Transcriptional effect of the LH surge in bovine granulosa cells during the peri-ovulation period

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Abstract

The LH surge induces a multitude of events that are essential for ovulation and corpus luteum formation. The transcriptional responses to the LH surge of preovulatory granulosa cells (GCs) are complex and still poorly understood. In this study, a genome-wide bovine oligo array was used to determine how the gene expression profile of GCs is modulated by the LH surge. GCs from three different stages were used to assess the short- and long-term effects of this hormone on follicle differentiation: 1) 2 h before induction of the LH surge, 2) 6 h and 3) 22 h after the LH surge. The results obtained were a list of differentially expressed transcripts for each GC group. To provide a comprehensive understanding of the processes at play, biological annotations were used to reveal the different functions of transcripts, confirming that the LH surge acts in a temporal manner. The pre-LH group is involved in typical tasks such as cell division, development, and proliferation, while the early response to the LH surge included features such as response to stimulus, vascularization, and lipid synthesis, which are indicative of cells preparing for ovulation. The late response of GCs revealed terms associated with protein localization and intracellular transport, corresponding to the future secretion task that will be required for the transformation of GCs into corpus luteum. Overall, results described in this study provide new insights into the different transcriptional steps that GCs go through during ovulation and before luteinization.

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Introduction

In the ovary, the chronological and spatial events that occur during folliculogenesis are very important for the formation of a developmentally competent oocyte. Folliculogenesis is a tightly regulated process, controlled by the endocrine system and intraovarian factors. In cattle, a mono-ovulatory species, the FSH and LH play a significant role in the growth, selection, and dominance of the preovulatory follicle. Each estrous cycle usually consists of two or three follicular waves. For each wave, the increase in FSH initiates the growth of a cohort of sensitive follicles. As a growing follicle is selected to become dominant, the other subordinate follicles cease to grow and undergo atresia. The dominant follicle is characterized by the acquisition of LH receptors on its granulosa cells (GCs) and the switch of its dependency from FSH to LH. During the luteal period, the dominant follicle regresses and a new wave occurs. When the corpus luteum reaches the end of its functional life, a surge in circulating LH occurs and the dominant follicle becomes the ovulatory follicle (for a review, see [Ginther \(2000\)](#)). Regarded as a pivotal event triggering follicular rupture and ovulation, the preovulatory LH surge is also

essential for inducing the meiotic resumption of oocytes, cumulus expansion, and the luteinization of GCs (for a review, see [Richards *et al.* \(1998\)](#)).

Efforts have been made to describe the signal transduction events induced by the LH receptor in the ovary using the limited knowledge available on signaling cascades and few gene targets (for a review, see [Richards & Hedin \(1988\)](#)). The introduction of transcriptomics creates a new level of complexity to analyze. The diversity of intraovarian factors involved and of molecules activated by LH reflects the major reorganization that takes place during this event. The classic linear model, which presents gene transcription as a result of the activation of the CREB–CBP complex through protein kinase A (PKA), is in fact much more complex and should be substituted by a mosaic model with many interactions ([Richards 2001](#)). Moreover, the distribution of the LH receptors fluctuates during folliculogenesis. Theca cells carry functional LH receptors throughout follicular growth while GCs acquire them only in their dominant phase ([Peng *et al.* 1991](#), [Robert *et al.* 2003](#)). Induction will therefore be influenced by the presence of the receptor on cells rather than by the presence of the hormone. It is thus not

surprising that genes induced by the ovulatory LH surge or during the dominance phase are predominantly found in GC (Espey & Richards 2002, Skinner *et al.* 2008).

Although previous studies have looked at the LH response in GC, the chronological context of the rapid and late effects of LH/human chorionic gonadotrophin (hCG) on gene expression have not been examined in cows. In mice, the rapid effect of LH on gene expression revealed an upregulation of many growth and transcription factors required for downstream events (Carletti & Christenson 2009). In rats, Espey & Richards (2002) measured the expression of 20 ovarian genes at different times after hCG injection. The temporal expression of these genes during the ovulatory period varies greatly. Some genes, such as *Egr1*, a transcription factor, begin to increase 30 min after hCG injection, while others, such as *PTGS2* (*COX2*), epiregulin, and *TNFAIP6* (*TSG6*), dramatically increase near the 4 h time point. *ADAMTS1* and *TIMP1*, both implicated in extracellular matrix remodeling, increase nearly 12 h into the ovulation period (Espey & Richards 2002). These results indicate that the LH surge triggers an activation sequence.

The working hypothesis is that the survey of transcript abundance prior to and following the LH surge will allow the determination of the typical transformation in GC gene expression profile in preparation for ovulation and luteinization. Thus, the discovery of key genes and biological functions of the short- and long-term response following the LH surge will lead to a better understanding of the different events induced by the LH surge. Since the LH signaling cascade operates in a spatio-temporal manner, this study looks at the influence of the LH surge on mural GC gene expression using a chronological approach. In this study, we used a global transcriptional method to identify genes present 2 h before the LH surge as well as early- (6 h post-LH) and late-response genes (22 h post-LH) following an LH ovulatory dose. Identification of the gene expression cascade that follows the LH surge should lead to a better understanding of the molecular determinants involved in ovulation and in the last stage of GC differentiation.

Results

Correlation analysis between each GC group in the microarray analysis

As a quality assessment step, the reproducibility of microarray hybridizations for each GC categories was evaluated using a between-group analysis (BGA). BGA also allows identifying a similarity between the different GC groups. All significantly expressed probes ($P < 0.05$) were used in the analysis. As shown in Fig. 1A, all GC biological samples ($n = 3$) and their technical replications were discriminated in distinct groups and classified according to their CG categories, and no particular outliers within the experimental groups were observed. Furthermore, to determine the similarity between the -2 h pre-LH, 6 h post-LH, and 22 h post-LH GC groups, an unsupervised hierarchical clustering was performed (Fig. 1B). Hierarchical clustering is useful to examine the relationship between samples and indicates which GC groups is more similar; the result is illustrated by a dendrogram in which similar samples were grouped together to form a cluster. Figure 1B demonstrates that the -2 and 6 h GC groups shared more similarity in their related expression patterns than other pairs and are joined together. The 22 h post-LH GC group is more distant and was clustered in a different branch of the dendrogram. Since all GC groups share a large number of similar transcripts and have a comparable number of stage-specific transcripts (Fig. 2), the 22 h post-LH group seems to express the same set of transcripts but at different levels.

