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Cumulus cell gene expression following the LH surge in bovine preovulatory follicles: potential early markers of oocyte competence

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Abstract

Cumulus cells (CCs) are essential for oocytes to reach full development competency and become fertilized. Many major functional properties of CCs are triggered by gonadotropins and governed by the oocyte. Consequently, cumulus may reflect oocyte quality and is often used for oocyte selection. The most visible function of CCs is their ability for rapid extracellular matrix expansion after the LH surge. Although unexplained, LH induces the final maturation and improves oocyte quality. To study the LH signaling and gene expression cascade patterns close to the germinal vesicle breakdown, bovine CCs collected at 2 h before and 6 h after the LH surge were hybridized to a custom-made microarray to better understand the LH genomic action and find differentially expressed genes associated with the LH-induced oocyte final maturation. Functional genomic analysis of the 141 overexpressed and 161 underexpressed clones was performed according to their molecular functions, gene networks, and cell compartments. Following real-time PCR validation of our gene lists, some interesting pathways associated with the LH genomic action on CCs and their possible roles in oocyte final maturation, ovulation, and fertilization are discussed. A list of early potential markers of oocyte competency *in vivo* and *in vitro* is thereafter suggested. These early biomarkers are a preamble to understand the LH molecular pathways that trigger the final oocyte competence acquisition process in bovine.

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Introduction

In large mammals, oocyte maturation is a prerequisite to fulfill the subsequent steps of embryo development (Krisher 2004). The ability to achieve such development, known as oocyte competence, is acquired mainly during the antral phase of folliculogenesis through well-coordinated molecular processes, including proper nuclear maturation and final cytoplasmic maturation (Sirard *et al*. 2003, Krisher 2004, Gilchrist & Thompson 2007, Kimura *et al*. 2007). Successful oocyte final maturation is therefore a complex process that implies many factors such as intrinsic oocyte quality (Lonergan *et al*. 2003, Wang & Sun 2007), dialog with neighboring somatic compartment (Matzuk *et al*. 2002, Tanghe *et al*. 2002, Yokoo & Sato 2004, Gilchrist *et al*. 2008, Li *et al*. 2008), and functional gap junctions (Thomas *et al*. 2004, Ali *et al*. 2005, Lodde *et al*. 2007). It has been established that, inside the follicle, the oocyte is surrounded by granulosa cell (GC) populations that, through folliculogenesis, differentiate into both mural GCs and cumulus cells (CCs). CCs are closer to the oocyte and were shown to maintain a proximity relationship with the oocyte, providing nutrients, maturation-enabling factors, and an optimal microenvironment to ensure successful maturation and further developmental competence (Eppig 1991, Pangas & Matzuk 2005, Gilchrist *et al*. 2008). The layers of differentiated GCs that surround the oocyte are essential for oocyte maturation, ovulation, as well as fertilization (Tanghe *et al*. 2002). Premature rupture of the communication between CCs and oocyte affected the competence level (Modina *et al*. 2001). Such communication is ensured via cytoplasmic extensions of corona radiata cells that pass through the zona pellucida and exchange with the oocyte during the whole maturation process (Allworth & Albertini 1993). The inhibition of these communicative junctions considerably affects bovine oocyte cytoplasmic maturation and hence the blastocyst rate *in vitro* (Ali *et al*. 2005). This is likely due to the lack of transfer of specific molecular signals that coordinate oocyte final maturation (Gilchrist *et al*. 2004, Lodde *et al*. 2007). Moreover, extracellular matrix (ECM)

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formation, CC differentiation, gene expression, metabolic activity, and steroidogenesis in the preovulatory stage were reported to be governed by the oocyte in many mammalian species like the mouse and the pig (Salustri 2000, Lucidi *et al*. 2003, Su *et al*. 2008, Paradis *et al*. 2010). Conversely, inhibition of transcription and/or translation in the cumulus–oocyte complex (COC) impaired oocyte maturation and fertilization (Motlik *et al*. 1989, Sirard *et al*. 1989, Kastrop *et al*. 1991, Tatemoto & Terada 1995). Optimal and reciprocal exchanges between oocyte and its CCs are therefore a key factor to successful maturation, fertilization, and early embryo development. Based on this, it is thought that CCs could be considered as a mirror that reflects the oocyte's level of competence and could thus be used as one of the main criteria for COC selection (Tanghe *et al*. 2002, McKenzie *et al*. 2004). This led us to focus on CCs in order to increase our knowledge concerning the gene expression patterns in CCs and contribute to the establishment of a non-invasive approach for oocyte quality prediction.

Other parameters, in particular the number of cumulus layers, their degree of expansion and/or apoptosis, oocyte diameter, and ooplasm homogeneity, are also used in COC selection (Blondin & Sirard 1995, Patel *et al*. 2007). However, these criteria are subjective and lack the required precision to select highly competent oocytes that allow successful embryo development. Finding reliable tools that efficiently assess the oocyte's quality is a prerequisite to predict its developmental potential. Alternative approaches were suggested to define molecular markers of oocyte competence, which are expressed in the oocyte itself (Lonergan *et al*. 2003, Paradis *et al*. 2005) or in the surrounding somatic compartments (Robker *et al*. 2000, Ochsner *et al*. 2003*b*, McKenzie *et al*. 2004, Assidi *et al*. 2008, Hamel *et al*. 2008). Investigation of the molecular pathways of competence through the identification of potential candidates expressed in CCs and associated with the oocyte quality is a key step to demystify the complex pathway of oocyte final maturation and competence acquisition. CCs gene expression patterns have the advantage of being a specific (for each oocyte) and non-invasive method that preserves oocyte integrity and allows its fertilization, early development, and even subsequent transfer.

The capacity of the cumulus to support oocyte maturation during antral follicle growth requires two types of stimulation: stimulation of oocyte factors GDF9 and BMP15 (Lucidi *et al*. 2003, Pangas & Matzuk 2005), and gonadotropin stimulation with FSH and/or LH (Adriaens *et al*. 2004, Ali & Sirard 2005, Sirard *et al*. 2007). Additionally, gonadotropin signal transduction by the somatic compartment requires *de novo* mRNA synthesis within CCs (Meinecke & Meinecke-Tillmann 1993). Most assisted reproductive technologies, including ovarian stimulation and *in vitro* maturation (IVM), are based on gonadotropins' actions. The investigation of LH differential gene expression *in vivo* and its correlation with oocyte competence is the focus of our present study. In fact, LH was reported to be important to oocyte final maturation, ovulation, as well as optimal fertilization and early embryo development (Dieleman *et al*. 2002). This is achieved through activation of intrafollicular signaling and gene expression pathways leading to CCs expansion, meiosis resumption, oocyte final maturation, and ovulation in most mammalian species including mouse (Panigone *et al*. 2008), pig (Kawashima *et al*. 2008), cow (Hyttel *et al*. 1997, Dieleman *et al*. 2002), and human (Filicori 1999, Feuerstein *et al*. 2007). Although it has been established that LH triggers key functions in both oocyte and cumulus, there are questions about the presence of LH receptor proteins in CCs membrane, suggesting an indirect induction pathway (Peng *et al*. 1991) probably through the EGFR (epidermal growth factor receptor) pathway (Panigone *et al*. 2008, Reizel *et al*. 2010).

In order to accurately explore the effect of LH, we used suppressive subtractive hybridization (SSH) of cDNA as a powerful strategy to both isolate and identify cDNAs of differentially expressed genes. SSH also allows the enrichment of low-abundance transcripts that are differentially expressed in the tester population (Diatchenko *et al*. 1996). A custom-made library of differentially expressed genes revealed by SSH and associated with confirmed competence contexts was made (Assidi *et al*. 2008, Hamel *et al*. 2008) and used in our microarray study.

The process of competence, which includes well space- and time-coordinated sequences of molecular events required for the oocyte to fulfill maturation and gain the ability to pass through the stages of early embryo to blastocyst, is poorly understood. Its proper achievement requires the contribution of CCs (Dieleman *et al*. 2002, Tanghe *et al*. 2002, Kimura *et al*. 2007). Herein, we investigated the genomic patterns of two cumulus treatments collected *in vivo* from superstimulated cows at 2 h before and at 6 h after the LH surge. At 2 h pre-LH, the oocyte is still in prophase I and is, with its somatic environment, ready to start the final maturation step induced by the LH peak. It corresponds to the *in vivo* prematuration stage (Dieleman *et al*. 2002). At this moment, we assume that gene expression machinery of the CCs is set to receive the last major induction of oocyte final maturation (the LH surge). The 6 h post-LH time point corresponds to the beginning of the germinal vesicle breakdown (GVBD) of the oocyte and the transcriptional arrest (Dieleman *et al*. 1983, Sirard *et al*. 1989, Hyttel *et al*. 1997). From the LH surge until the GVBD, we believe that CCs express key factors that are inducers or consistent markers of oocyte final maturation and therefore competency. The genomic study of gene expression pattern evolution from 2 h before to 6 h after the LH surge should yield insights

about CCs contribution to the final maturation process and provide powerful markers of oocyte quality. These CCs at 6 h post-LH are considered to be associated with high competent oocytes. In fact, our control treatment in Dr Dieleman's laboratory using the same superovulation protocol yielded more than 60% of expanded blastocysts as reported previously (Dieleman *et al.* 2002). This high competence level could indicate that our *in vivo* CCs are the sites of expression of final markers of oocyte quality. The identification of these candidates may be a valuable contribution in our understanding of the molecular events of oocyte competence fulfillment and may serve as predictors of high-quality oocytes.

