REPRODUCTION
RESEARCH

Changes in granulosa cells' gene expression associated with increased oocyte competence in bovine

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Abstract

One of the challenges in mammalian reproduction is to understand the basic physiology of oocyte quality. It is believed that the follicle status is linked to developmental competence of the enclosed oocyte. To explore the link between follicles and competence in cows, previous research at our laboratory has developed an ovarian stimulation protocol that increases and then decreases oocyte quality according to the timing of oocyte recovery post-FSH withdrawal (coasting). Using this protocol, we have obtained the granulosa cells associated with oocytes of different qualities at selected times of coasting. Transcriptome analysis was done with Embryogene microarray slides and validation was performed by real-time PCR. Results show that the major changes in gene expression occurred from 20 to 44 h of coasting, when oocyte quality increases. Secondly, among upregulated genes (20–44 h), 25% were extracellular molecules, highlighting potential granulosa signaling cascades. Principal component analysis identified two patterns: one resembling the competence profile and another associated with follicle growth and atresia. Additionally, three major functional changes were identified: i) the end of follicle growth (*BMPR1B*, *IGF2*, and *RELN*), involving interactions with the extracellular matrix (*TFPI2*); angiogenesis (*NRP1*), including early hypoxia, and potentially oxidative stress (*GFPT2*, *TF*, and *VNN1*) and ii) apoptosis (*KCNJ8*) followed by iii) inflammation (*ANKRD1*). This unique window of analysis indicates a progressive hypoxia during coasting mixed with an increase in apoptosis and inflammation. Potential signaling pathways leading to competence have been identified and will require downstream testing. This preliminary analysis supports the potential role of the follicular differentiation in oocyte quality both during competence increase and decrease phases.
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Introduction

The understanding of the variability of oocyte quality is particularly important in large mono-ovulating species such as cows, horses, and humans. There is a general consensus that oocyte quality is best during a natural cycle, although several good-quality oocytes can sometimes be obtained during stimulated treatments. The bovine species coupled with an efficient *in vitro* maturation–IVF system offer a good research tool to address the effects of follicular growth and dynamics on oocyte quality. Indeed, it is ethically possible to hormonally manipulate the different parameters of follicular maturation and then assess the related quality of the oocytes obtained using the same females repeatedly and the same bull.

In cattle, the follicle status has a significant effect on oocyte quality, and through several protocols, it has been demonstrated that hormonal pretreatment of ovaries with gonadotropins can improve oocyte quality in a time-regulated manner (Blondin *et al*. 1996, 1997, 2002). In parallel, we and others have observed that granulosa/follicular gene expression may be associated with developmental competence of the enclosed oocyte in bovines (Robert *et al*. 2001), rats (Jiang *et al*. 2010), and humans (Assidi *et al*. 2008, 2010, Hamel *et al*. 2008, 2010). In our previous experiment, it was observed that an improved developmental rate was obtained with a hormonal treatment consisting of 3 days of stimulation using FSH plus a coasting period of 44 h (Blondin *et al*. 2002).

More recently, it was shown that the best period for ovum pick up (OPU) ranges from 44 to 68 h after the last FSH injection and is associated with an average blastocyst rate of 70% in cows, with some 33% of animals reaching 100% (Nivet *et al*. 2011; Supplementary Table 1). In the above experiment, early (20 h) and late (92 h) recovery time illustrates that the competence window is maximal between two states of lower competence. Based on this phenotypic observation, the current experiment was designed to analyze the granulosa cell transcriptome in relation to the three periods of competence: early, optimal, and late recovery.

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Results

Microarray analysis and principal component analysis

Principal component analysis (PCA) was performed based on intensity data (log2) at each treatment (20, 44, 68, and 92 h). In this PCA, genes are considered as variables and coasting periods as samples. Considering the log2 (signal intensity) for each gene symbol, three new variables, called principal components, were identified (linearly, Supplementary Figure 1A, and 3D view, Supplementary Figure 1B, see section on supplementary data given at the end of this article). These new variables were not correlated and permitted the definition of several general patterns significant for global changes in gene expression. One clear observation is that two patterns resembled the blastocyst or development pattern while the third one was not related to competence, thereby allowing interesting groupings of related genes.

Microarray-positive probes and fold change analysis

Using data with a significant ANOVA P value <0.05, a fold change of 1.5, and a false discovery rate of 0.1, there were 820 probes showing differential expression from 20 to 44 h (2.6% of the potential positive probes) (Fig. 1). Among them, 508 probes were over-expressed and 312 were under-expressed. This represented the biggest change in 24 h during the experiment, as there were 509 and 498 probes for 44–68 and 68–92 h respectively. This indicates that granulosa cells are significantly affected by the coasting regimen and that most changes occur within the first 2 days of FSH withdrawal. Between 44 and 68 h of coasting, as well as between 68 and 92 h, the proportion of probes associated with transcripts differentially expressed (fold change >1.5 and false discovery rate <0.1) decreased to 1.6%.

