and fading away pattern as observed for *Has2* and *Ptgs2* is in line with earlier reports by others [42–46]. One paper reports a second upregulation for *Ptgs2* from 8 to 12 h [47], which we did not observe in our QPCR setting. The early occurrence and disappearance of these genes might indicate that their main role is prior to ovulation. This is in line with the fact that the HA synthesis by mice COCs is first detected 2–3 h post hCG and increases to a maximum between 4–10 h [48].

The *Vcan* and *Grem1* pattern reported here is to our knowledge the first quantitative expression pattern in CC. The *Alcam* expression pattern is in line with an earlier study in mice [7]. The observed expression pattern fits in with the fact that *Alcam* protein was detectable at the CC surface from 8 to 16 h post hCG [7]. Since recombinant Gremlin inhibits BMP4 activity—a theca cell factor involved in androgen production—it was hypothesized that Gremlin blocks steroidogenesis and luteinisation in CC [49]. In this perspective, its persistence in CC would make sense.

Finally, there are the 2 last wave genes (*Sdc4* and *Ptgs1*), which are only induced post ovulation, and whose expression pattern in CC is also poorly documented in current literature. In mouse ovary, *Sdc4* was expressed by GC from type 4 follicles (large preantral follicle) [50] onwards. In situ hybridization also indicated that the GC lining the antrum in type 6 and 7 follicles and the Corpus Luteum were positive. It was also suggested that increased *Sdc4* expression was related to increased apoptosis [40], but the main increase in *Sdc4* occurred from ovulated GVBD to optimal PB CC (5-fold) where apoptosis in CC is mainly observed from 5 h post ovulation onwards [51], which corresponds most with the transition from optimal PB to post-ovulatory aged PB oocyte CC, which only had a 2-fold increase in the current study. *Sdc4* was found in follicle fluid aspirates of ART patients and was 10-fold increased at 36 h post hCG compared to pre hCG (patients undergoing follicle reduction prior to hCG). The protein levels also seemed to be negatively correlated to follicle fluid estradiol and progesterone concentrations [52].

The pattern observed for *Ptgs1* suggests that *Ptgs1* is complementary to *Ptgs2*. These findings confirm the compensatory upregulation of *Ptgs1* described in CD1 *Ptgs2* null mice, where it resulted in the partial rescue of ovulation, fertilization, and implantation capacity [53].

Although the expression level of *Ptgs1* was much lower than that for *Ptgs2*, the occurrence of *Ptgs1* around the time of ovulation could make it a useful marker at pick up in an ART-setting.

Can an up or down regulation be related to oocyte maturity or intrinsic oocyte quality?

There is a within patient heterogeneity in the COCs/oocytes collected at pick up in human ART. This heterogeneity is reflected in the COC morphology, the oocyte maturity, and the oocyte developmental competence. The underlying cause of this heterogeneity might be related to a difference in the vascularisation of the follicles and the maturity of the follicle/oocyte at the start of stimulation. Both might result in a speeding up or slowing down of the ovulatory response in the CC of the respective COCs and the heterogeneity observed at the fixed time point of pick up. In this perspective, more knowledge as to whether one specific gene is a fast response gene like *Has2* or hardly undergoes any change in the CC of PB oocytes like *Grem1* is useful information when we compare expression levels in human CC.

Completion of nuclear maturation (PB) coincided with an increase in expression for all genes studied here except for *Ptgs2*. *Vcan*, *Grem1* and *Alcam* were high from GVBD on *Ptgs1* and *Sdc4* from PB on.

Care should be taken when extrapolating these results to expression differences found in human ART CC. One argument is the fact that all COCs in the clinic are collected 36 h post hCG injection from large antral follicles (usually >17 mm) and are thus most likely to be exposed to some hCG. Bearing this in mind, we should consider the expression levels in the activated GV population of the current study. Compared to activated GV CC, PB oocyte CC have an increased *Alcam*, *Sdc4*, *Ptgs1* and decreased *Has2* expression.

In human oocytes, nuclear maturity correlated positively to *PTGS2* [14, 54], *GREM1* [14] and negatively to *VCAN* [13, 14] expression in CC. Overall, the expression differences found in human in relation to oocyte nuclear maturity ( $\leq 2$ -fold) are smaller than those observed in the current study (5-fold and more), which also suggests the different nature of the samples compared. The results from the current setting are therefore of limited use to validate the observations made in relation to oocyte maturity CC of human oocytes after ART.

Post-ovulatory ageing as in the current setting up to 12 h (the 24 h time point) does not occur in a clinical setting, but allowed to make a clear distinction between two populations of morphologically identical COCs. The dynamics observed might give an indication as to how expression evolves and might underlie the subtle differences observed

in human CC at pick up. In the mouse model, post-ovulatory ageing resulted in the disappearance of *Has2* expression (below the detection limit), a decreased expression of *Vcan* and *Alcam*, and an increased *Sdc4*.

In human ICSI samples, increased *HAS2*, *ALCAM*, *PTGS2*, *SDC4* and decreased *VCAN* were reported in PB oocyte CC resulting in higher grade embryos or blastocysts after ICSI [13, 23, 55]. The *Has2* and *Alcam* observations seem to be in line with our findings since post-ovulatory aged oocytes proved to have less *Has2* and *Alcam*.

The CC analyzed and compared for developmental competence in human ART are CC of PB oocytes collected prior to ovulation. In the mice setting, all CC of PB oocytes were collected post ovulation. In this perspective, the expression level in the CC of mice GVBD oocytes might be relevant. Considering also the GVBD expression levels suggests that a decrease in *Ptgs2* would be detrimental to the oocyte competence, which is in line with reports in human CC. The *Vcan* and *Sdc4* regulation could not be confirmed.

Higher *GREM1* expression in the CC of ICSI patients has been described as a marker of better blastocyst forming capacity [23, 55]. *Grem1* seems to be on steady state level from ovulation on; a higher expression of *Grem1* might result from the intrinsic oocyte factors and/or might be determined by follicular development stage at the time of ovulation induction.

On the other hand, stimulating patients with a gonadotrophin preparation containing an hCG action resulted in increased *SDC4* expression in the CC [13]. Given the specific pattern of *Sdc4* expression, with its gradual increase, it could be hypothesized that pre-exposing follicle to a low hCG/LH concentration during the follicular phase results in a more advanced ovulatory response, which would be characterised by an increased *Sdc4*.

## Conclusion

In the current study, genes reported to be differentially expressed in relation to oocyte competence in human CC were investigated during the ovulatory response. Overall expression differences were quite large compared to the differences currently described in human CC at time of pick up. While all genes proved to be regulated, *Vcan*, *Grem1* and *Alcam* expression were quite stable around the time of ovulation (the time of pick up in human). This makes them interesting markers since expression changes observed for these genes in human CC might than primarily originate from intrinsic oocyte factors. Immediately prior to ovulation CC of GVBD oocytes have 5× less *Sdc4* and *Ptgs1* and 5× more *Ptgs2* when compared to the CC of freshly

ovulated PB oocytes. The post-ovulatory aged PB oocytes had a 2× reduced blastocyst forming capacity and their CC expressed 2× more *Sdc4* and 6× less *Alcam* when compared to the freshly ovulated PB oocytes. Morphologically identical mouse cumulus oocyte complexes with different developmental competence could thus be differentiated by CC gene expression. We cannot exclude that the dynamics in CC gene expression would differ between human and mice, but decreased *HAS2*, *ALCAM* and *PTGS2* seems to coincide with reduced oocyte competence in both species.

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