Results

Microarray data analysis

Ovarian stimulation was achieved in order to increase the number of follicles that reach maturity. After COC aspiration and CCs isolation, total RNA of CCs was extracted and amplified, and hybridizations were performed. Our hybridization design consisted of 12 replicates ($3 \times 2 \times 2$) to be analyzed: 3 biological replicates, 2 technical replicates (dye-swap design), and 2 technical sub-replicates or blocks (SSH clones were printed twice on our custom-made microarray). Hybridization analysis of overexpressed and under-expressed clones at 6 h post-LH peak compared to 2 h pre-LH peak was therefore based on these 12 replicates. Correlation values between the log intensities of the 12 replicates for each group were very high (all above 0.85). These findings were also confirmed by hierarchical clustering of replications (data not shown). A minimum cutoff limit of 2.25 allowed us to filter data, lower the error variance, and select candidates with higher probabilities to be true positives (fold change >1.5). Using the National Institute on Aging (NIA) Array Analysis Tool at 5% false discovery rate (FDR), we obtained two lists of 141 overexpressed and 161 underexpressed clones that were affected by the LH surge. These clones represent respectively 6.18 and 7% of the 2278 transcripts printed on our custom-made array. These clones represent respectively 63 up-regulated and 112 down-regulated separate genes whose expression level vary significantly at 6 h post-LH compared to the 2 h pre-LH. The complete gene lists are provided (Supplementary Tables 1 and 2, see section on supplementary data given at the end of this article).

Functional genomic analysis

Using the Ingenuity Pathway Analysis (IPA) software, functional genomic analysis was performed to investigate the main molecular and cellular functions (Fig. 1; Table 1) related to our gene lists. Among the molecular pathways triggered by our differentially expressed genes,

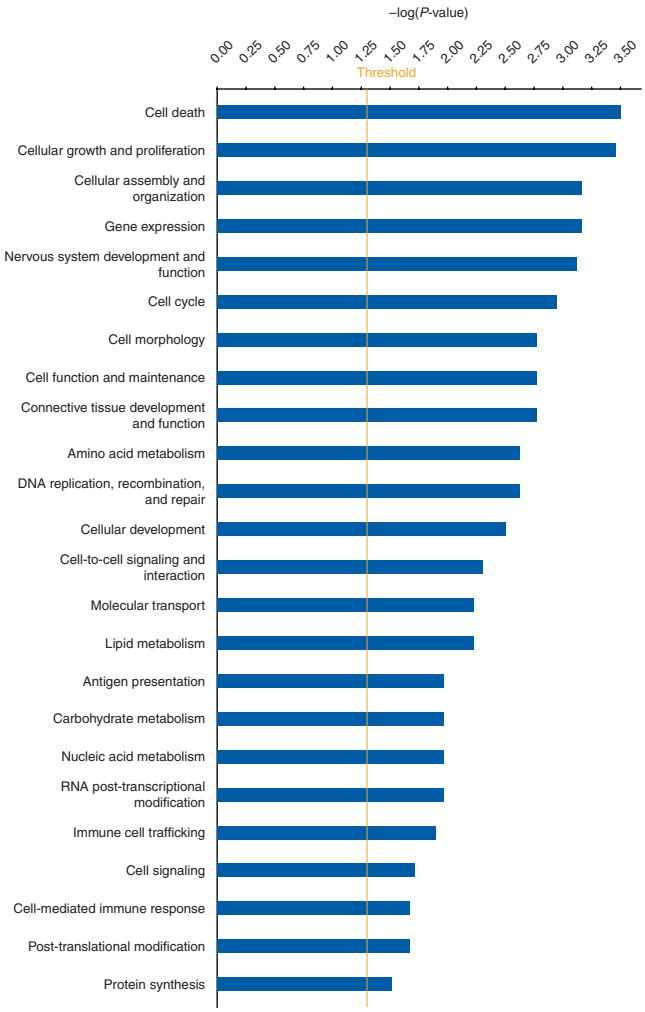

Figure 1 Summary of the main molecular and cellular functions triggerred by the LH surge-induced genes. Only significant functions (over the threshold, $P<0.05$) are shown.

protein biosynthesis and maturation (gene expression, protein synthesis, protein folding, and post-translational modification), cell differentiation (cell morphology, cell function and maintenance, and cell signaling), molecular transport, and cell-to-cell interactions (cellular assembly and organization, cell-to-cell signaling and interaction, cell-mediated immune response, nervous system function, and antigen presentation) were the main general functions (Fig. 1).

Using the IPA software, three (3) gene pathway networks, with the highest scores, were selected for further analysis. The first network includes mainly candidate genes that were overexpressed (red color) following LH/human chorionic gonadotropin (hCG) action (Fig. 2). On the other hand, the second network is composed of candidate genes (green color) that were down-regulated following the LH surge (Fig. 3). Concerning the third gene network selected, it included both overexpressed and underexpressed genes (Fig. 4).

Table 1 Main molecular and cellular functions regulated by the LH surge in bovine cumulus cells according to the Ingenuity Pathway Analysis software.

Function	*P* value	Percentage of genes
Underexpressed genes		
Protein synthesis	4.35×10^{-6}–4.78×10^{-2}	29.8
Cellular movement	5.14×10^{-4}–4.78×10^{-2}	31.6
Cell signaling	7.58×10^{-4}–4.78×10^{-2}	10.5
Molecular transport	7.58×10^{-4}–4.78×10^{-2}	17.6
Nucleic acid metabolism	7.58×10^{-4}–3.61×10^{-2}	10.5
Overexpressed genes		
Cellular growth and proliferation	4.17×10^{-6}–9.60×10^{-3}	33.3
Cell cycle	1.04×10^{-5}–1.03×10^{-2}	19.7
Cell morphology	1.55×10^{-5}–1.05×10^{-2}	19.7
Carbohydrate metabolism	7.27×10^{-5}–7.04×10^{-3}	6.1
Cellular assembly and organization	7.27×10^{-5}–7.04×10^{-3}	21.2

Real-time PCR validation

To validate both our positive and negative gene lists, seven candidate genes (four overexpressed and three underexpressed) were selected for real-time PCR validation. This quantitative real-time PCR (QPCR) validation was achieved on the initial biological replicates (pools of CCs) of the two (2 h before and 6 h after the LH surge) treatment groups (Fig. 5). Following QPCR analysis, all the four overexpressed candidates assessed were statistically very significant ($P<0.01$; Fig. 6A). For the three underexpressed candidates tested, two were statistically significant ($P<0.05$; Fig. 6B). The significant candidates are thrombospondin 1 (*THBS1*; $P<0.0001$), epiregulin (*EREG*; $P=0.0002$), ubiquitin-conjugating enzyme E2N (*UBE2N*; $P=0.0043$), tumor necrosis factor, α-induced protein 6 (*TNFAIP6*; $P=0.0068$), tribbles homolog 2 (*TRIB2*; $P=0.008$), and ERBB receptor feedback inhibitor 1 (*ERRFI1*; $P=0.050$; Fig. 6).

Discussion

Study approach

The choice of the 2 h pre- and 6 h post-LH peak time points was based on previous studies that showed that oocytes exposed to LH *in vivo* had better chances of becoming viable embryos after fertilization (Blondin *et al.* 2002, Dieleman *et al.* 2002). The gonadotropins' signal transduction, mainly LH, through the somatic compartment contributes to provide the oocyte with the required molecular tools and ingredients reserve to attain competence and undergo fertilization (Dieleman *et al.* 2002, Sirard *et al.* 2003, 2007).

It is also to note that the ovarian stimulation used herein to collect our samples is an established stimulation protocol previously used in Dr Dieleman's laboratory. This protocol always yields around 60% of expanded blastocysts (Dieleman *et al.* 2002). This high rate of blastocyst with best grade allowed us to assume that our collected *in vivo* tissues are associated with high competent oocytes.