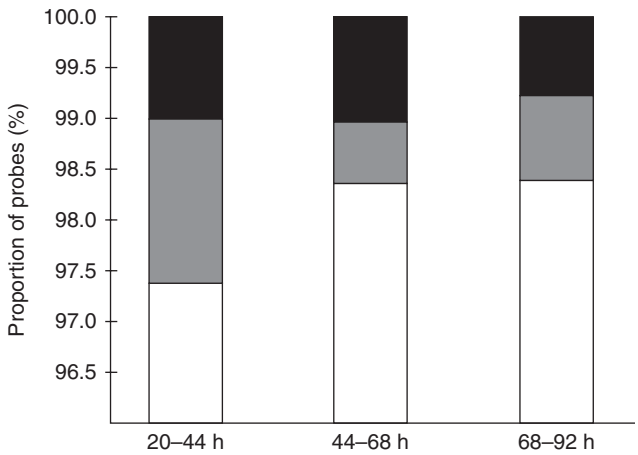

Figure 1 Proportion of probes differentially expressed over time. White bars: probes associated with transcripts not differentially expressed; gray bars: probes associated with positive fold changes; black bars: probes associated with negative fold changes.

Real-time PCR

Confirmation of the microarray data analysis was performed by real-time PCR with 11 different transcripts selected according to their significant changes during the coasting period, their profiles, and known function in or out of the ovary. Genes were also selected for being potential biomarkers of the competence period. The chosen candidate genes were transferrin (*TF*), insulin-like growth factor 2 (*IGF2*), reelin (*RELN*), potassium inwardly rectifying channel, subfamily J, member 8 (*KCNJ8*), tissue factor pathway inhibitor 2 (*TFPI2*), bone morphogenetic protein receptor, type IB (*BMP1RB*), neuropilin 1 (*NRP1*), ankyrin repeat domain 1 (*ANKRD1*), annexin A1 (*ANXA1*), glutamine-fructose-6-phosphate transaminase 2 (*GFPT2*), and vanin 1 (*VNN1*). Real-time PCR results confirmed the fluctuation profiles observed in microarray data analysis, although some changes were not significant for the four different time points (Fig. 2A and B). Functions associated with these potential markers were identified and are summarized in Fig. 3.

DAVID functional annotation and ingenuity pathway analysis

Data upload into the ingenuity pathway analysis (IPA) program permitted the identification of extracellular molecules upregulated at 44 h compared with 20 h, i.e. during the period of increasing competence. Some of them have known receptors that are reported to be expressed in the ovary (Table 1).

IPA also allows the identification of predicted activated transcription factors. We have performed this analysis with the differentially expressed genes for the following contrasts: 20–44, 44–68, and 68–92 h (Supplementary Figure 2, see section on supplementary data given at the end of this article). Using information from the literature (Sirotkin 2011), we could assign ovarian function for some of those genes. The functions associated with each contrast (20–44, 44–68, and 68–92 h) are cell death, cellular movement, cellular growth and proliferation, cell-to-cell signaling, and interaction (Supplementary Figure 2).

For each contrast (20–44, 44–68, and 68–92 h), gene symbols associated with fold changes (FC) >1.5 or <-1.5 ($P<0.05$ and false discovery rate (FDR) <0.1) were uploaded to the Database for Annotation, Visualization and Integrated Discovery (DAVID) functional annotation tool. For the 20–44, 44–68 and 68–92 h contrasts, seven groups of functions were similar. These global groups are inflammation (relative DAVID scores (significant when >1.3) from 7.9 to 9.9), angiogenesis (5.6–7.7), Smad/transforming growth factor 1 (SMAD/TGFB1) signaling (1.7–4.7), cell death/apoptosis (2.4–3.9), coagulation (3.5–5.7), cell movement (2–3.3), and extracellular matrix (3.5–6.1). These