Transcription profile of positive signals and differential gene expression in 2 h pre-LH versus 6 and 22 h post-LH surge

Microarray analyses generate vast amount of data, which can be visualized simply in a Venn diagram in which the intersection represents transcripts shared by the different groups, whereas the outside of the junction represents the exclusive mRNA for a group. Hybridization of the GC samples revealed more than 8000 positive signals

Figure 1 Visualization of the different GC groups after microarray analysis. (A) Between-group analysis (BGA) of the statistically significant transcripts ($P < 0.05$) performed with the biological and technical replicates from all GC stages (-2, 6, and 22 h). All sample categories show a clear separation for each experimental group setup. The circle represents the group mean and the different lines connected to the center represent the outliers. (B) Hierarchical clustering of samples shows that the gene expression profile of the 2 h pre-LH GC and the 6 h post-LH GC are more closely related and that the 22 h group is more distant.

Figure 2 Venn diagram summarizing microarray analysis results between -2, 6, and 22 h granulosa cell categories. (A) Venn diagram representing all positive signals on the microarray hybridization. (B–D) Venn diagram showing the number of early (B), late (C), and chronic (D) response transcripts using a filter of 1.5-fold change and a P value <0.05 . The number of upregulated (colored in green) and downregulated (colored in red) genes for each chronological group is illustrated in the right and left panels respectively.

(Fig. 2A), representing around 30% of the probes spotted on the array. Of these signals, 7084 (~87%) are shared between the three stages, indicating that most transcripts were present in every type of the GC group studied. Despite the large amount of mRNA shared between the three GC groups, some transcripts were expressed in a specific manner for each group. To identify all transcripts influenced by the preovulatory LH surge, direct comparisons between the GC groups were performed using one of them as the reference. Three different sets of chronological comparisons were done: the early-response transcripts (comparing the 6 h group with both the -2 and 22 h groups, Fig. 2B), the late-response transcripts (comparing the 22 h group with both -2 and 6 h groups, Fig. 2C), and the chronic changes (comparing the -2 h group with both 6 and 22 h groups, Fig. 3D). The upregulated and downregulated genes for each chronological group are presented in the right and left

panels respectively. Among the positive signals shown in these Venn diagrams, the majority are known to encode a protein or to correspond to a sequence containing an open reading frame region. These transcripts were then used for functional classification and to profile molecular and cellular processes. For each cell type, a list of the 15 most upregulated transcripts, on the basis of fold change, is given in Table 1.

LH-induced alteration in gene expression

Principal component analysis (PCA) is a statistical method, which is used to simplify complex data sets and summarize transcripts reaction to a treatment. To identify temporal elements influenced by the LH surge in GC, a PCA was performed in order to categorize the trend with which genes vary in each GC group. The PCA analysis generated six different clusters; for four clusters

Figure 3 Principal component analysis (PCA). All genes on the array with a minimum of 1.5-fold change (correlation threshold 0.7) were used to perform the PCA. Six different clusters were identified for the three GC groups (-2, 6, and 22 h). The effect of the LH surge is illustrated in (C–F) where a fluctuation in gene expression patterns is observed and represents 43% of transcripts variation. Clusters (A) and (B) are represented by linear upregulated and down-regulated gene patterns (57%), indicating the chronic changes of LH on transcripts. Gray lines represent transcript intensities, and the darker line is the average intensity of the cluster.

Table 1 Top 15 upregulated transcripts encoding for a protein for each granulosa cell group.

Gene symbol	Description	Fold change
2 h pre-LH		
<i>CXCL14</i>	Chemokine (C-X-C motif) ligand 14	30.91
<i>TRIB2</i>	Tribbles homolog 2	19.03
<i>MAL2</i>	Mal, T-cell differentiation protein 2	16.68
<i>GNL1</i>	Guanine nucleotide binding protein-like 1	13.00
<i>LGR4</i>	Similar to leucine-rich repeat-containing G-protein-coupled receptor 4	7.31
<i>NUF2</i>	NUF2, NDC80 kinetochore complex component, homolog	6.92
<i>LOC618339</i>	Similar to cytochrome c oxidase subunit IV	6.19
<i>ASPN</i>	Asporin	5.78
<i>MSL3</i>	Similar to male-specific lethal 3-like 1	5.31
<i>STAR</i>	STAR	4.99
<i>EEF1G</i>	Eukaryotic translation elongation factor 1 gamma	4.86
<i>RADIL</i>	Rap GTPase interactor	4.50
<i>ALDH1A2</i>	Similar to aldehyde dehydrogenase 1A2	4.32
<i>TNFRSF10A</i>	Tumor necrosis factor receptor superfamily, member 10a	4.32
<i>GKN1</i>	Gastrokine 1	4.26
6 h post-LH		
<i>PCBD1</i>	Pterin-4 alpha-carbinolamine dehydratase/dimerization cofactor of hepatocyte nuclear factor 1 alpha	27.67
<i>FGF2</i>	Fibroblast growth factor 2	22.01
<i>FATE1</i>	Fetal and adult testis expressed 1	17.88
<i>DEFB</i>	Defensin, beta	17.63
<i>SYT4</i>	Synaptotagmin IV	13.55
<i>TBC1D8B</i>	Similar to TBC1 domain family, member 8B (with GRAM domain)	12.38
<i>SLC39A8</i>	Similar to solute carrier family 39 (zinc transporter), member 8	11.79
<i>SLC27A6</i>	Solute carrier family 27 (fatty acid transporter), member 6	8.63
<i>NSDHL</i>	NAD(P)-dependent steroid dehydrogenase like	7.94
<i>HSPH1</i>	Heat shock 105 kDa/110 kDa protein 1	7.31
<i>PLK2</i>	Similar to serum-inducible kinase, transcript variant 1	6.63
<i>FDPS</i>	Farnesyl diphosphate synthase	6.59
<i>BAMBI</i>	BMP and activin membrane-bound inhibitor homolog	6.28
<i>LOC782568</i>	Similar to tumor necrosis factor receptor superfamily member 10B precursor (death receptor 5) (TRAIL receptor 2)	5.78
<i>LOC786015</i>	Similar to HIG1 domain family, member 1A	5.74
22 h post-LH		
<i>WASF1</i>	WAS protein family, member 1	53.45
<i>TIMM13</i>	Translocase of inner mitochondrial membrane 13 homolog	30.06
<i>CSRP3</i>	Cysteine and glycine-rich protein 3 (cardiac LIM protein)	27.28
<i>LOC515253</i>	Similar to phospholipase A2, group IVA (cytosolic, calcium-dependent)	18.90
<i>CLGN</i>	Calmeglin	18.90
<i>ARHGAP24</i>	Rho GTPase-activating protein 24	17.88
<i>MRO</i>	Maestro	16.45
<i>SLMAP</i>	Similar to sarcolemma-associated protein	15.14
<i>RHOBTB1</i>	Rho-related BTB domain containing 1	14.93
<i>TSPO</i>	Translocator protein (18 kDa)	11.39
<i>FAM129A</i>	Family with sequence similarity 129, member A	10.85
<i>PLIN2</i>	Adipose differentiation-related protein	10.70
<i>GUSB</i>	Glucuronidase, beta	9.38
<i>LOC534839</i>	Similar to high-risk human papilloma viruses E6 oncoprotein-targeted protein E6TP1 alpha; putative GAP protein alpha, transcript variant 8	9.25
<i>ITPRIP</i>	Inositol 1,4,5-triphosphate receptor interacting protein	8.94

(Fig. 3C–F), we observed that 43% of the transcripts responded to the LH surge. Moreover, 39% of these mRNAs (Fig. 3C and D) fluctuated precisely in the 6 h GC group and seemed to represent the short transcriptomic response that follows the LH surge. Around 4% (Fig. 3E and F) of the candidates shifted at the 22 h time point, thus representing the long-term response to the LH surge. However, the first two clusters (Fig. 3A and B), in which no disruption in the gene trend was observed at the 6 h post-LH time point, seem to represent chronic changes.