Additionally, finding efficient tools to measure oocyte competence and successful embryo development necessitates a better understanding of the *in vivo* molecular pathways induced following the LH surge. It is established that CCs are beneficial for the oocyte maturation, fertilization, and later embryo development

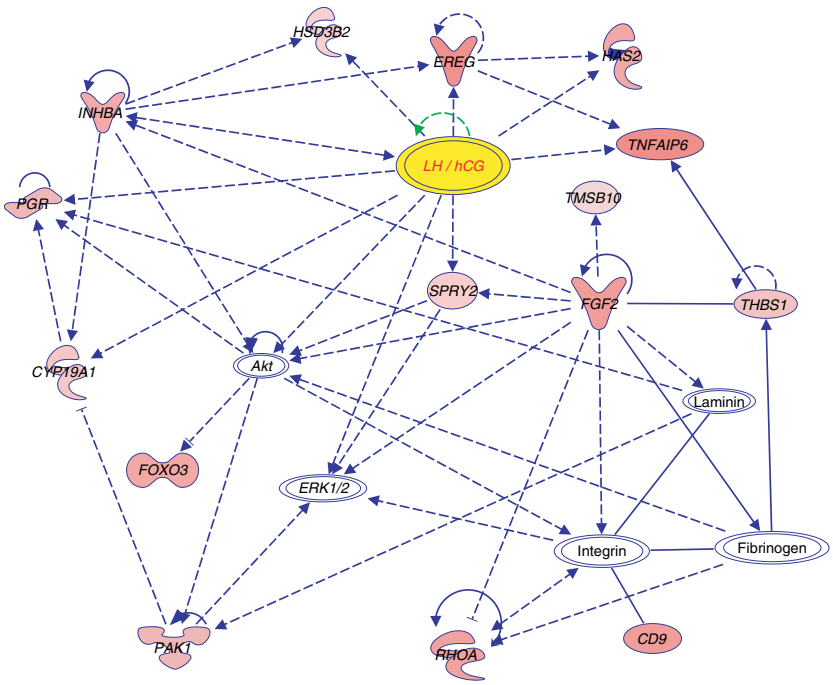

Figure 2 Ingenuity network 1 generated from the overexpressed genes (red) 6 h after the LH surge in bovine CCs. The genes in this first network are epiregulin (*EREG*), tumor necrosis factor, α-induced protein 6 (*TNFAIP6*), hyaluronan synthase 2 (*HAS2*), LH/human chorionic gonadotropin (*LH/hCG*), hydroxy-delta-5-steroid dehydrogenase, 3β- and steroid delta-isomerase 2 (*HSD3B2*), inhibin, βA (*INHBA*), BMP and activin membrane-bound inhibitor homolog (*Xenopus laevis*) (*BAMBI*), progesterone receptor (*PGR*), cytochrome P450, family 19, subfamily A, polypeptide 1 (CYP19A1), *Akt* (protein kinase B), sprouty homolog 2 (*Drosophila*) (*SPRY2*), thymosin β-10 (*TMSB10*), fibroblast growth factor 2 (*FGF2*), thrombospondin 1 (*THBS1*), *ERK1/2*, forkhead box O3 (*FOXO3*), CD9 molecule (*CD9*), ras homolog gene family, member A (*RHOA*), and p21 protein (Cdc42/Rac)-activated kinase 1 (*PAK1*).

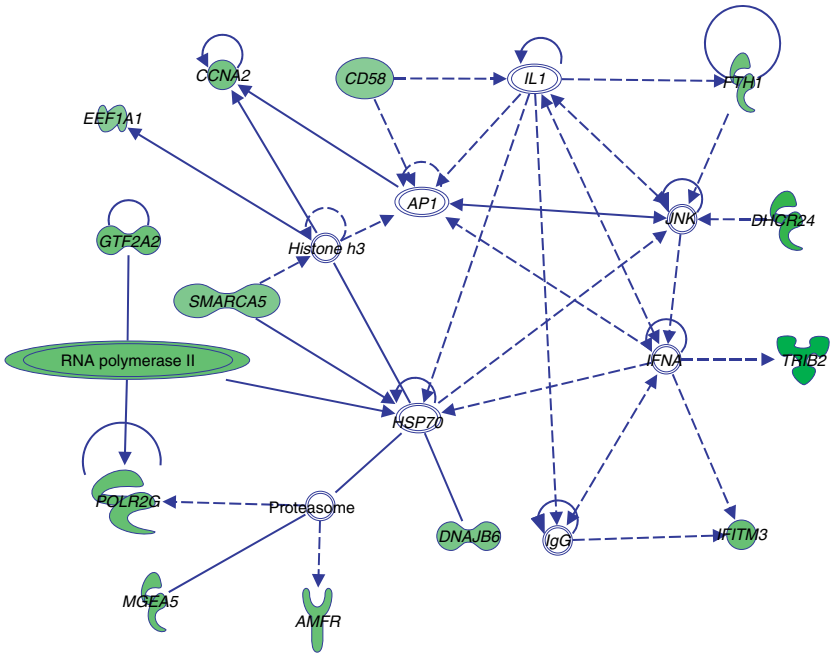

Figure 3 Ingenuity network 2 generated from the underexpressed genes (green) following the LH surge in bovine CCs. The genes in this second network are interleukin 1 (*IL1*), ferritin, heavy polypeptide 1 (*FTH1*), c-Jun N-terminal kinase (*JNK*), 24-dehydrocholesterol reductase (*DHCR24*), tribbles homolog 2 (*Drosophila*) (*TRIB2*), interferon induced transmembrane protein 3 (1-8U) (*IFITM3*), *IgG*, DnaJ (Hsp40) homolog, subfamily B, member 6 (*DNAJB6*), transcription factor AP1 (*AP1*), CD58 molecule (*CD58*), cyclin A2 (*CCNA2*), eukaryotic translation elongation factor 1 α 1 (*EEF1A1*), SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5 (*SMARCA5*), general transcription factor IIA, 2, 12 kDa (*GTF2A2*), polymerase (RNA) II (DNA directed) polypeptide G (*POLR2G*), meningioma expressed antigen 5 (hyaluronidase) (*MGEA5*), and autocrine motility factor receptor (AMFR).

in many mammalian species including mouse (Zhou *et al*. 2010), bovine (Assidi *et al*. 2008), and human (Russell & Robker 2007, Anderson *et al*. 2009). Keeping in mind that most of the transcriptional activity of the oocyte occurred during meiotic arrest before the GVBD (Hyttel *et al*. 2001), the study of the CCs gene expression patterns at the GVBD stage (6 h post-LH) could reflect the oocyte's quality. In fact, the oocyte at this stage has acquired most if not all the transcript stockpile needed for its final maturation and could therefore, through its continuous and intimate communication with its neighboring cells, trigger the expression of some potential and indirect biomarkers in CCs. In addition to their usefulness in improving COC selection, the study of these potential biomarkers' pathways is in the scope of this study in order to yield insights into the complex molecular process of oocyte competence that remains ambiguous.

Following a solid statistical design based on 12 highly correlated replicates, the microarray data analysis yielded 141 overexpressed and 161 underexpressed clones (Supplementary Tables 1 and 2). RNA profiles from 2 h pre- versus 6 h post-LH peak were analyzed using a functional approach according to their cellular and molecular functions, as well as the corresponding cell compartments.

Real-time PCR analysis

In order to strengthen our gene lists provided by microarray analysis, seven candidates were chosen for real-time PCR validation. The relative expression level of each candidate in each treatment (2 h before versus 6 h after LH) is summarized in Fig. 6. Among the candidates selected, over 85.7% of them were statistically significant. This high level of fidelity between microarray and QPCR data could be explained by our hybridization design based on 12 replicates (3 biological replicates $\times$2 technical replicates (dye-swap design) $\times$2 technical sub-replicates (2 blocks/chip)). Correlation values between the log intensities of microarray replicates were very high ($r^2 > 0.95$). Our results are in line with what is recommended in microarray analysis to get relevant data that highly correlate with further real-time PCR validation and therefore true biological differences (Gupta *et al*. 2008).

Cellular and molecular function analysis

Many cellular and molecular functions were statistically significant (over the threshold) following the LH surge. As expected, CCs start showing high degree of cell differentiation accompanied by slight apoptosis. In fact, the CCs are very specialized cells that undertake final differentiation after the LH peak. This differentiation phenotype included cell morphology, cellular maintenance, and development (Fig. 1 and Table 1), and it was required to CCs contribution at oocyte final maturation and successful ovulation (Tanghe *et al*. 2002). In this context, the cell morphology pathway at the GVBD was associated with a change in CCs from round to polarized shape. Cytoskeleton arrangements were marked by the assembly of actin microfilaments leading to cytoplasmic projections' formation that may serve cell-to-cell communications despite intensive mucification (Allworth & Albertini 1993, Sutovsky *et al*. 1994).