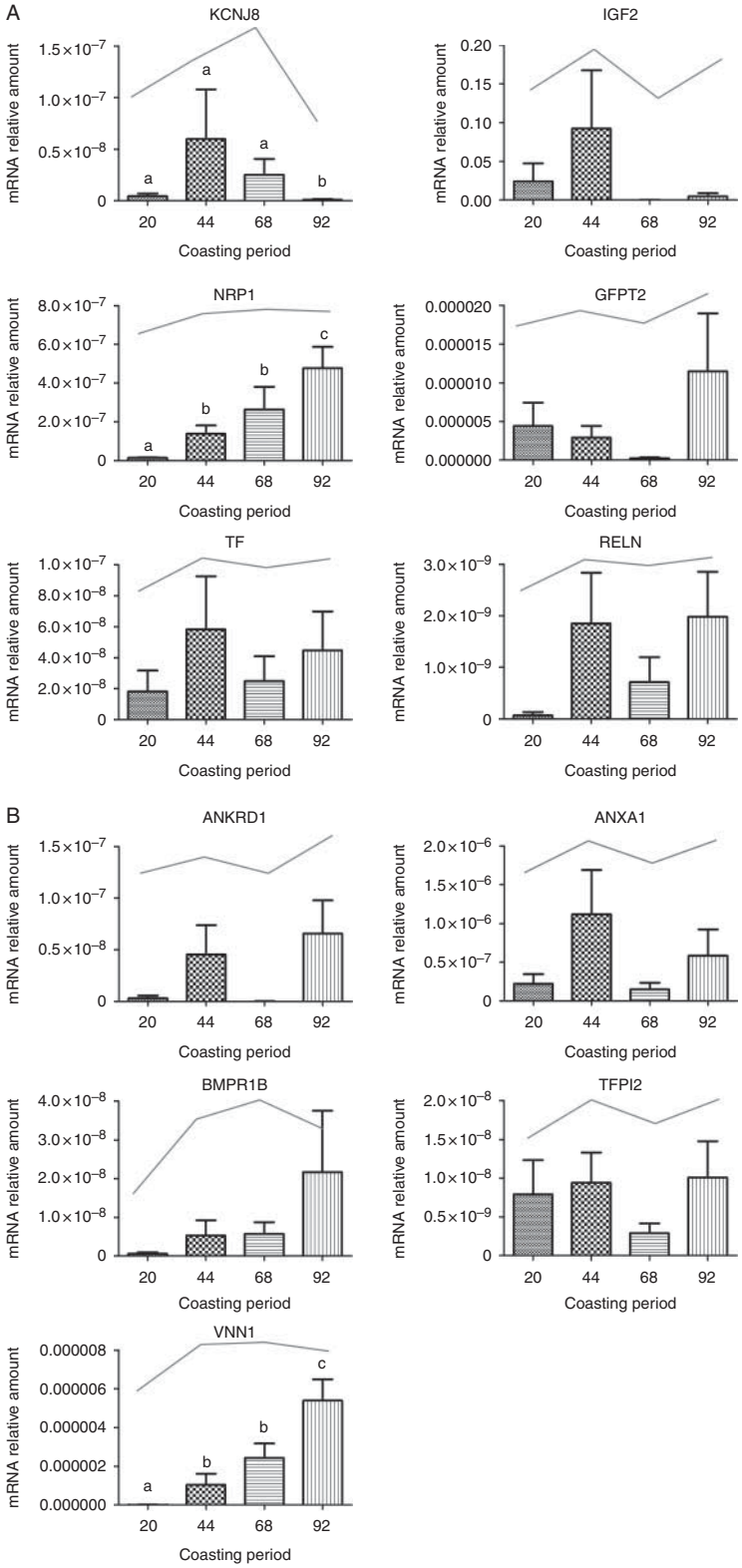

Figure 2 (A and B) Real-time PCR of selected potential markers (24 samples from six animals). Relative mRNA amounts are presented with S.E.M. Different superscripts represent significant differences ($P<0.05$) after ANOVA analysis and Newman–Keuls *post hoc* test. Lines: corresponding microarray hybridization profiles.

functions are globally overrepresented at 44 h of coasting compared with 20 h, then under-represented at 68 h compared with 44 h, and finally overrepresented at 92 h compared with 68 h.

Genes associated with cell death from the previous analysis were uploaded in the DAVID functional annotation chart tool. Apoptosis ($P=3.7\times10^{-2}$) and tumor protein 53 (TP53) signaling ($P=2.3\times10^{-2}$)

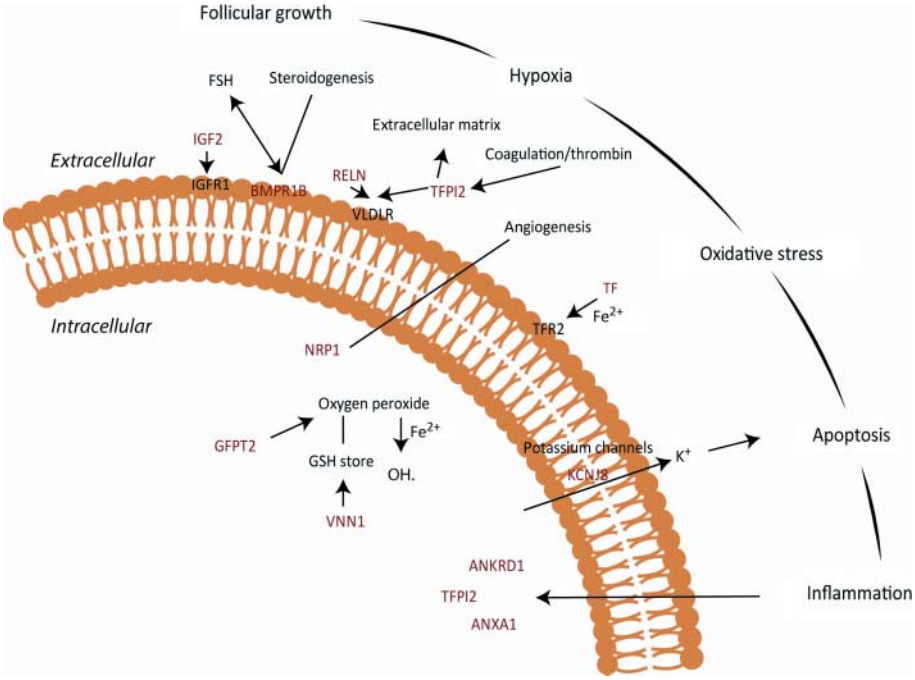

Figure 3 Functional roles of the selected markers. Red: the selected markers; black: functions and other molecules; orange: the plasma membrane delimiting the intra- and extracellular compartments. Interdependent global functions represented in bigger front size are linked by black lines, the black lines indicates 'is associated with', A, arrow; B, indicates; A acts on B.