Real-time PCR validation

Confirmation of the microarray data analysis was performed by real-time PCR with nine different transcripts (Fig. 4). Candidate genes were selected according to their previously described PCA profiles (Fig. 3). Among the chosen candidate genes, some mRNAs, such as *TIMP1*, *TIMP2*, *TNFAIP6*, *INHBA*, and *FST*, are well known for their involvement in folliculogenesis, while others, such as *OGN*, *SRGN* (*SRG*), *TRIB2*, and *CSRP3*, have no known function in the ovary. For all

Figure 4 Quantitative PCR validation. Reactions were performed in triplicates with nonamplified cDNA. Significant differences are indicated by different superscript letters. Array profiles are represented with dash lines. CSRP3, cysteine and glycine-rich protein 3 (cardiac LIM protein); TIMP2, TIMP metalloproteinase inhibitor 2; TNFAIP6, tumor necrosis factor, alpha-induced protein 6; INHBA, inhibin, beta A; FST, follistatin; TIMP1, TIMP metalloproteinase inhibitor 1; TRIB2, tribbles homolog 2; OGN, osteoglycin; SRGN, serglycin.

selected candidates, real-time PCR results confirmed the same fluctuation profile in each GC stage than observed in microarray analysis.

Functional classification of transcripts with increased expression in preovulatory GCs

To facilitate the overall interpretation of the different gene expression profiles, the diversity of biological processes associated with the transcripts in 2 h pre-LH, 6 h post-LH, and 22 h post-LH GC groups, upregulated candidates were grouped according to their known biological function annotations using GO term annotation clustering in the DAVID software. In the pre-LH

reference group (-2 h, Fig. 5A), numerous terms were associated with cell cycle, including cell cycle process, cell cycle checkpoint, and cell growth, all of which are indicative of a typical cell undergoing division and proliferation. The transcriptional profile at 6 h post-LH surge (Fig. 5B) highlighted the biological terms such as response to chemical stimulus, extracellular matrix, lipid synthesis, and blood vessel development, indicating that cells are in preparation for a major transformation similar to the one observed during ovulation. Since the terms associated with cell cycle and cell growth were present in the pre-LH group but were not found at the 6 h time point, this suggests that the LH surge turns off these functions. At 22 h, the late response of GC to the

Figure 5 Classification of upregulated transcripts of the three GC stages into functional annotations. (A) 2 h before LH surge, (B) 6 h post-LH surge, and (C) 22 h post-LH surge. Bar chart shows the 15 most significant terms for each GC group. The GO analysis was performed using DAVID Bioinformatics Resources (<http://david.abcc.ncifcrf.gov/>).

LH surge was characterized by terms related to intracellular transport, protein localization, and protein folding. A global observation of these annotations points to different and specialized functions for each GC group.

Molecular and cellular profiles of GC groups before and after the LH surge

To illustrate the fluctuation in the biological function for every GC group, upregulated transcript data files were analyzed for molecular and cellular functions and compared using the Ingenuity Pathways Analysis (IPA) software in order to obtain a metabolic profile (Fig. 6). In Fig. 6, only the significant functions (log value > 1.30) that varied between the GC stages are represented. The results obtained from this analysis are in agreement with the previous GO term analysis (Fig. 5) in which the -2 h stage was enriched with genes involved in cellular development, growth, and proliferation. At the 6 h time point, lipid metabolism movement, assembly, and organization were highlighted and at 22 h, protein synthesis and trafficking function were predominant. Interestingly, specific functions corresponding to energy production, protein synthesis, and trafficking were only observed at 22 h, indicative of the distinct nature of these GC at 22 h and consistent with hierarchical clustering

(Fig. 1B) in which CGs at 22 h were clustered in a separate group from the -2 and 6 h groups.

Network and pathways analysis

To identify interacting transcripts, we used the IPA to analyze genes that were affected by the LH surge and were also found to be affected by the LH surge in the PCA (Fig. 4C and D). Several networks were proposed: Fig. 7A represents transcripts with a rapid response to the LH surge and Fig. 7B corresponds to a later response. It is important to mention that the networks generated by the IPA are based on published results; the interactions presented in these networks are thus compiled from several research areas and are not specific to the reproduction field. The first network assembled 25 molecules and shows AKT, progesterone receptor (PGR), and insulin-like growth factor-binding protein 3 in a central position. FSH, hCG, and the estrogen receptor were also found in this pathway, reflecting LH action. The second network also contains 25 molecules, but for the majority of these genes, such as T-cell acute lymphocytic leukemia 1 (*TAL1*), dinactin (*DCTN1*), Rho guanine nucleotide exchange factor 1 (*ARHGEF1*), 2'-5'-oligoadenylate synthetase 2 (*OAS2*), and tripartite motif-containing 2 (*TRIM2*), there is no or little information about their involvement in ovarian physiology.

Figure 6 Transcript classification into biological functions according to the GC stages. The upregulated and downregulated genes specific for each GC stage were classified by biological function categories using the Ingenuity Pathways Analysis (Ingenuity Systems, Mountain View, CA, USA). The horizontal dash lines represent the significant threshold for biological function. Right-tailed Fisher's exact test was used to calculate a *P* value, determining the probability that the association of each biological function into a data set is due to chance alone and represented as $-\log(P \text{ value})$; $-\log$ values exceeding 1.30 were significant ($P < 0.05$).

Figure 7 Interacting networks among molecules affected by the LH surge in GC. Genes identified in the principal component analysis as being influenced by the LH surge were uploaded into the Ingenuity Pathway Analysis. (A) Network of genes found to be rapidly upregulated after the LH surge (Fig. 4D). (B) Network of late-activated molecules in response to the LH surge (Fig. 4C). The difference in the red color intensity of the molecules involved in the pathway shows the degree of upregulation. Uncolored nodes represent eligible molecules provided by the Ingenuity knowledge base and combined with the user's molecules to maximize connectivity.