Protein translation and maturation is another interesting phenotype selectively exposed by the CCs. In fact,

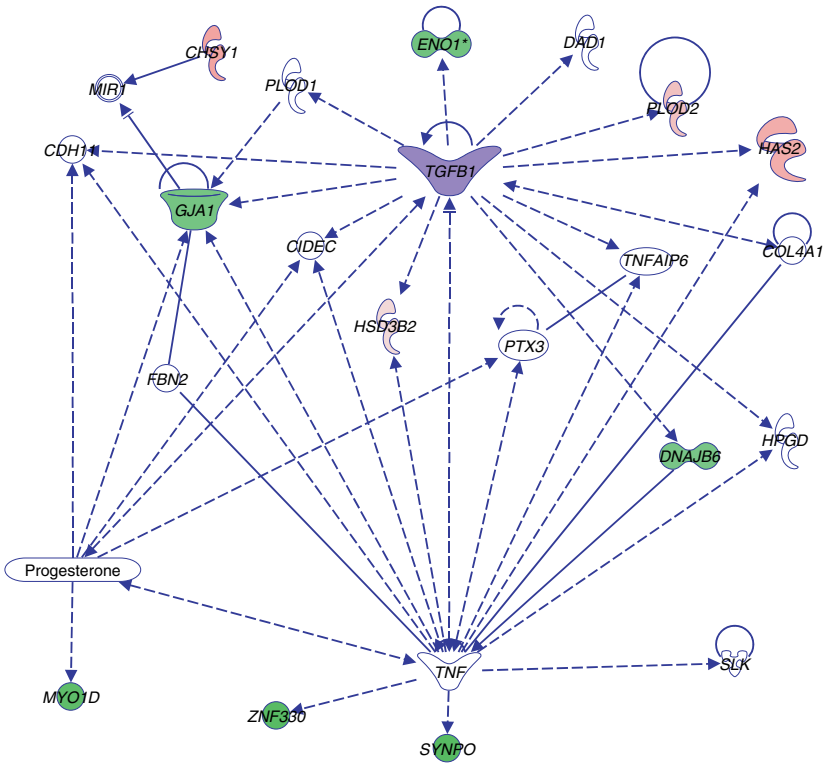

Figure 4 Ingenuity network 3 generated from both the over- and underexpressed gene candidates 6 h after the LH surge in bovine CCs. Overexpressed genes (red) are tumor necrosis factor, α-induced protein 6 (*TNFAIP6*), hyaluronan synthase 2 (*HAS2*), hydroxy-delta-5-steroid dehydrogenase, 3β- and steroid delta-isomerase 2 (*HSD3B2*), procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (*PLOD2*), and chondroitin sulfate synthase 1 (*CHSY1*). Underexpressed genes (green) are enolase 1, (α) (*ENO1*), DnaJ (Hsp40) homolog, subfamily B, member 6 (*DNAJB6*), gap junction protein, α 1, 43 kDa (*GJA1*), synaptopodin (*SYNPO*), zinc finger protein 330 (*ZNF330*), and myosin ID (*MYO1D*). The other genes (uncolored), recommended by the IPA software to achieve the network, are transforming growth factor, β1 (*TGFB1*), tumor necrosis factor (TNF superfamily, member 2) (*TNF*), pentraxin-related gene, rapidly induced by IL1β (*PTX3*), the defender against apoptotic cell death 1 (*DAD1*), procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1 (*PLOD1*), collagen, type IV, α 1 (*COL4A1*), cell death-inducing DFFA-like effector c (*CIDEC*), cadherin 11, type 2, OB-cadherin (osteoblast) (*CDH11*), fibrillin 2 (*FBN2*), hydroxyprostaglandin dehydrogenase 15-(NAD) (*HPGD*), and STE20-like kinase (yeast) (*SLK*).

despite the important rise in gene expression and translation occurring at this stage (GVBD), the CCs are the site of slight and selective decrease of some pathways/functions related to proliferation and cell growth including cellular movement (probably due to active expansion), protein synthesis, and nucleic acid metabolism (Table 1). These findings reinforce our knowledge of the CCs aforementioned differentiation status marked by fine regulation of protein synthesis, nucleic and amino acids' metabolism, as well as post-translational modifications (Hernandez-Gonzalez *et al.* 2006). This fine regulation is supported by the over-expression of genes associated with the transcription machinery (gene expression; BMP and activin membrane-bound inhibitor homolog (*BAMBI*), progesterone receptor (*PGR*), etc.) and the cell cycle (forkhead box O3 (*FOXO3*), fibroblast growth factor 2 (*FGF2*), etc.; Fig. 1). Moreover, expression of apoptosis-related genes in CCs reported herein was also associated with oocyte final maturation progression in several previous studies in many mammalian species (Host *et al.* 2002, van Montfoort *et al.* 2008), and it was even suggested as an indicator to predict human oocyte quality (Lee *et al.* 2001). However, at this stage (GVBD), the CCs tissue maintains a proliferative phenotype (cell cycle and cellular growth functions) that prepares the prerequisite structure for rapid expansion and oocyte competence acquisition support. These findings do not match with the conclusions of Okazaki *et al.* (2003) during their *in vitro* assays in the porcine species where the LH was reported to decrease the proliferative activity of CCs in culture. This may be due to timing of tissue analysis, the *in vitro* bias, and species differences. Interestingly, the expression of some proliferative genes was reported to be induced by the oocyte (Hussein *et al.* 2005), and we can assume that it may compensate/counteract the early apoptosis signs. This survival behavior was also reinforced by the activation of cell morphology and cellular assembly functions. These functions involved the expression of the *ECM* genes that prevent or delay the follicular cells apoptosis (Kaneko *et al.* 2000).

This 'mucification' process that distinguishes CCs is induced by the LH surge. Our differentially expressed genes at 6 h post-LH triggered many molecular and cellular functions associated with this mechanism including cell morphology, cellular assembly and organization, connective tissue development, as well as cell-to-cell signaling and interaction (Fig. 1). They include crucial genes reported to be essential for ECM formation and oocyte competence like *TNFAIP6*, *EREG*, sprouty homolog 2 (*SPRY2*), *PGR*, and hyaluronan synthase 2 (*HAS2*; Chen *et al.* 1993, Fulop *et al.* 2003, Ochsner *et al.* 2003*a*, Cillo *et al.* 2007).

Another remarkable pathway induced by LH is the metabolic activity. This activity includes the metabolism of amino acids, lipids, nucleic acids, as well as carbohydrates. It appears to be crucial to CCs activity and their dialog with the oocyte in order to reach its full developmental potential (Thompson *et al.* 2007, Su *et al.* 2008). This metabolic pathway includes mainly

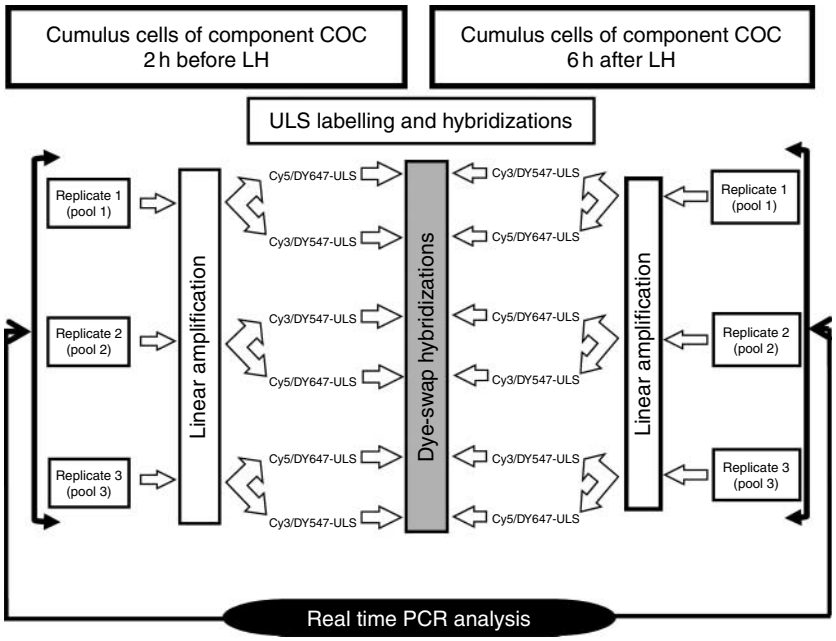

Figure 5 Experimental design of linear amplifications, dye-swap hybridizations, and real-time PCR validation. Each pool/biological replicate corresponded to the cumulus cells of ten healthy follicles collected from cows with regular superovulatory response.

glycolysis, steroidogenesis, and carbon metabolism (Krisher & Bavister 1999, Lucidi *et al*. 2003, Kwong *et al*. 2010, Sutton-McDowall *et al*. 2010).

Concurrently with their active differentiation, CCs are the site of intensive signaling pathways (both intra- and intercellular; Fig. 1). These signaling pathways, including many protein kinases such as protein kinase C, protein kinase B, protein kinase A (PKA), phosphatidylinositol 3-kinase (PI3K), and ERK1/2, are activated in CCs *in vitro* or following the LH surge and are required to the oocyte final maturation as well as ovulation (Mattioli & Barboni 2000, Shimada & Terada 2001, Fan *et al*. 2004, Russell & Robker 2007).

Molecular transport is another important function that is overrepresented by our LH-induced genes in CCs. This pathway includes mainly genes involved in intracellular organelles' motility and molecular transport (*TUBA1B*, *TMED5*, *TSNAX*, *LMAN1*, *BCAP29*, and *NRP1*; Supplementary Tables 1 and 2). The expression of these factors looks to be necessary for CCs membrane and cellular organelles to maintain their morphology and their content and to ensure their functions regardless of the rapid molecular events occurring following the LH surge. The transported cargo may be proteins, anions, and cations. Interestingly, this cytoplasmic streaming may drive CCs polarization required during mucification and thereafter as reported by Sutovsky *et al*. (1994). In fact, authors have shown that following the gonadotropins' stimulation, bovine CCs microtubules were associated with the Golgi, whereas the intermediate filaments combine with the lipid droplets close to the nucleus. Similar findings were also reported elsewhere (Hernandez-Gonzalez *et al*. 2006, Assou *et al*. 2008, van Montfoort *et al*. 2008, Racedo *et al*. 2009, Tesfaye *et al*. 2009).