pathways were significant. Genes of our data set represented in these pathways were respectively interleukin 1A (IL1A), caspase 8 (CASP8), nuclear factor of kappa light polypeptide gene enhancer in B-cells (NFKBIA), and phosphatase and tensin homolog (PTEN), PERP, and TP53 apoptosis effector (PERP). PTEN is implicated in the inhibition of IGF1/mechanistic target of rapamycin ((IGF1)/MTOR)) pathway (DAVID database), IL1A, PERP, and CASP8 positively regulate apoptosis. Fifty gene symbols were positively or negatively related to apoptosis.

IPA and DAVID bioinformatics analysis permitted us to identify that prolactin (PRL) and GH1 signaling pathways were associated with the optimal developmental competence period. Most of the molecules of these pathways are expressed or over-expressed at 44 h of coasting.

The number of genes expressed at 44 and 68 h (log (signal intensity)) and not expressed at 20 h nor at 92 h reached 205. DAVID cluster functional analysis was performed on this group and the only significant cluster was ion-binding function with a score of 1.42. There were

Table 1 Extracellular molecules over-expressed from 20 to 44 h of coasting.

Gene	Entrez gene name	Known receptor(s) in ovary
BMP2	Bone morphogenetic protein 2	BMPR1A, BMPR1B, and ACVR1 (Glister *et al.* 2010)
BMP6	Bone morphogenetic protein 6	BMPR2, ACVR2A, and ACVR2B (Glister *et al.* 2010)
CCL2	Chemokine (C-C motif) ligand 2	CCR2 (Luo *et al.* 2011)
COL1A2	Collagen, type I, α2	α2β1, α3β1, and α9β1 (Monniaux *et al.* 2006)
COL3A1	Collagen, type III, α1	α2β1, α3β1, and α9β1 (Monniaux *et al.* 2006)
CTGF	Connective tissue growth factor	FGFR2 (Gasperin *et al.* 2012)
CXCL2	Chemokine (C-X-C motif) ligand 2	CCR2 (Luo *et al.* 2011)
DCN	Decorin	HGFR (Parrott & Skinner 1998)
DCN	Decorin	EGFR (Wang & Ge 2004)
EFEMP1	EGF containing fibulin-like extracellular matrix protein 1	EGFR (Wang & Ge 2004)
FN1	Fibronectin 1	α3β1, α5β1, and αVβ3 (Monniaux *et al.* 2006)
IGF2	Insulin-like growth factor 2 (somatomedin A)	IGF2R (Echternkamp *et al.* 2012)
JAG1	Jagged 1	NOTCH1 (Trombly *et al.* 2009)
NTS	Neurotensin	NTSR2 (Gendron *et al.* 2004)
RELN	Reelin	LRP8 (Fayad *et al.* 2007)
RELN	Reelin	VLDLR (Cherian-Shaw *et al.* 2009)
SPP1	Secreted phosphoprotein 1	CD44 (Assidi *et al.* 2008)
SPP1	Secreted phosphoprotein 1	αVβ3, αVβ5, α5β1, and α9β1 (Monniaux *et al.* 2006)
TF	Transferrin	TFRC (Gohin *et al.* 2010)
TF	Transferrin	TFR2 (Ikuta *et al.* 2010)
TGFB3	Transforming growth factor β3	TGFBR2 (Nilsson *et al.* 2003)