Discussion

Events occurring during the peri-ovulation period induce a profound transformation in follicular cells, which results in ovulation and luteinization. The preovulatory LH surge is the endocrine signal that causes this transformation by switching the growing and dividing

cells into organized cells that will release a competent oocyte and sustain early pregnancy. In this study, we sought to examine the short- and long-term messages provided by LH in mural GCs. To achieve this goal, we chose a genome-wide microarray approach with GC collected at three different time points: 2 h before the LH

surge and 6 and 22 h after the administration of LH. Given the amount of data generated by this type of analysis, we focused our gene lists by performing biological function annotations and by connecting molecules into networks.

To obtain the different GC groups at specific time points, ovarian stimulation treatments were administered to cows. The ovarian stimulation protocol using FSH with controlled release of the LH surge has been examined by one other group since 1999 (Knijn *et al.* 2011). This method was proven to be efficient for the collection of GCs and oocytes/embryos at specific time points, since the accuracy in determining the time of the LH surge was effective in 92% of cases ($n=238$; Knijn *et al.* 2011). In mono-ovulatory species such as humans and cows, ovarian stimulation with gonadotropins is widely used to stimulate the growing cohort of follicles to reach the ovulatory phase and produce an increased number of fertilizable oocytes as opposed to only one. The multiple FSH injections followed by a coasting period and an LH injection have been reported to generate developmentally competent oocytes and good to high embryonic developmental rates (Blondin *et al.* 2002, Chaubal *et al.* 2007, Knijn *et al.* 2011). Although some differences in gene expression compared with natural cycles are expected, it has been demonstrated that stimulation with FSH, compared with those performed with eCG, has a profile of 17β -estradiol similar to those observed in a natural cycle (Soumano *et al.* 1996, Knijn *et al.* 2011). However, little was previously known about the gene expression cascade that follows the LH surge and the molecular determinants involved in ovulation and the last stage of GC differentiation.

It is important to mention that the LH surge induces a cascade of events observed through the action of intermediate molecules. The immediate response to the LH surge is known to be mediated by the activation of the LH receptor, which leads to the production of cAMP. This second messenger can then activate many intracellular signaling cascades (Richards 2001, Espey & Richards 2002). These intermediate signals can induce the expression of other molecules and represent a longer response following the LH surge (Richards 2001, Espey & Richards 2002). This concept is well illustrated in the follicles with the epidermal growth factors (EGFs) where LH activates cAMP in the GC, which in turn activates the expression of EGF-like growth factors. The EGF-like molecules such as epiregulin, amphiregulin, and beta-cellulin will act as an intermediate signal and induce cumulus expansion and oocyte nuclear maturation (for a review, see Conti *et al.* (2006)).

Annotations allowed the association of gene profiles obtained from our samples with biological functions. Our results demonstrated that following the LH surge, the status of GC changed dramatically into a specific profile for each of the stages studied. Although the number of upregulated genes was similar for the three

groups of granulosa, the genes upregulated in the 22 h post-LH group did not provide as many annotations compared to the -2 and 6 h groups. The GO term vocabulary is a type of hierarchy: a gene is associated with many terms due to the association between the gene and the parent term (Rhee *et al.* 2008). Therefore, a general term will encompass more genes than a specific one. However, the specificity comes at a cost: as specificity increases, the coverage of the gene list decreases. Furthermore, $\sim 37\%$ of the human genes have no documentation on their functions (Wren 2009). The upregulated genes for the 6 h post-LH group are well known and a lot of function clusters were found. However, for the 22 h post-LH group, such analyses were limited because little information or annotations were available.

The rapid and long-term responses following LH surge have been assessed by biological function and network analyses. In the biological function, most of the categories were significant (>1.3 threshold) across all the three GC categories, but a few are significant in one or two stages and reflected the cell response to the LH surge. These functions can be placed in a logical continuum where prior to the LH surge, the cells are characterized by protein degradation activities, followed (after the LH surge) by increased amino acid metabolism that seems to translate at the preovulatory stage into protein synthesis and trafficking. These results raise questions about whether the increase in protein degradation (at -2 h pre-LH) will serve for amino acid production (at 6 and 22 h post-LH) and whether these amino acids will be recycled to synthesize proteins (at 22 h) to modify the cell structure or whether it might also serve as signaling function for ovulation. Other roles have also been attributed to the accumulation of amino acids during folliculogenesis. In mice, it has been reported that they can feed the oocyte by transferring amino acid through the gap junctions (Colonna & Mangia 1983, Haghghat & Van Winkle 1990, Eppig *et al.* 2005). In cattle, complementary observations that may reflect this link between follicular-cell-secreted amino acids and the gamete have shown that the concentrations of some amino acids in follicular fluid change, depending on follicle dominance status and estrous cyclicity (Orsi *et al.* 2005). Others have reported that some amino acids are involved in the physiology of the oocyte and are required for oocyte maturation, fertilization, and preimplantation development and can be used, in many species, as a marker of embryo developmental competence or fertility outcome (D'Aniello *et al.* 2007, Hong & Lee 2007, Sinclair *et al.* 2008, Pinero-Sagredo *et al.* 2010). Taken together, these results suggest that protein degradation occurring before the LH surge could be utilized for amino acid bioavailability. In the short-term LH surge response, these amino acids can be transferred to the oocyte to modify its environment before ovulation. For the long-term response, they may be required for

protein synthesis for the conversion of GC into the corpus luteum. Obviously, more work is required in this area to better understand the complexity of this system.

The network representing the rapid response to the LH surge shows AKT (also known as protein kinase B) and PGR in a central position, which is in agreement with evidence accumulated on ovarian physiology. FSH and LH exert their effect on proliferation and steroidogenesis via a G-protein-coupled receptor that causes an increase in cAMP and an activation of PKA. It has also been demonstrated in rodent follicle that the activation of LH and FSH is induced by other signaling pathways, including the AKT pathway (Carvalho *et al.* 2003). Moreover, AKT is predominant in cattle and sheep dominant follicle compared to the subordinates and acts on progesterone secretion (Evans & Martin 2000, Ryan *et al.* 2007, 2008). The correlation of the molecules involved in the network with different studies on folliculogenesis increases the confidence in our microarray results.

DICER1 has been found in our analysis as being preferentially expressed in 6 h post-LH GC, and is also found in the short-term response to LH network. *DICER* plays a role in posttranscriptional regulation; this endonuclease is implicated in the cleavage of double-stranded RNA for the biogenesis of microRNAs (miRNA) and small interfering RNAs (siRNA). Recently, two miRNAs (miR-132 (Mirn132) and miR-212 (Mirn212)) were found to be upregulated 4 h after the induction of the LH/hCG surge in mice GC. This time point corresponds to the germinal vesicle breakdown (similar to the 6 h time point in cows; Fiedler *et al.* 2008). However, contrary to our results, no change in *Dicer* mRNA levels has been detected in mouse before and after the LH surge (Fiedler *et al.* 2008). Moreover, *Dicer* knockout mice have shown a downregulation of mmu-mir503 in their ovaries and a divergence in gene expression for some follicle-related genes in comparison to wild-type (Lei *et al.* 2010). Evidence from different *Dicer* knockout mice indicates a direct link between LH and *DICER*. The alteration in *DICER* expression results in a defect in ovarian function, a luteal deficiency, and a trapped oocyte within luteinizing follicles (Nagaraja *et al.* 2008, Otsuka *et al.* 2008). Since our results show a rapid activation of *DICER* following the LH surge, *DICER* may be useful in the follicular cell development by regulating gene expression to allow ovulation.