Finally, we should highlight that in addition to the LH stimulation, these changes in transcriptome patterns of CCs are also influenced by the oocyte–cumulus reciprocal dialog documented by other previous studies (Eppig *et al*. 1997, Russell & Salustri 2006, Kimura *et al*. 2007, Su *et al*. 2008).

Gene network analysis

To investigate CCs genomic behavior following the LH surge, four important IPA gene networks of both overexpressed and underexpressed genes are discussed (Figs 2–4). We aim at finding relationships between identified gene candidates, the cumulus functions, and the established pathways in the COC. The analysis of such pathways reveals interesting pathways in CCs that are crucial to the oocyte final maturation process. Moreover, the analysis of these networks has allowed the identification of several key functions accomplished by CCs according to their gene expression profile at the GVBD stage.

ECM formation and stabilization

Following the LH surge, several genes involved in ECM formation including *EREG*, *THBS1*, *TNFAIP6*, *HAS2*, procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (*PLOD2*), and pentraxin (*PTX3*) and other cell adhesion molecules (CD9, integrins, laminin, and fibrinogen) were overexpressed. These candidate genes, illustrated mainly in Figs 2 and 4 pathways, are good illustration

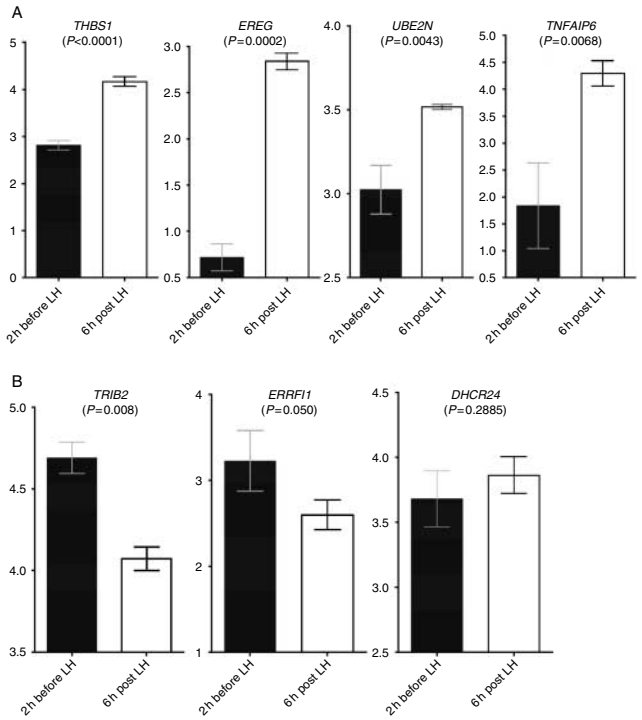

Figure 6 Real-time PCR analysis of some differentially expressed genes in original biological samples of bovine CCs at 6 h after versus 2 h before the LH surge. (A) Gene candidates overexpressed at 6 h after the LH surge. (B) Underexpressed genes at 6 h after compared to 2 h before the LH surge. The analyzed genes are thrombospondin 1 (*THBS1*), epiregulin (*EREG*), ubiquitin-conjugating enzyme E2N (*UBE2N*), tumor necrosis factor, α-induced protein 6 (*TNFAIP6*), tribbles homolog 2 (*Drosophila*) (*TRIB2*), ERBB receptor feedback inhibitor 1 (*ERRFI1*), and 24-dehydrocholesterol reductase (*DHCR24*). Candidates were ranked according to their *P* values, which were determined following a *t*-test analysis achieved on normalized data at $\alpha=0.05$.

of the signaling pathways that occur in CCs following the gonadotropin (LH/hCG and FSH) stimulation. In fact, LH induced some key genes known to be involved in CCs proper expansion mainly *TNFAIP6*, *EREG*, and *HAS2*. It has been established that *TNFAIP6* is essential for the ECM stabilization through its covalent links between hyaluronan (HA; backbone of the ECM) and inter-α-trypsin inhibitor (Carrette *et al*. 2001). Its expression downstream of the LH/hCG preovulatory surge has been confirmed in most mammals including mice (Hernandez-Gonzalez *et al*. 2006), bovine (Tesfaye *et al*. 2009), and human (Haouzi *et al*. 2009). Moreover, *TNFAIP6*-deficient mice ($TNFAIP6^{-/-}$) failed to form a stable ECM and are infertile (Fulop *et al*. 2003).

HAS2 is also a key enzyme for ECM structure through its induction of HA production. Its expression correlates with the HA production and the ECM kinetic formation, and was differentially expressed in human competent CCs (McKenzie *et al*. 2004). Our network also shows that *HAS2* and *TNFAIP6* are activated by the EGF-like factors' pathway (mainly EREG in our case, Fig. 2) during oocyte final maturation. This LH stimulation of the EGF pathway is established in mammals (Park *et al*. 2004), and it is considered as a main playmaker in the gonadotropins' signal transduction and amplification in CCs (Edson *et al*. 2009). The proper ECM construction requires additional immune-like factors such as *PTX3* (Bottazzi *et al*. 2006) and glucoproteins such as laminin, fibronectin, and integrins that are involved in cell differentiation and migration, as well as cell-to-cell adhesion (Sutovsky *et al*. 1995).

CD9 is also another member of the tetraspanins family. It is known to be an important protein in the fertilization process through its involvement in egg–sperm fusion (Ito *et al*. 2010, Lefevre *et al*. 2010) and sperm–CCs interactions (Mattioli *et al*. 2009, Sutovsky 2009). Taken together, these constituents provide ECM viscoelastic properties that are crucial to oocyte ovulation and further fertilization (reviewed by Zhuo & Kimata (2001), Tanghe *et al*. (2002) and Russell & Salustri (2006)).

Ovulation preparation and inflammatory-like response

Ovulation is one of the main functions associated with the LH preovulatory surge. It is a complex process marked by the rupture of the ovarian epithelium and the release of the COC into the fallopian tube (Hunter 2003). In fact, the LH surge induces rapid changes, mainly CC expansion, the ECM's water attraction, and hence the increase in antrum volume (Hunter 2003, Russell & Salustri 2006). Many crucial genes involved in this process are overexpressed in our data sets like *TNFAIP6*, *HAS2*, and *PGR* (Figs 2 and 4). These candidates are involved in both CCs expansion and proteolysis, which are required for ovulation (Tsafriri 1995, Tsafriri & Reich 1999, Espey & Richards 2002). The knockout of these genes in mice resulted in impaired mucification and the inhibition/reduction of ovulation rates and therefore fertility (Lydon *et al*. 1995, Hess *et al*. 1999, Fulop *et al*. 2003, Mittaz *et al*. 2004, Sugiura *et al*. 2009). Moreover, *PGR* was even reported to be induced by LH (through MAPK), and it was suggested as a crucial playmaker (hormone receptor and transcription factor) upstream of the ovulatory process pathway (Robker *et al*. 2000, Richards 2005, 2007, Kawashima *et al*. 2008, Kim *et al*. 2009).

Additionally, we report herein the expression of several inflammatory-like factors such as *CD58*, *IL1*, *FTH1*, *THBS1*, *DNAJB6*, *IFNA*, *TGFB*, and *TNF* (Figs 3 and 4). These results are in agreement with previous studies where ovulation was considered as an inflammatory-like process finely regulated through CCs (Hernandez-Gonzalez *et al*. 2006, Richards 2007, Russell & Robker 2007). Recently, it was reported that *IFNA*, whose expression in follicular cells is induced by LH, is involved in the rat preovulatory follicular differentiation (through PI3K/ERK pathway; Lee da *et al*. 2009).

Additionally, our gene networks showed that *FGF2*, which contributes to both the expression of these immune-like factors and CCs expansion (Fig. 2), was

recently suggested as a biomarker of high-quality human oocytes that lead to successful pregnancy (van Montfoort *et al*. 2008), and it was therefore involved in the developmental competence acquisition process.

Uterine milk protein (*UTMP*) is an interesting candidate that is reported for the first time to be up-regulated following the LH surge (fold change >7). It is a protein known to be expressed in the endometrium downstream of the progesterone pathway. It is associated with important protease inhibition functions (protease functions similar to SERPIN's superfamily), and is believed to support early pregnancy through nutritional supply and autoimmune protection of the embryo (Moffatt *et al*. 1987, Ing & Roberts 1989, Khatib *et al*. 2007). It also seems to be involved in protein metabolism (Fig. 1) by reducing the negative impact of the inflammatory-like environment (required for ovulation) in the immediate vicinity of the oocyte and therefore preserving its quality.