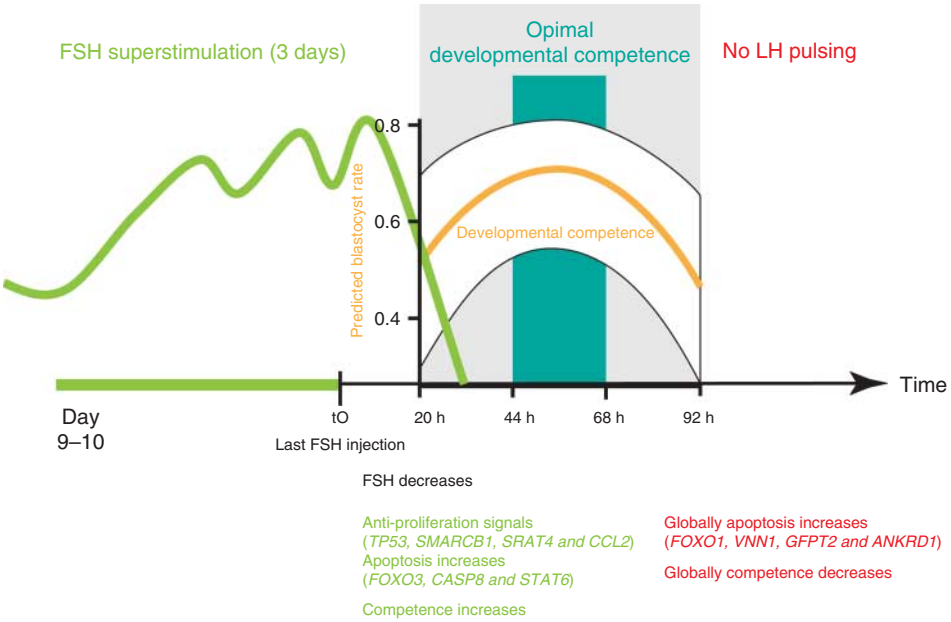

Figure 4 Following FSH (green line) superstimulation, FSH withdrawal is associated with oocyte developmental competence (orange line) increase. The granulosa cell expression during this period of increasing competence is associated with expression of genes leading to apoptosis increases (*FOXO3*, *STAT6*, and *CASP8*) and cellular proliferation decreases (*TP53*, *SMARCB1*, *STAT4*, and *CCL2*). In this context, there is no LH pulse increasing during coasting. The developmental competence (orange line) consequently decreases. Granulosa cell expression during the period of decreasing competence is associated with expression of genes leading to apoptosis and eventually to atresia (*FOXO1*, *VNN1*, *GFPT2*, and *ANKRD1*).

570 genes expressed at 44 h (log (signal intensity)) and not expressed at 20 h nor at 92 h. DAVID cluster functional analysis yielded three significant clusters: the complement function (score 1.81), response to ions (score 1.68), and cell projections (score 1.58). There were 287 genes expressed at 68 h (log (signal intensity)) and expressed neither at 20 h nor at 92 h. DAVID cluster functional analysis was performed and there was only one significant cluster, complement function (score 1.44).

Finally, DAVID bioinformatics protein binding site analysis was performed and, using the University of California Santa Cruz, Transcription Factor Binding sites (UCSC_TFBS) database, potential transcription factors linked to 20–44 h contrast (FC >1.5) were identified. Interestingly, analysis of the gene list associated with the potential transcription factor identified revealed that the first two canonical pathways were GH1 (signal transducer 1 (STAT1), STAT3, STAT5, serum response factor (SRF)) and PRL (STAT1, STAT3, and STAT5), interferon regulatory factor 1 (IRF1)). FBJ murine osteosarcoma viral oncogene homolog (FOS) and protein kinase C (PKC) are in the PRL pathway and were part of the 20–44 FC >1.5 gene list. Parts of the molecules of this pathway were positive at 20 h and almost all the PRL pathway molecules were positive at 44 h. The microarray expression data showed that *FOS* had a significant competence expression profile ($P=0.007$, FDR <0.1). Alpha-2-macroglobulin (A2M), IGF2, and FOS are in the GH pathway and *A2M*, *IGF2*, and *FOS* were part of the 20–44 FC >1.5 gene list ($P<0.05$, FDR 0.1). Part of the molecules in this pathway was positive at 20 h and almost all the GH pathway molecules were positive at 44 h. FOS is common at the end of these two pathways (GH and PRL pathways).

Discussion

The unique genomic comparison undertaken in this study represents a significant step in the molecular dissection of the follicle's role in oocyte competence in a large mammalian species. Modulating oocyte competence via FSH support followed by a withdrawal period before OPU is a new and powerful tool to elucidate the cause of variations in oocyte quality. The use of genomics, although still limited by the information available for each gene, is revealing potential granulosa cell's signaling that could explain how developmental competence is acquired.

The challenge of this study is the proximity of the physiological status of the follicles being compared. The application of all time periods to each individual animal was designed to minimize individual variations in the genomic response. This design is even more important in the context of known variability in the response to hormones both across and within individuals (Kawamata 1994, Cushman *et al.* 1999, Rico *et al.* 2009). The physiological observations of oocyte quality as reflected by the blastocyst rates across coasting duration indicates an initial increase (20–68 h) followed

by a general decrease after 68 h for most animals (Nivet *et al*. 2011). The six animals used in this experiment had neither the exact same pattern nor the same number of follicles nor the same size distribution. Despite the fact that these phenotypic variations raise the background noise, the genes that emerge above such background have an even more significant value and are more likely to be reproducible phenotypes in a larger population.

When looking at all the transcripts expressed, some general trends can be observed. Through a 3-dimensional representation, the PCA analysis highlighted higher similarities between 44 and 68 h of coasting compared with before or after (Supplementary Figure 1B). These results are interesting as the 44–68 h temporal window is linked to developmental competence in terms of blastocyst rate (Nivet *et al*. 2011). The 20 and 92 h time points appeared distinctly different. With the 2-dimensional PCA representation, three types of expression profiles emerged over the coasting period. Two patterns of gene expression were similar to the blastocyst or development pattern while the third one was not related to competence, allowing interesting groupings of related genes.