In the late response to LH surge, a large number of transcripts are associated with transport, localization, and metabolic processes. In this network, actin is in relation with tropomyosin and three of its variants (TPM1, TPM2, and TPM3), constituting a section of the network. Very few papers have studied the role of these molecules in the follicle. In *in vitro* GC cultures, a study has shown that the organization of microfilaments was dependent on the different isoforms of tropomyosin and allowed cell differentiation (Baum *et al.* 1990). In other

contexts, including embryonic development and wound repair, tropomyosin variants can act on actin filaments to influence cell migration and speed (Bach *et al.* 2010). Since cell speed and migration are important for cell organization (Bach *et al.* 2010), the upregulation of the tropomyosin variants in the late response to LH suggests a possible role of these molecules in cytoskeleton rearrangement for the transformation of GCs into luteal cells.

Unlike the transient responses that are usually observed during the signaling process, the cellular response following the LH surge results in an undeviating change for follicular cells. The transformation of GCs into luteal cells leads to a permanent change and cells will never return to their original state. Epigenetic modifications could play a role in this permanent change but still very little information is available on chromatin modification during folliculogenesis. Our results show differential expressions for some transcripts implicated in chromatin modification such as the HAT1 and CPB/P300 acetyltransferases, which are upregulated in the 22 h post-LH GC group (data not shown). Seneda *et al.* (2008) have demonstrated, in pigs, that before eCG/hCG, the signal for H3-K4 methylation is strong in mural GC, that it drastically decreases near the ovulation period, and that the signal reappears in large luteal cells. However, the role of epigenetic mechanisms during the peri-ovulation period has to be further investigated.

Taken together, the spatio-temporal information on transcriptomic and posttranscriptomic events, such as miRNA, siRNA, long ncRNA, and epigenetic modifications, brings the level of complexity of the effect of the preovulatory LH surge to a higher level and highlights the need for additional studies.

Materials and Methods

Tissue collection

GCs were provided by Dr S J Dieleman (Utrecht University, The Netherlands). The ovarian stimulation treatment and ovariectomy protocol were described in detail by Knijn *et al.* (2002, 2011). Briefly, normally cyclic Holstein Friesian cows ($n=30$) were presynchronized using an ear implant for 9 days (3 mg norgestomet, Crestar; Intervet International BV, Boxmeer, The Netherlands) and a single administration of 3 mg norgestomet and 5 mg estradiol valerate (Intervet International BV). Two days before implant removal, prostaglandin was administered (15 mg Prosolvin, Intervet International BV) to induce a new estrous cycle. Nine days after estrus, the animals received another Crestar implant for 5 days. From day 10 of the estrous cycle, cows were treated with oFSH (Ovagen ICP, Auckland, New Zealand) twice a day in decreasing doses over 4 days. Prostaglandin was administered with the fifth dose of FSH. The ear implant was removed 49–50 h later and a GNRH dose (Intervet International BV) was given to induce the LH surge. In the 2 h pre-LH group, cows were ovariectomized 2 h after removal of the ear implant. In the 6 and 22 h post-LH groups,

ovariectomy was performed at 58 and 74 h after prostaglandin administration, which is 8 and 24 h after GNRH; the LH surge occurs on average 2.2 h after GNRH (Knijn *et al.* 2011) and ovulation 24 h after the LH surge (Dieleman *et al.* 1983). For the post-LH group, animals received GNRH at the time of removal of the implant and were ovariectomized 8 or 24 h after receiving GNRH, which itself occurs 6 and 22 h after the induced LH. After ovariectomy, ovaries were transported to the laboratory in 0.9% NaCl at 37 °C. After removal of the cumulus-oocyte complex by aspiration, the follicles were cut and turned inside out. GCs were scraped off in a small volume of PBS and centrifuged. This isolation procedure recovers mainly mural GCs but minor contamination by theca cells occurring during scraping was demonstrated by PCR amplification of the *CYP17a1* gene (data not shown). The supernatant was removed and the GC pellet was frozen at -80 °C until RNA extraction. Cows were assigned randomly to the three experimental groups (2 h pre-LH surge, 6 h post-LH surge, and 22 h post-LH surge) and four pools of GC were prepared. For each group, pools 1, 2, and 3 were used for microarray hybridization and pools 1, 2, and 4 for real-time PCR validation.

Total RNA extraction

Total RNA extraction of each pool of GC was performed using the Absolutely RNA Miniprep kit (Stratagene, La Jolla, CA, USA) and RNA was recovered into a 30 µl elution volume using the provided buffer. The RNA extraction procedure included an on-column DNase I treatment to remove genomic DNA. Total RNA integrity and concentration were evaluated using the 2100-Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) with the RNA NanoLab Chip (Agilent Technologies).

Sample labeling, hybridization, and microarray scanning

Five hundred nanograms of total RNA from three pools of 2 h pre-LH, 6 h post-LH, and 22 h post-LH CG were amplified using T7 RNA polymerase (RiboAmp RNA Amplification kit; Molecular Devices, Sunnyvale, CA, USA) according to the manufacturer's indications. A fixed amount of 2.5 µg aRNA was labeled indirectly using ULS aRNA Fluorescent Labeling (Kreatec Biotechnology, Amsterdam, The Netherlands) according to the manufacturer's protocol. Labeling efficiency was measured using the NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). Three biological replicates were used for each GC stage with a technical dye swap replicate. A total of nine hybridizations were performed in a loop design.

The bovine oligo array used contains a total of 25 200 oligonucleotides including unknown sequences and negative and positive controls, and 16 846 oligo sequences correspond to protein-encoding transcripts taken from the Bovine Oligo Microarray Consortium database (BOMC, <http://www.bovineoligo.org>). The sequence probes on the array are 70-mer oligos and there is 1 oligo per probe sequence. The bovine oligo microarray was printed in singlet using an Arrayjet Supermarathon instrument onto Corning GAPS II slides.

Hybridizations were performed in Slidehyb buffer #1 (Ambion, Austin, TX, USA) at 50 °C for 18 h in the SlideBooster hybridization station (Advalytix, San Francisco, CA, USA). Slides were then washed twice with 2× SSC-0.5% SDS at 50 °C for 15 min and twice with 0.5× SSC-0.5% SDS at 50 °C for 15 min. The slides were dipped three times in 1× SSC and three times in H₂O. Finally, the slides were dried by centrifugation at room temperature at 1200 g for 5 min.