Steroid biosynthesis

The analysis of our gene networks at 6 h following the LH peak revealed many steroidogenesis-related genes including *HSD3B2*, *INHBA*, *PGR*, *HPGD*, and *DHCR24* (Figs 2–4). These patterns are indicative of increased steroidogenic activity within CCs at the GVBD stage, and may reflect an oocyte control of this process (Vanderhyden *et al*. 1993). The synthesis of steroids was significantly higher in the late preovulatory stage COC (Vanderhyden & Macdonald 1998). The presence/absence of steroids in IVM–IVF protocols may affect the expected oocyte quality and therefore its developmental competence both *in vivo* and *in vitro* (Ali & Sirard 2002, Dieleman *et al*. 2002). *PGR* (Fig. 2) could play an essential role especially in CCs (Li *et al*. 2004) through an increased sensitivity to progesterone-induced genes. The *PGR* is expressed in CCs and associated with the preovulatory stage in many mammals including mice (Hernandez-Gonzalez *et al*. 2006), bovine (Mingoti *et al*. 2002, Schoenfelder *et al*. 2003), pig (Lucidi *et al*. 2003), and human (Chian *et al*. 1999). The changes in steroid output and sensitivity, especially in progesterone, could be required not only for mammalian oocyte final maturation, but also for ovulation and subsequent embryo survival (Wise *et al*. 1994, Richards 2005, Christian & Moenter 2010, Lynch *et al*. 2010). Interestingly, progesterone is also reported to be useful to the human sperm acrosome reaction (Teves *et al*. 2009) and therefore could be useful for COCs in the fallopian tube. The fine regulation of steroid profile is suggested to influence the granulosa differentiation and to increase IVF yields (Lucidi *et al*. 2003).

Cell signaling

Cell signaling is one of the main functions governed by the LH-activated genes as shown in Fig. 1. The cellular signaling pathway included mainly signal transduction and intracellular signaling. *TNFAIP6*, *EREG*, *INHBA*, *PGR* (Fig. 2), and *UTMP* (Supplementary Tables 1 and 2) were the major redundant candidates in our gene networks. While *TNFAIP6*, *INHBA*, *EREG*, and *TNF* were reported previously as potential candidates induced by LH in CCs in association with competence (Ben-Ami *et al*. 2006, Feuerstein *et al*. 2007), we reported herein an interesting gene network (Fig. 4) where the *TNF* seems to play a crucial and central role in CCs expansion and steroidogenesis via its interactions with several known genes as *PTX3*, *TNFAIP6*, gap junction protein α1 (*GJA1*), and progesterone. Moreover, *TNF* and its receptor type II were previously reported to be present in both human CCs and oocyte (Naz *et al*. 1997). More studies on this candidate are needed to better understand its contribution in the follicle differentiation.

It is widely known that CCs–oocyte communications occurred mainly through the gap junctions (Szollosi 1978, Carabatsos *et al*. 2000). These communications played important roles in the oocyte–somatic compartment cell signaling and decreased at the GVBD (Sutovsky *et al*. 1993, Mattioli & Barboni 2000). Breakdown of such connections was suggested to be gonadotropin-dependent in pig COCs (Sasseville *et al*. 2009). Our data confirmed these findings in the cow at 6 h post-LH by the down-regulation of a fundamental protein of these cell-to-cell connections: GJA1.

PGR is probably involved as a coordinator in CC intracellular signaling. It is an essential factor that is expressed through the LH/MAPK pathway and required for the oocyte final maturation and subsequent ovulation. It acts as a hormone receptor as well as a transcription factor (Richards 2007, Kawashima *et al*. 2008). PGR knockout mice have impaired reproductive functions as an anovulatory phenotype, troubles in sexual behavior, and even inflammatory symptoms (Lydon *et al*. 1995). It is suggested to control the expression and/or the activity of key genes needed in the ovulatory process such as key transcription factors, proteases, cell adhesion molecules, and inflammation factors (Robker *et al*. 2000, Li *et al*. 2004, Kim *et al*. 2009).

The LH surge is believed to induce multiple intracellular signaling and second messengers in the follicle, including the PKA, phospholipase C, and inositol triphosphate pathways. It is also known to increase the intracellular calcium (reviewed in Russell & Robker (2007)). AKT (Fig. 2) becomes phosphorylated in the follicular cells in rat and bovine species following LH treatment (Carvalho *et al*. 2003, Fukuda *et al*. 2009).

Additionally, the ERK1/2 pathway (Fig. 2) is involved in the response to LH by the maturational events in mouse COC including cumulus expansion and oocyte meiosis resumption (GVBD; Su *et al*. 2003). ERK1/2 is also suggested to phosphorylate several transcriptional factors implicated in the final maturation and the ovulation processes, including FOS, MYC, STAT3, and

AP1 (Sharma & Richards 2000, Roux & Blenis 2004, Russell & Robker 2007). AP1, for example (Fig. 3), was confirmed as an essential transcription factor for the expression of the *TNFAIP6* gene in bovine GCs just prior to ovulation (Sayasith *et al*. 2008).

EREG gene, which is an EGF-like factor (Fig. 2), is considered as a major factor in competence acquisition via the amplification of the gonadotropin signal in CCs (particularly LH *in vivo* and FSH *in vitro*) that leads to the oocyte's final maturation and ovulation (Park *et al*. 2004, Ashkenazi *et al*. 2005, Assidi *et al*. 2008, Conti 2010, Su *et al*. 2010). Following the LH surge, it was demonstrated that EREG activates the EGFR in order to spread the ovulation signal (Park *et al*. 2004).

The transforming growth factor, β1 (*TGFB1*) gene seems to trigger interesting pathways that should be explored further (Fig. 4). This gene is a member of the TGFB superfamily and was not present in our gene list. This gene candidate may therefore represent the oocyte's control of the major molecular processes in CCs through some oocyte-derived paracrine factors, mainly the TGFB members (BMP15, GDF9, FGF8, TGFB1, etc.; Galloway *et al*. 2000, Pangas & Matzuk 2005, Sugiura *et al*. 2007, Su *et al*. 2008). Recently, it was reported that $Tgfb^{-/-}$ mice suffered from severe perturbation including the LH synthesis, oocyte incompetence, and early embryo arrest (Ingman & Robertson 2009), which is a further confirmation of its importance in the oocyte's acquisition of developmental competence.

Neuronal-like functions of cumulus cells

Following the LH surge, we showed up the overexpression of several neuronal-related genes such as *THBS1* (Liauw *et al*. 2008), *FGF2* (Chen *et al*. 2010), *MYO1D* (Brown & Bridgman 2004), *SYNPO* (Vlachos *et al*. 2009), and *CD9* (Ishibashi *et al*. 2004; Figs 2 and 4). Strikingly, the common functions ensured by these genes were mainly neuron plasticity, neurogenesis (including the dendrogenesis and the axogenesis), and synaptogenesis. These pathways were also reported to be finely controlled by steroids and particularly estradiol (E_2; Fester *et al*. 2009). Some synaptic proteins, such as synaptosomal-associated protein; 25 kDa, were previously reported in the pre-ovulatory and luteal GC as well as in the oocyte. This protein, which is a crucial element of the molecular machinery required for the neurotransmitter exocytosis, was overexpressed by FSH and E_2, and maintained by the LH (Grosse *et al*. 2000). Moreover, it was demonstrated that some of the ECM proteins of CCs were also involved in neuronal development (Moore *et al*. 2009).

In addition to their immune-like phenotype discussed before, CCs were also reported herein to have some neuronal-like functions, confirming the previous study of Hernandez-Gonzalez *et al*. (2006). Other cytokines and neuronal factors were also shown to be differentially expressed within CCs, and therefore suggested as a mediator of the ovulatory process in many mammal species including humans (McKenzie *et al*. 2004, Zhang *et al*. 2005, Feuerstein *et al*. 2007), mice (Shimada *et al*. 2006, Richards 2007), rats (Espey *et al*. 2000), and cattle (Assidi *et al*. 2008).

Based on these gene expression reports, CC plasticity could be clearly highlighted, leading to crucial questions concerning the real pathways of communications between the oocyte and the surrounding CCs, restricted until now to the gap junctions. Considering both the cytoplasmic extensions that meet the oocyte and these immune- and neuronal-like factors, we can assume the possibility of exchange of signaling vesicles through phagocytosis and/or synapses between CCs and oocyte. CCs might need an active transport process to carry specific molecules to their polarization site of action to ensure efficient and rapid biological effects. This hypothesis needs more investigation in future studies to enlighten the intricate and mysterious cumulus–oocyte communication pathways.

Potential* in vivo *markers of oocyte competence

In previous work in our laboratory, we reported a set of potential markers of oocyte competence expressed in bovine CCs *in vitro* with three different IVM treatments (FSH; phorbol myristate acetate (PMA); FSH + PMA; Assidi *et al*. 2008). Using the oocyte meiotic state (GVBD) as a reference, we compared herein the reported potential genes associated with oocyte competence and regulated by all three *in vitro* treatments (Assidi *et al*. 2008), with the genes overexpressed herein *in vivo* at 6 h after the LH surge. Potential candidates associated with oocyte competence, which were up-regulated in three IVM treatments and confirmed *in vivo*, should offer more efficient markers to assess oocyte quality and maturation fulfillment. Table 2 summarizes the common candidates between the two contexts.