Gene analysis

In this study, the genes are not analyzed in a standard contrast where a control condition is compared with a treatment but in a dynamic process of terminal folliculogenesis (see the synthesis Fig. 4). In this context, the first thing to look at is the modulation of transcription factors that control cell fate. The first obvious observation is the initial rise and then decrease of several transcription factors associated with follicular differentiation: *CDKN2A* (cell division inhibition, Huang *et al*. (2009)), *STAT3* (induction of competence in the cumulus, van Tol *et al*. (2008)), *STAT4*, *STAT6*, *SMARCB1*, and *TP53* (anti-proliferation/pro-apoptosis (Lee *et al*. 2002, Sirotkin *et al*. 2009), *CREBBP*, and *FOXO3* (pro-apoptosis, Huang & Tindall (2007), Richards & Pangas (2010) and Matsuda *et al*. (2012)). Among the genes and/or functions associated with the developmental competence plateau (44–68 h), only one transcription factor (Supplementary Figure 2), smad family member 7 (*SMAD7*), is predicted to be activated specifically from 44 to 68 h and inhibited from 20 to 44 h and from 68 to 92 h. Only two transcription factors, *STAT1* and CCAAT/enhancer binding protein, alpha (*CEBPA*), are predicted to be inhibited specifically from 20 to 44 h and from 68 to 92 h. The transcription factors *TP53*, CREB binding protein (*CREBBP*), smad family member 4 (*SMAD4*), and forkhead box O3 (*FOXO3*) seem specific to the competence-acquisition period (20–44 h), and three of them (*TP53*, *CREBBP*, and *FOXO3*) are implicated in the mediation of the effect of hormones in the ovary (Sirotkin 2011). TP53 is upstream of the selected candidates *TFPI2*, *IGF2*, *NRP1*, and *ANXA1*, which will be addressed below.

The second series of genes that are interesting to analyze are the potential signaling molecules that could reach the oocyte–cumulus complex. Interestingly, an important subset (25%) of genes differentially expressed between 20 and 44 h of coasting encodes extracellular proteins (IPA data base) (Table 1). This is consistent with the follicular context, in which communication and dialogue between the different cellular compartments (granulosa, cumulus, and oocyte) are critical for optimal follicular differentiation (Gilchrist *et al*. 2004). Among these extracellular molecules, approximately half have known receptors. The secreted molecules that have known receptors could act in an autocrine manner on granulosa cells or in a paracrine manner on cumulus cells. Four of these molecules have been chosen for real-time PCR validation: *IGF2*, *RELN*, *TF*, and *TFPI2* (Fig. 4). Approximately one third of the extracellular molecules that we have found are known to interact with the extracellular matrix and remodeling enzymes or molecules associated with collagen, indicating the potential importance of this follicular component in terminal differentiation.

Therefore, the molecules associated with maximal competence (44–68 h) led to the identification of two major signaling pathways, i.e. PRL and GH. These two signaling pathways have in common the end point molecule FBJ murine osteosarcoma viral oncogene homolog (FOS) (IPA database). This molecule has an expression profile similar to the competence profile. Among genes specifically activated during the plateau phase, two are common to the 44 and 68 h time points: IL6 and macrophage stimulating 1 (MST1). Interestingly, IL6 is involved in the LH response and could be signaling to the cumulus (Mack *et al*. 2012). The ovarian role of MST1, a hepatocyte-growth-factor-like molecule expressed notably in epithelial cells, remains to be defined, but in liver, it stimulates the FOX family (Huang & Tindall 2007), which is upregulated during the competence activation process. Interestingly, PRL could act positively on IGF2 expression. IGF2 (Fig. 3) is associated with cell survival and *IGF2* has been linked to developmental competence and validated by QPCR. In our study, *IGF2* has a developmental competence expression profile and would probably act differently from *IGF1*, which is associated with FSH response in bovine folliculogenesis (Lucy 2011). In fishes, IGF2 stimulates follicles and oocyte maturation (Reinecke 2010) but the mouse KO shows no infertility (Chandrashekar *et al*. 2004). Another potential actor on oocyte competence is BMP2, which has been associated with better oocytes in humans (Sugiyama *et al*. 2010). Not only is *BMP2* upregulated but its receptor *BMPR1B* is also increased. BMPR1B is a BMP family member that regulates follicular development by affecting granulosa cell proliferation and steroidogenesis. BMPs modulate

FSH actions in the ovary and reciprocally (Miyoshi *et al.* 2006). *BMPR1B* is expressed from secondary to atretic follicles and is the receptor for BMP2, -4, -6, and -7. *BMPR1B* gene KO and mutations have an adverse effect on fertility (compromised cumulus expansion) in mice and in humans (Tanwar & McFarlane 2011). This gene, which responds to BMP15, is also a good candidate for a granulosa signaling back to the oocyte. The final part of the analysis will focus on the changes within granulosa cells during the coasting period. These changes are summarized in Fig. 2.