The slides were scanned using the VersArray ChipReader System (Bio-Rad) and visualized with the ChipReader software

Table 2 Primer sequences used for real-time PCR validation.

Name	Forward sequence	Reverse sequence	Annealing temperature (°C)	Fluorescence acquisition temperature (°C)	Product size (bp)	Accession number
<i>CSRP3</i>	5'-acgcagaagaatccagtcg-3'	5' ctccgattctccaaactcg-3'	57	89	299	NM_001024689
<i>FST</i>	5'-gacttcaaggttggcagagg-3'	5'-catcttctctctgtcttcg-3'	57	88	214	NM_175801
<i>HPRT1</i> ^a	5'-ggctcgagatgtgatgaagg-3'	5'-gcaaagtctgcattgtctcc-3'	57		293	NM_001034035
<i>HSPA5</i>	5'-ggaaggaggagaagaacatcc-3'	5'-tctgcacagctctgtgtcc-3'	57	82	218	BT030726
<i>INHBA</i>	5'-gaagggaagaagaggatgg-3'	5'-agtcattccagccaatgtcc-3'	57	89	208	NM_174363
<i>OGN</i>	5'-caatgccttggaaatctgtgc-3'	5'-gatgtttccaggatgacg-3'	57	81	182	BT030567
<i>C4orf49</i> (OSAP)	5'-cagaggtcagcactgagacg-3'	5'-agattagccttgggctgagg-3'	57	86	218	BC148137
<i>PTGS2</i>	5'-ccaggaagtcttggctctgg-3'	5'-agccactcaagtgtctacg-3'	57	84	200	NM_174445
<i>RPL13A</i> ^a	5'-tgaggttggctggaagtacc-3'	5'-tgaggacctctgtgaattgc-3'	57	83	161	NM_001076998
<i>SRGN</i>	5'-cagtgcaatctgacagtc-3'	5'-tctgctccagaacctgatcc-3'	57	82	197	NM_001025326
<i>TIMP1</i>	5'-gacatccgggtcatctacacc-3'	5'-accagcagcataggtcttgg-3'	57	86	201	NM_174471
<i>TIMP2</i>	5'-ctctggcagcagcatctacg-3'	5'-gcacgatgaagtcacagagg-3'	57	84	227	NM_174472
<i>TNFAIP6</i>	5'-caagggcagagttgatacc-3'	5'-tgtgccagtagcagattgg-3'	57	81	230	BC151789
<i>TRIB2</i>	5'-gtggcatgtatgtcagacc-3'	5'-acaggacaaagcaccagagg-3'	57	84	300	NM_178317
<i>YWHAZ</i> ^a	5'-ccagtcacagcaagcatacc-3'	5'-cttcagcttctgtccttgg-3'	57	82	287	NM_174814

CSRP3, cysteine and glycine-rich protein 3 (cardiac LIM protein); FST, follistatin; HPRT1, hypoxanthine phosphoribosyltransferase 1; HSPA5, heat shock 70 kDa protein 5; INHBA, inhibin, beta A; OGN, osteoglycin; C4orf49 (OSAP), ovary-specific acidic protein; PTGS2, prostaglandin-endoperoxide synthase 2; SRGN, serglycin; RPL13A, ribosomal protein L13a; TIMP1, TIMP metalloproteinase inhibitor 1; TIMP2, TIMP metalloproteinase inhibitor 2; TNFAIP6, tumor necrosis factor alpha-induced protein 6; TRIB2, TRB-2 protein; YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase.

^aIndicates the housekeeping primers used for the real-time PCR normalization.

(Media Cybernetics, San Diego, CA, USA). After acquisition, scanned images were analyzed using ArrayPro Analyzer software (Media Cybernetics).

Data normalization and statistical analysis

Signal intensity data files were normalized and analyzed using the WebArray DB software (<http://www.webarraydb.org/webarray>), which is implemented with functions from Bioconductor (Xia *et al.* 2005, 2009). In the preprocessing step, the background of the intensity files was removed using Minimum Background Subtraction. Data were transformed in log2 and normalized for dye bias using a within-array loess. A between-array quantile normalization was then performed in order to obtain a similar distribution across the entire array.

Determination of the positive signals on the array was calculated on normalized data using the procedure described by Vallee *et al.* (2005) and Gilbert *et al.* (2007). Uninformative data were removed from the analysis by establishing a significant threshold of cutoff based on a degree of confidence associated with the variability of the negative controls. This cutoff threshold was calculated as follows: $T = M + 2 \times \text{s.d.}$, where T is the calculated threshold for cutoff, M is the average of negative controls present on the slides. Moreover, all the data equal or lower to the cutoff threshold determined previously were not considered in the analysis. A transcript was regarded as positive for the analysis and included in the gene list if its signal was higher than the background noise determined and if it was present in all biological replicates and both of their technical replications.

To identify early, late, and chronically regulated genes, normalized data were assessed using e-Bayes moderated t -test (LIMMA) included in the Webarray software. First, a cutoff threshold on minimum log intensity was applied on the data; this threshold was determined using the average of log intensities of the negative controls present on the slide. Probe signals with an average log-intensity (log2) value over 9 were considered present. Finally, to be considered as differentially expressed, transcripts were filtered on the basis of M value (log ratio) and P value. All probes with an M signal $> \pm 0.66$ (1.58-fold) and P value < 0.05 were determined to be differentially expressed.

Data processing, including BGA and hierarchical clustering dendrogram, was also performed with the Webarray software. Data clustering with the PCA was performed using the NIA microarray analysis tool (<http://lgsun.grc.nia.nih.gov/ANOVA>). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al.* 2002) and are accessible through GEO Series accession number GSE23900 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE23900>). The list of differentially expressed transcripts is also available in Supplementary Table 1, see section on supplementary data given at the end of this article.

Data mining: functional annotation and pathway analysis

Bovine gene annotations were retrieved from human gene orthologs using the g:profiler software (<http://biit.cs.ut.ee/gprofiler>). Genes that were found to be differentially expressed

(ratio > 1.58 , $P < 0.05$) and specific for each GC stage were functionally annotated and classified by using database for annotation, visualization, and integrated discovery (DAVID, <http://david.abcc.ncifcrf.gov>; Dennis *et al.* 2003). The GO term annotation was done using the functional annotation chart and a minimum count threshold of five terms, and an ease value (corresponding to P value) of < 0.05 was applied in order to provide an adequate number of annotations. Differentially expressed genes were also used for pathway analysis and were imported into the IPA software (Ingenuity Systems, Mountain View, CA, USA). The IPA was used to generate networks of genes and to perform a functional analysis associated with biological functions and molecular processes.