Interestingly, among the 25 candidates that were overexpressed in competence contexts *in vitro*, 16 were also overexpressed *in vivo* (64%). Some of these candidates were reported in other species and/or contexts as potential markers of competence, e.g. *TNFAIP6* in mouse, porcine CCs (Fulop *et al*. 2003, Nagyova *et al*. 2009), *HAS2* in human cumulus GCs (McKenzie *et al*. 2004), *PGR* in mouse GCs (Shao *et al*. 2003), and *INHBA* in bovine GCs (Fayad *et al*. 2004).

SPRY2 is another interesting gene candidate that is overexpressed following the LH surge (Fig. 2). Its overexpression in follicular cells was differentially associated with developmentally competent oocytes in mammals, mainly in the bovine (Robert *et al*. 2001) and human (Hamel *et al*. 2008) species. This gene was furthermore shown to be induced by the FGF pathway and acts to reduce cell proliferation (Edwin *et al*. 2006) and to amplify the EGFR pathway (through MAPK/ERK1/2; Fig. 2; Egan *et al*. 2002). It may be assumed that *SPRY2*

Table 2 Common differentially expressed genes in bovine cumulus cells between up-regulated candidates in three *in vitro* maturation treatments (FSH; phorbol myristate acetate (PMA); FSH + PMA) and the overexpressed candidates *in vivo* at 6 h after the LH surge: possible biomarkers of oocyte developmental competence.

Gene name	Full name of gene/protein	Accession number
ADFP	*Bos taurus* adipose differentiation-related protein	NM_173980
ATP6V1C1	*Bos taurus* ATPase, H+ transporting, lysosomal 42 kDa, V1 subunit C1	NM_176676
HSPA8	*Bos taurus* heat shock 70 kDa protein 8	NM_174345
HAS2	*Bos taurus* hyaluronan synthase 2	NM_174079
INHBA	*Bos taurus* inhibin, β A (activin A, activin AB α polypeptide)	NM_174363
SELK	*Bos taurus* selenoprotein K	NM_001037489.2
TNFAIP6	*Bos taurus* tumor necrosis factor, α-induced protein 6	NM_001007813
UTMP	*Bos taurus* uterine milk protein precursor	NM_174797
AKAP7	*Bos taurus* A kinase (PRKA) anchor protein 7	NM_001102266.1
EREG	*Bos taurus* epiregulin (EREG)	XM_596732
HIGD1A	*Homo sapiens* HIG1 domain family, member 1A	BC070277
THBS1	*Bos taurus* thrombospondin 1	NM_174196.1
HSPA5 (*GRP-78*)	*Bos taurus* heat shock 70 kDa protein 5 (78 kDa glucose-regulated protein)	NM_001075148
SLC18A2	*Bos taurus* solute carrier family 18 (vesicular monoamine), member 2	NM_174653
SLC39A10	Predicted: *Bos taurus* similar to solute carrier family 39 (zinc transporter), member 10	XM_599261
PGR	*Bos taurus* progesterone receptor	XM_583951.4

could be a biomarker of oocyte quality that contributes to CCs final differentiation and is involved in the EGF-like factors' amplification of the gonadotropins' signals.

Taken together, these results reinforce our findings and yield insights concerning the main factors involved in CCs contribution to the oocyte competence acquisition. These biomarkers will serve as a precious tool for a non-invasive assessment of oocyte quality and therefore competence. The interactions of these factors and other yet unidentified candidates will be a precious itinerary to explore in future studies in order to complete the oocyte competence puzzle.

This non-exhaustive inventory of the main up- and down-regulated molecular functions and gene networks involved in the CCs is a preamble to enrich our comprehension of molecular contribution of CCs in oocyte final maturation, ovulation, and subsequent fertilization. To our knowledge, this is the first study that analyzed these molecular events around the LH surge (GVBD) in the bovine cumulus *in vivo*. Many potential candidates (see Supplementary Tables 1 and 2) expressed hours prior to ovulation are reported for the first time. More exploratory studies are required to draw the chronology of the whole molecular events in CCs – starting before the LH surge and ending at ovulation – and leading to oocyte final maturation.

Materials and Methods

Animals

Cyclic, non-lactating, and clinically healthy Holstein-Friesian cows were selected as described previously (Knijn *et al.* 2002). Animals were synchronized using a 9-day ear implant (3 mg Norgestomet, Crestar, Intervet International BV, Boxmeer, The Netherlands) followed by prostaglandin (PG; Prosolvin, Intervet International BV). The detailed protocol was approved by the Ethical Committee of the Veterinary Faculty of Utrecht University.

Superovulation schedule

Selected cows ($n=20$) were ~4 year old and 185 days *post partum*. They were randomized to ten cows per treatment and then selected for regular superovulatory response and synchronous cycle using progesterone measurement three times a week. The aim was to avoid possible effects of asynchronous cycles on the LH surge (Atkins *et al.* 2008). A crestar/GNRH-controlled LH surge was used in our superovulation protocol (Knijn *et al.* 2002). The LH concentration was monitored to determine the time of maximum LH and ovariectomy. The few cows with deviating LH profiles were excluded. Following estrus synchronization, cows were kept in similar conditions of housing, food (silage and concentrate), as well as water *ad libitum*. Superstimulatory treatments were initiated on the 9th day of the new estrus cycle (day 0 = estrus) by implanting another ear implant Norgestomet. Cows then received a total dose of ovine FSH (Ovagen ICP, Auckland, New Zealand) equivalent to 299 IU NIH-FSH-S1 units in eight decreasing doses administered at 12 h intervals from day 10 to day 13 (3.5, 2.5, 1.5, and 1 ml; Knijn *et al.* 2002). PG (22.5 mg) was given simultaneously with the fifth dose of FSH. Fifty hours after PG treatment, the ear implant was removed, and GNRH (0.021 mg Receptal i.m., Intervet International BV) was administered to induce the LH peak. Ovaries were collected immediately after cow ovariectomy (by laparotomy through flank incision and under local anesthesia) at 50 and 58 h following PG administration, which respectively corresponded to 2 h before and 6 h after the highest level of LH. They were immediately transported to the laboratory in saline solution at 37 °C.

Collection of cumulus cells

Two experimental groups were set up as follows: 1) 2 h before LH peak and 2) 6 h after LH peak to explore the genomic events of the final maturation steps of COC *in vivo*, using the LH surge as a reference. For each treatment (group of 4 cows/treatment), follicular contents were aspirated from

ovaries and stored at 4 °C. For each follicle, collected follicular fluid (FF) was used to predict follicular diameter using the volume formula ($=4/3\pi r^3$ with $2r=$diameter). Since the FF underestimates the follicle size, 1 mm is added to accurately predict the follicular diameter. Only follicles with 9–16 mm diameter were used in this study. COCs were then retrieved under a stereomicroscope and washed twice in PBS–5% polyvinyl alcohol (Sigma–Aldrich). COCs were individually incubated with 5% (w/v) hyaluronidase (Sigma–Aldrich) for 1 min, and CCs were denuded by gentle pipetting, washed, and classified on the basis of satisfactory cumulus and oocyte morphology (Blondin & Sirard 1995), as well as on the basis of steroid concentrations of the FF. For the 2 h pre-LH group, the healthy follicles selected have a diameter >9 mm and E_2 concentration $>1.41\pm0.1$ μmol/l (progesterone level is not considered at this stage since it is very low). Concerning the 6 h post-LH group, follicle size was between 9 and 16 mm with E_2 and progesterone concentrations greater than 0.3 ± 0.2 and 0.46 ± 0.05 respectively. Using steroid concentration patterns, and follicle fluid volume as a reference (Dieleman *et al.* 1983, van de Leemput *et al.* 1999, Dieleman *et al.* 2002), ten and eight cows had regular superovulatory response respectively at 2 h before and 6 h after the LH surge compared to the normal steroid profile of non-stimulated, cyclic cows used as a control. Cumulus of selected follicles were pooled together to get four biological replicates, and stored at −80 °C for total RNA extraction. Each replicate corresponded to the cumulus of ten healthy follicles collected from five of ten cows at 2 h before LH and from four of eight cows at 6 h after LH.