Appropriate follicle growth involves angiogenesis to oxygenate cell layers that reach a thickness of 110 μm or more. Even if the granulosa layer is avascular, it can stimulate angiogenesis in the surrounding stroma. In our experiment, some genes increased expression as a function of time as well as follicle diameter, such as *VNN1* and *NRP1* (Fig. 2). When VNN1 and NRP1 reach their maximal expression, at 92 h, the majority of follicles are larger than 10 mm in diameter. These genes are thus potential indicators of follicular growth and/or differentiation. They are necessary to reach the maximal competence status but are not linear competence markers, as their expressions continue to increase in the decreasing-competence period. VNN1 is known to act as a regulator of tissue response to oxidative stress by modulating the GSH store in mouse (Berruyer *et al.* 2004). *VNN1*$^{-/-}$ mice display downregulated inflammation (Berruyer *et al.* 2004). Our results are consistent with these previous studies.

Angiogenesis needs to be fine-tuned in the follicle; NRP1 is upstream of vascular endothelial growth factor (VEGF) signaling. NRP1 is a VEGFA co-receptor, and the sema domain, immunoglobulin domain (Ig), short basic domain, and secreted (semaphorin) 3A (SEMA3A) receptor (Cariboni *et al.* 2011). Interestingly, VEGFA is lower in Frizzled-4 (*FZD4*) KO mice associated with an infertile phenotype (failure to become pregnant) (Hsieh *et al.* 2005). NRP1 forms a complex with PlexinD1 (linked to semaphorin) regulating angiogenesis and vascular growth (Yamamizu *et al.* 2011). Granulosa cells could then influence the degree of expansion of follicular capillaries in the stroma and increase the follicle oxygen level, consistent with previous observations in mice (Antczak & Van Blerkom 2000). For angiogenesis and follicular growth, extracellular matrix remodeling (ECM) is necessary.

TFPI2 plays a role in the regulation of plasmin-mediated matrix remodeling (Neaud *et al.* 2004). It is interesting to note that full-length TFPI2 as well as RELN are very-low-density lipoprotein receptor (VLDLR) ligands, with an antiproliferative activity (Hembrough *et al.* 2001).

Globally, these two last aspects (angiogenic action and extracellular matrix (ECM) degradation) are similar in tumors. In fact, as the tumor cell grows, the needs for O_2 and nutrients increase, leading to hypoxia. In order to avoid hypoxia, tumor cells signal ECM degradation/remodeling and angiogenesis. Follicle growth could be compared with this: the follicle is growing, nutrients and O_2 have to reach all the follicle cells. But follicles are not tumor cells, as they have to stop and/or regulate their proliferation. Consistent with this, *TP53* transcription factor, which has an anti-oncogenic effect, is specific to the acquisition of competence period (from 20 to 44 h). TP53 is upstream of ANXA1.

ANXA1 expression is stimulated by 17β-estradiol, in the anterior pituitary *in vivo* (Davies *et al.* 2007*a*, 2007*b*). ANXA1 has an effect on cell growth, differentiation, apoptosis, membrane fusion, endocytosis, and exocytosis (John *et al.* 2004). Furthermore, ANXA1 is implicated in secretion and signaling and acts negatively on inflammation (Brancaleone *et al.* 2011).

In our study, gene expression associated with inflammation (Fig. 4) (*TFPI2*, *ANXA1*, and *ANKRD1* induced by IL1 and tumor necrosis factor (TNF) α induced by TLR4), angiogenesis (*TFPI2*), and oxidative stress (*GFPT2* and *TF*) had a triangular profile (increasing competence (20–44 h) and decreasing competence (68–92 h)). The first increase, from increasing competence (20–44 h), is associated with competence acquisition and possibly preparation for ovulation while the second increase in the triangular expression, from decreasing competence (68–92 h), could be associated with onset of atresia and less competent follicles.

The equilibrium between growth proliferation and apoptosis is regulated at various levels, such as angiogenesis, cell proliferation, and inflammation, but also at the oxidative stress level with GFPT2 or iron transport with TF. GFPT2 is known to reduce peroxide toxicity associated with oxidative stress (Zitzler *et al.* 2004). TF is implicated in iron transport that is positively related to oxidative stress (conversion of peroxide to OH.) and transferrin receptor 2 (*TFR2*) is upregulated at 68 h in granulosa cells in our experiment. ANKRD1 is induced by IL1 and TNFα, which are associated with inflammation and apoptosis signaling. In fact, TNFα increases intracellular superoxide and hydrogen peroxide (Xia *et al.* 2010), and hydrogen peroxide increases expression of ANKRD1 (Kyng *et al.* 2003).

Globally, at 92 h of coasting, the follicle appears to be overwhelmed by apoptosis-promoting signals and developmental competence decrease signals. GFPT2, VNN1, and ANKRD1 (Fig. 3) are linked to apoptosis and/or oxidative stress, which is associated with insufficient oxygen supply leading to atresia. Even if early atresia has been associated with developmental competence (Blondin & Sirard 1995, Li *et al.* 2009), it has a negative effect if it is a long-lasting process.