Real-time PCR

Five hundred nanograms of total RNA was reverse transcribed using the Transcriptor reverse transcriptase (Roche) with a mix of oligo dt (Applied Biosystems, Streetsville, ON, Canada) and decamer (Applied Biosystems) according to the manufacturers' instructions. All reactions were performed in triplicate with nonamplified cDNA in a final volume of 20 μ l. The primers for each gene were designed using the Primer3 web interface (<http://frodo.wi.mit.edu/primer3>). Sequences, size of amplified product, GenBank accession numbers, and annealing temperatures are presented in Table 2. For each candidate gene tested, a standard curve, consisting of PCR products purified with the QIAquick PCR Purification kit (Qiagen) and quantified with a spectrophotometer (NanoDrop ND-1000, NanoDrop Technologies), was included in the run. The standard curve consisted of five standards of the purified PCR products diluted from 0.10 pg to 0.1 fg. Real-time PCR was performed on a LightCycler apparatus (Roche Diagnostics) using SYBR green incorporation for real-time monitoring of amplicon production. The reaction was performed in glass capillaries in a final volume of 20 μ l (Roche Diagnostics). Target transcripts were normalized with the tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ), hypoxanthine phosphoribosyltransferase 1 (HPRT1), and ribosomal protein L13A (RPL13A) transcripts (Table 2) using Geometric averaging normalization (GeNorm, <http://medgen.ugent.be/~jvdesomp/genorm/>). One-way ANOVA and Tukey's multiple comparison tests were performed using Prism 4.0 (GraphPad Software, La Jolla, CA, USA) to determine the statistically significant differences in mRNA levels between each GC stage.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-10-0381>.

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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References

- Bach CT, Schevzov G, Bryce NS, Gunning PW & O'Neill GM 2010 Tropomyosin isoform modulation of focal adhesion structure and cell migration. *Cell Adhesion & Migration* **4** 226–234. (doi:10.4161/cam.4.2.10888)
- Baum G, Suh BS, Amsterdam A & Ben-Ze'ev A 1990 Regulation of tropomyosin expression in transformed granulosa cell lines with steroidogenic ability. *Developmental Biology* **142** 115–128. (doi:10.1016/0012-1606(90)90155-C)
- Blondin P, Bousquet D, Twagiramungu H, Barnes F & Sirard MA 2002 Manipulation of follicular development to produce developmentally competent bovine oocytes. *Biology of Reproduction* **66** 38–43. (doi:10.1095/biolreprod66.1.38)
- Carletti MZ & Christenson LK 2009 Rapid effects of LH on gene expression in the mural granulosa cells of mouse periovulatory follicles. *Reproduction* **137** 843–855. (doi:10.1530/REP-08-0457)
- Carvalho CR, Carnevali JB, Lima MH, Zimmerman SF, Caperuto LC, Amanso A, Gasparetti AL, Meneghetti V, Zimmerman LF, Velloso LA *et al.* 2003 Novel signal transduction pathway for luteinizing hormone and its interaction with insulin: activation of Janus kinase/signal transducer and activator of transcription and phosphoinositol 3-kinase/Akt pathways. *Endocrinology* **144** 638–647. (doi:10.1210/en.2002-220706)
- Chaubal SA, Ferre LB, Molina JA, Faber DC, Bols PE, Rezamand P, Tian X & Yang X 2007 Hormonal treatments for increasing the oocyte and embryo production in an OPU-IVP system. *Theriogenology* **67** 719–728. (doi:10.1016/j.theriogenology.2006.07.022)
- Colonna R & Mangia F 1983 Mechanisms of amino acid uptake in cumulus-enclosed mouse oocytes. *Biology of Reproduction* **28** 797–803. (doi:10.1095/biolreprod28.4.797)
- Conti M, Hsieh M, Park JY & Su YQ 2006 Role of the epidermal growth factor network in ovarian follicles. *Molecular Endocrinology* **20** 715–723. (doi:10.1210/me.2005-0185)
- D'Aniello G, Grieco N, Di Filippo MA, Cappiello F, Topo E, D'Aniello E & Ronsini S 2007 Reproductive implication of D-aspartic acid in human pre-ovulatory follicular fluid. *Human Reproduction* **22** 3178–3183. (doi:10.1093/humrep/dem328)
- Dennis G Jr, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC & Lempicki RA 2003 DAVID: database for annotation, visualization, and integrated discovery. *Genome Biology* **4** P3. (doi:10.1186/gb-2003-4-5-p3)
- Dieleman SJ, Kruip TA, Fontjine P, de Jong WH & van der Weyden GC 1983 Changes in oestradiol, progesterone and testosterone concentrations in follicular fluid and in the micromorphology of preovulatory bovine follicles relative to the peak of luteinizing hormone. *Journal of Endocrinology* **97** 31–42. (doi:10.1677/joe.0.0970031)
- Edgar R, Domrachev M & Lash AE 2002 Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Research* **30** 207–210. (doi:10.1093/nar/30.1.207)
- Eppig JJ, Pendola FL, Wigglesworth K & Pendola JK 2005 Mouse oocytes regulate metabolic cooperativity between granulosa cells and oocytes: amino acid transport. *Biology of Reproduction* **73** 351–357. (doi:10.1095/biolreprod.105.041798)
- Espey LL & Richards JS 2002 Temporal and spatial patterns of ovarian gene transcription following an ovulatory dose of gonadotropin in the rat. *Biology of Reproduction* **67** 1662–1670. (doi:10.1095/biolreprod.102.005173)
- Evans AC & Martin F 2000 Kinase pathways in dominant and subordinate ovarian follicles during the first wave of follicular development in sheep. *Animal Reproduction Science* **64** 221–231. (doi:10.1016/S0378-4320(00)00210-4)
- Fiedler SD, Carletti MZ, Hong X & Christenson LK 2008 Hormonal regulation of microRNA expression in periovulatory mouse mural granulosa cells. *Biology of Reproduction* **79** 1030–1037. (doi:10.1095/biolreprod.108.069690)
- Gilbert I, Bissonnette N, Boissonneault G, Vallee M & Robert C 2007 A molecular analysis of the population of mRNA in bovine spermatozoa. *Reproduction* **133** 1073–1086. (doi:10.1530/REP-06-0292)
- Ginther OJ 2000 Selection of the dominant follicle in cattle and horses. *Animal Reproduction Science* **60–61** 61–79. (doi:10.1016/S0378-4320(00)00083-X)
- Haghighat N & Van Winkle LJ 1990 Developmental change in follicular cell-enhanced amino acid uptake into mouse oocytes that depends on intact gap junctions and transport system Gly. *Journal of Experimental Zoology* **253** 71–82. (doi:10.1002/jez.1402530110)
- Hong J & Lee E 2007 Intrafollicular amino acid concentration and the effect of amino acids in a defined maturation medium on porcine oocyte maturation, fertilization, and preimplantation development. *Theriogenology* **68** 728–735. (doi:10.1016/j.theriogenology.2007.06.002)
- Knijin HM, Wrenzycki C, Hendriksen PJ, Vos PL, Herrmann D, van der Weijden GC, Niemann H & Dieleman SJ 2002 Effects of oocyte maturation regimen on the relative abundance of gene transcripts in bovine blastocysts derived *in vitro* or *in vivo*. *Reproduction* **124** 365–375. (doi:10.1530/rep.0.1240365)
- Knijin HM, Fokker W, van der Weijden GC, Dieleman SJ & Vos PL 2011 Effects of superovulation with oFSH and norgestomet/GnRH-controlled release of the LH surge on hormone concentrations, and yield of oocytes and embryos at specific developmental stages. *Reproduction in Domestic Animals* [in press]. (doi:10.1111/j.1439-0531.2008.01305.x)
- Lei L, Jin S, Gonzalez G, Behringer RR & Woodruff TK 2010 The regulatory role of Dicer in folliculogenesis in mice. *Molecular and Cellular Endocrinology* **315** 63–73. (doi:10.1016/j.mce.2009.09.021)
- Nagaraja AK, Andreu-Vieyra C, Franco HL, Ma L, Chen R, Han DY, Zhu H, Agno JE, Gunaratne PH, DeMayo FJ *et al.* 2008 Deletion of Dicer in somatic cells of the female reproductive tract causes sterility. *Molecular Endocrinology* **22** 2336–2352. (doi:10.1210/me.2008-0142)
- Orsi NM, Gopichandran N, Leese HJ, Picton HM & Harris SE 2005 Fluctuations in bovine ovarian follicular fluid composition throughout the oestrous cycle. *Reproduction* **129** 219–228. (doi:10.1530/rep.1.00460)
- Otsuka M, Zheng M, Hayashi M, Lee JD, Yoshino O, Lin S & Han J 2008 Impaired microRNA processing causes corpus luteum insufficiency and infertility in mice. *Journal of Clinical Investigation* **118** 1944–1954. (doi:10.1172/JCI33680)
- Peng XR, Hsueh AJ, LaPlante PS, Bjersing L & Ny T 1991 Localization of luteinizing hormone receptor messenger ribonucleic acid expression in ovarian cell types during follicle development and ovulation. *Endocrinology* **129** 3200–3207. (doi:10.1210/endo-129-6-3200)
- Pinero-Sagredo E, Nunes S, de Los Santos MJ, Celda B & Esteve V 2010 NMR metabolic profile of human follicular fluid. *NMR in Biomedicine* **23** 485–495. (doi:10.1002/nbm.1488)
- Rhee SY, Wood V, Dolinski K & Draghi S 2008 Use and misuse of the gene ontology annotations. *Nature Reviews. Genetics* **9** 509–515. (doi:10.1038/nrg2363)
- Richards JS 2001 New signaling pathways for hormones and cyclic adenosine 3',5'-monophosphate action in endocrine cells. *Molecular Endocrinology* **15** 209–218. (doi:10.1210/me.15.2.209)
- Richards JS & Hedin L 1988 Molecular aspects of hormone action in ovarian follicular development, ovulation, and luteinization. *Annual Review of Physiology* **50** 441–463. (doi:10.1146/annurev.ph.50.030188.002301)
- Richards JS, Russell DL, Robker RL, Dajee M & Alliston TN 1998 Molecular mechanisms of ovulation and luteinization. *Molecular and Cellular Endocrinology* **145** 47–54. (doi:10.1016/S0303-7207(98)00168-3)
- Robert C, Gagne D, Lussier JG, Bousquet D, Barnes FL & Sirard MA 2003 Presence of LH receptor mRNA in granulosa cells as a potential marker of oocyte developmental competence and characterization of the bovine splicing isoforms. *Reproduction* **125** 437–446. (doi:10.1530/rep.0.1250437)