Total RNA extraction

CCs were subjected to total RNA extraction using the Absolutely RNA Microprep kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. Briefly, total RNA was protected from RNases using a mixture of 0.7% (v/v) β-mercaptoethanol added to 100 μl of lysis buffer. After homogenization and addition of an equal volume of 70% ethanol, the mix was transferred to a column microcentrifuge and spun for 30 s. Column product was washed with 600 μl of 1× low-salt wash buffer for 30 s, then immediately for 2 min to air dry the spin cup. To prevent contamination, DNase digestion of the retained RNA was then performed for 15 min at 37 °C according to the kit recommendations. Extracted RNA was washed once in 1× high-salt wash buffer and twice in 1× low-salt wash buffer, and resuspended in 30 μl of Elution buffer provided in the kit. The concentration and quality of the RNA were assessed using Agilent 2100 bioanalyzer (Agilent Technologies, Waldbronn, Germany) according to the manufacturer's protocol.

mRNA linear amplification

Based on RNA quality, the three best biological replicates of CCs collected both at 2 h before and 6 h after the LH surge were amplified using two-round *in vitro* transcription (IVT) following the instructions of the RiboAmp RNA Amplification kit (Arcturus, Mountain View, CA, USA). Briefly, 10 ng total RNA of each biological replicate of each treatment was added to 1 μl of RiboAmp primer A for 5 min at 65 °C, reverse transcribed in a total volume of 20 μl containing 7 μl of first strand master mix and 2 μl of first strand enzyme mix at 42 °C for 2 h, and then put on ice. Two microliters of first strand nuclease mix were added to the reaction for 30 min at 37 °C, followed by 5 min at 95 °C. After incubation with 1 μl of RiboAmp primer B for 2 min at 95 °C, second strand synthesis was achieved using 29 μl of second strand master mix and 1 μl of second strand enzyme following this thermocycling program (5 min at 25 °C, 10 min at 37 °C, and 5 min at 70 °C), hold at 4 °C (on ice), subsequently purified with the columns provided, and eluted with 11 μl of DNA elution buffer. The IVT of round 1 was carried out by successive addition of 8 μl of IVT buffer, 12 μl of IVT master mix, and 4 μl of IVT enzyme mix for 6 h at 37 °C. The IVT product was DNase-treated, purified, and eluted in 12 μl of RNA elution buffer. One microliter of this elution was used for NanoDrop (Wilmington, DE, USA) quantification of the first round yield, whereas the rest served as a template for the second round.

As for the first round, the second round started with the addition of 1 μl of RiboAmp primer B and incubation for 5 min at 65 °C; then, the complete first strand was synthesized using the same mix of the first round for 10 min at 25 °C, followed by 45 min at 37 °C. Second strand synthesis was performed using the RiboAmp primer A and the same mix as the second strand synthesis of the first round using this program (30 min for 37 °C and 5 min for 70 °C), purified, and the amplification product was eluted in 30 μl of RNA-eluted buffer and 1 μl was used to quantify the RNA amplification yield using NanoDrop as before.

mRNA direct labeling and hybridization

Amplified mRNA of each replicate was divided into two sub-replicates to get dye-swap design and labeled using the Universal Linkage System (ULS) aRNA Fluorescent Labelling Kit (KREATECH Biotechnology, Amsterdam, The Netherlands) according to the manufacturer's instructions. Briefly, for each sub-replicate, 2.5 μg of aRNA was labeled by incubation with 2.5 μl of Cy5/DY647-ULS or Cy3/DY547-ULS, and 2 μl of 10× Labeling solution in a 20 μl total volume at 85 °C for 15 min. Unbound dye was then removed, as recommended, using the KREApure columns provided in the kit. Labeled RNA was quantified on the NanoDrop. Each of the two differentially labeled sub-replicates (Cy5/DY647-ULS versus Cy3/DY547-ULS) used for hybridization on the same microarray slide (2 h before LH versus 6 h after LH) were then mixed together in equimolar proportions. Six hybridizations were performed in a dye-swap design (Fig. 5) on our custom-made array of differentially expressed transcripts described elsewhere (Hamel *et al.* 2008). Hybridizations were performed in the ArrayBooster using the Advacard AC3C (The Gel Company, San Francisco, CA, USA) for 18 h at 50 °C using Slide Hyb#1 (Ambion, Austin, TX, USA). The slides were then washed twice in 2×SSC/0.5% SDS buffer, and twice in 0.5×SSC/0.5% SDS buffer. After two quick final washes at room temperature in 1×SSC and water, slides were

spin-dried, scanned, and analyzed using the ChipReader and ArrayPro Analyzer software (Media Cybernetics, San Diego, CA, USA).

Microarray data analysis

Microarray data were subjected to Loess normalization using the ArrayPro software, and uninformative data that were below background were then removed. Data analysis of our dye-swap experiments was subsequently achieved through the free software NIA Array Analysis Tool (Baltimore, MD, USA; http://lgsun.grc.nia.nih.gov/ANOVA/) developed at NIA. The analysis was performed with our three biological replicates and the two technical replicates generated by the dye-swap design using the 2 h before LH as a reference and, at FDR = 5%, a minimum cutoff limit of 2.25. Since each clone was printed twice on our slide (Hamel *et al*. 2008), two additional technical sub-replicates that emerged from this design were taken into account. Two lists of overexpressed and underexpressed clones in the 6 h after LH treatment compared to 2 h before LH treatment were generated for further functional genomic analysis using mainly the IPA software (Ingenuity Pathways Analysis (IPA), v 8.0; Ingenuity Systems, Redwood City, CA, USA). Briefly, the candidate gene official names and fold change were uploaded into the IPA. Using its web-based database on previous studies, IPA is able to automatically find the potential connections between the uploaded candidates and to classify them into scored networks according to the molecular pathways. A score and a *P* value were therefore associated with each network, which is composed of selected genes from the uploaded list linked together and with other molecules (suggested by the software), and mapped in a whole signaling pathway. Each molecular relationship among the network members is represented in a conventional mapping that allows identification and therefore interpretation of the type of interactions. This genomic analysis aims at discovering some gene networks and pathways in CCs associated with the LH genomic effect and therefore with the oocyte final maturation.

Real-time PCR validation

Real-time PCR validation of some candidates was achieved on the three initial biological replicates (Fig. 5). Equal amounts of total RNA were taken from each CC pool of both 2 h before and 6 h after the LH surge treatments. To denature the RNA and remove secondary structures, the RNAs were heated at 65 °C for 5 min and then quenched rapidly on ice for at least 2 min. Samples were then reverse transcribed using the SensiScript reverse transcriptase kit (Qiagen) according to the manufacturer's recommendations. Real-time PCR was performed on the seven selected candidates (four overexpressed and three underexpressed) in LightCycler capillaries (Roche Applied Science) using the LightCycler FastStart DNA Master SYBR Green I (Roche) as detailed elsewhere (Assidi *et al*. 2008). For each candidate, a specific set of primers was designed using the NCBI/Primer-BLAST software and the candidates' specific sequences (NCBI; Table 3). Normalization was achieved using three housekeeping genes, *ACTB* (β-actin), *GAPDH*, and succinate dehydrogenase complex, subunit A (*SDHA*). Following the GeNorm software analysis, *SDHA* and *GAPDH* were successively the most stable control genes (*M* value = 0.121; $P > 0.05$). These two housekeeping genes were subsequently used in for QPCR data normalization. The real-time PCR product specificity of each candidate was confirmed by sequencing as well as through the analysis of the LightCycler melting curve (Roche). Each gene mRNA expression level was then divided by its normalization factor and log-transformed

Table 3 Sequences of real-time PCR-specific primers of gene candidates expressed in bovine cumulus cells.

Gene name	Primer set (5′–3′)	Genbank	Annealing temperature	Fluorescence acquisition temperature
UBE2N	Up 5′-AAGACCAATGAAGCCCAAGCCA-3′ Low 5′-GCAGCTAACCCTGACAACTACC-3′	NM_001076258.1	60	81
TRIB2	Up 5′-CACACATCTTGGCATCGCACTGTT-3′ Low 5′-AGCACCCAGGTTTCACATCAGTCT-3′	NM_178317.3	61	79
DHCR24	Up 5′-ACAAACCTGAGTCCAGTTCCCAGT-3′ Low 5′-AAGTGTCTTCTCCAAGCACACGGT-3′	NM_001103276.1	62	89
ERRFI1	Up 5′-TGCCTGCTTTAAGTCGTCCTGAGA-3′ Low 5′-ACCCACAACACACATCTCCACACA-3′	NM_001077930.1	61	83
EREG	Up 5′-GTGTGGCTCAAGTGTCAATAAC-3′ Low 5′-TCGATTTCTGTACCATCTGC-3′	XM_596732	53	81
TNFAIP6	Up 5′-GTCTGGCAAATACAAGCTCACCT-3′ Low 5′-GGATCTGTAAACACACCACCACA-3′	NM_001007813.1	57	84
THBS1	Up 5′-CTACATTGGCCACAAGACAA-3′ Low 5′-AGCAAGAGGTCCACTCAGAC-3′	NM_174196.1	53	89
GAPDH	Up 5′-CCAACGTGTCTGTTGTGGATCTGA-3′ Low 5′-GAGCTTGACAAAGTGGTCGTTGAG-3′	NM_001034034.1	57	86
ACTB	Up 5′-CGTGACATTAAGGAGAAGCTGTGC-3′ Low 5′-CTCAGGAGGAGCAATGATCTTGAT-3′	NM_173979.3	57	87
SDHA	Up 5′-GCAGAACCTGATGCTTTGTG-3′ Low 5′-CGTAGGAGAGCGTGTGCTT-3′	NM_174178.2	57	88

(Vandesompele *et al*. 2002). A *t*-test to compare gene expression levels between both treatments was then performed using the GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA) at $\alpha = 0.05$.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-10-0248.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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