- Ryan KE, Casey SM, Canty MJ, Crowe MA, Martin F & Evans AC 2007 Akt and Erk signal transduction pathways are early markers of differentiation in dominant and subordinate ovarian follicles in cattle. *Reproduction* **133** 617–626. (doi:10.1530/REP-06-0130)
- Ryan KE, Glistler C, Lonergan P, Martin F, Knight PG & Evans AC 2008 Functional significance of the signal transduction pathways Akt and Erk in ovarian follicles: *in vitro* and *in vivo* studies in cattle and sheep. *Journal of Ovarian Research* **1** 2. (doi:10.1186/1757-2215-1-2)
- Seneda MM, Godmann M, Murphy BD, Kimmins S & Bordignon V 2008 Developmental regulation of histone H3 methylation at lysine 4 in the porcine ovary. *Reproduction* **135** 829–838. (doi:10.1530/REP-07-0448)
- Sinclair KD, Lunn LA, Kwong WY, Wonnacott K, Linforth RS & Craigon J 2008 Amino acid and fatty acid composition of follicular fluid as predictors of *in vitro* embryo development. *Reproductive Biomedicine Online* **16** 859–868. (doi:10.1016/S1472-6483(10)60153-8)
- Skinner MK, Schmidt M, Savenkova MI, Sadler-Riggelman I & Nilsson EE 2008 Regulation of granulosa and theca cell transcriptomes during ovarian antral follicle development. *Molecular Reproduction and Development* **75** 1457–1472. (doi:10.1002/mrd.20883)
- Soumano K, Silversides DW, Doizé F & Price CA 1996 Follicular β -hydroxysteroid dehydrogenase and cytochromes P450 17α -hydroxylase and aromatase messenger ribonucleic acids in cattle undergoing superovulation. *Biology of Reproduction* **55** 1419–1426. (doi:10.1095/biolreprod55.6.1419)
- Vallee M, Gravel C, Palin MF, Reghenas H, Stothard P, Wishart DS & Sirard MA 2005 Identification of novel and known oocyte-specific genes using complementary DNA subtraction and microarray analysis in three different species. *Biology of Reproduction* **73** 63–71. (doi:10.1095/biolreprod.104.037069)
- Wren JD 2009 A global meta-analysis of microarray expression data to predict unknown gene functions and estimate the literature-data divide. *Bioinformatics* **25** 1694–1701. (doi:10.1093/bioinformatics/btp290)
- Xia X, McClelland M & Wang Y 2005 WebArray: an online platform for microarray data analysis. *BMC Bioinformatics* **6** 306. (doi:10.1186/1471-2105-6-306)
- Xia XQ, McClelland M, Porwollik S, Song W, Cong X & Wang Y 2009 WebArrayDB: cross-platform microarray data analysis and public data repository. *Bioinformatics* **25** 2425–2429. (doi:10.1093/bioinformatics/btp430)

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