Among developmental-competence-associated genes whose expression decreased from 68 to 92 h, *KCNJ8* (Fig. 3) was confirmed by real-time PCR to be a potential competence marker. Globally, the profile of this gene over time is positively associated with the proportion of

medium size follicles, although our analysis was done on pools of follicles. Therefore, progression of gene regulation cannot be directly linked to follicle size. KCNJ8 regulates the ATP response through potassium channels and could signal either protection, like in neurons, or predict atresia by stimulating caspases and apoptotic nucleases (Perez *et al.* 2000). *KCNJ8* could represent an early marker of terminal differentiation associated with developmental competence.

To conclude, this study provides a unique picture of the expression of granulosa genes associated with different degrees of oocyte competence. First, it permitted identification of transcription factors (*SMAD7*, *STAT1*, and *CEBPA*) and canonical pathways (PRL and GH signaling) associated with the optimal developmental competence period. Furthermore, it highlighted the importance of extracellular molecules expressed in granulosa cells that potentially reach the oocyte–cumulus complex. Finally, granulosa cell expression was modeled as a progressive gradual hypoxia associated with an increase in apoptosis and inflammation. Our study reveals granulosa cell signaling as the potential answer to explain how the oocyte acquires its developmental competence.

Materials and Methods

All reagents and media supplements used in these experiments were of tissue-culture grade and were obtained from Sigma Chemicals Co. unless otherwise indicated.

Granulosa cell's collection

Samples were obtained according to a previously published study protocol (Nivet *et al.* 2011). Briefly, commercial milking cycling cows ($n=6$) were stimulated with 3 days of FSH (6×40 mg NIH Folltropin-V given at 12 h intervals) followed by a coasting period of 20, 44, 68, or 92 h. Each animal was exposed to the four conditions and served as its own control (for a total of 24 OPU). The animals were used four times for ovarian aspiration and each session was separated by one or two estrous cycles (not 24 h).

Using the same animal sequentially is considered as equivalent if not better than several replicates with different animals. At each OPU scheduled time, transvaginal aspirations of immature oocytes were performed, followed by IVF and *in vitro culture* to assess the developmental competence of the oocytes. Our evaluation criteria for developmental competence were the ability of the oocyte to develop after fertilization into a transferable blastocyst. This experiment was realized in industry settings, the evaluation of the blastocyst quality was performed according to their standard for embryo transfer (International Embryo Transfer Society classification). At the same time, granulosa cells were collected in warm HEPES-buffered Tyrode's media (TLH) containing Hepalean (10 IU/ml) and transferred to the laboratory. Granulosa cells were selected under binocular observation and placed in RNAse-free tubes and were then washed three times with sterile PBS. Cell pellets were snap-frozen in nitrogen and stored at −80 °C. Out of the six animals, the same three cows (for all the four times of aspiration) were used for microarray analysis and the analysis was done with a within-cow contrast to minimize genetic noise. The theca cell-specific enzyme cytochrome P450, family 17, polypeptide (*CYP17A1*) was used to assess the possible theca cell contamination of the sample. The expression intensity was never considered positive from 20 to 92 h. Thus, theca cell contamination was considered negligible.

RNA extraction and amplification

Total RNA from each replicate was extracted and purified using PicoPure RNA Isolation Kit (Molecular Devices, Sunnyvale, CA, USA). After DNase digestion (Qiagen), the purity, quality, and concentration of extracted RNA were analyzed by Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). All extracted samples showed good quality with an RNA integrity number >6.9. For microarray purposes, 5 ng purified RNA was amplified from each individual sample ($n=12$) by *in vitro* transcription by T7 RNA amplification using the RiboAmp HS Plus RNA Amplification Kit (Molecular Devices). After two amplification rounds of 6 h each, the amplified RNA (aRNA) output was quantified using the NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA).

Sample labeling and microarray hybridization

For each sample, 2 μg aRNA were labeled using the ULS Fluorescent Labelling Kit for Agilent arrays (with Cy3 and Cy5) (Kreatech Diagnostics, Amsterdam, The Netherlands). The labeled product was then purified with the Pico-Pure RNA Isolation Kit but without DNase treatment. Labeling efficiency was measured using the Nano-Drop ND-1000. Samples ($n=12$) were hybridized individually on EmbryoGENE's bovine 44K microarray (Robert *et al.* 2011). Each hybridization was performed in the following design: for each cow individually, each coasting time was compared with others (i.e. 20 versus 44 h; 20 versus 68 h; 20 versus 92 h; 44 versus 68 h; 44 versus 92 h, and 68 versus 92 h) for a total of six comparisons. Overall, 36 hybridizations, corresponding to three cows and six comparisons in dye swap were done. A total of 825 ng of each labeled sample (Cy3 and Cy5) were incubated in a solution containing 2× blocking agent and 5× fragmentation buffer in a volume of 55 μl at 60 °C for 15 min and were put on ice immediately after. A 55 μl volume of 2× GEx Hybridization Buffer HI-RPM were added for a total volume of 110 μl. The hybridization mix (100 μl) was added onto the array and hybridization was performed at 65 °C for 17 h using an Agilent Hybridization chamber in a rotating oven. Slides were then washed with Gene Expression Wash Buffer 1 containing 0.005% Triton X-102 for 3 min at room temperature and then transferred to Gene Expression Wash Buffer 2 containing 0.005% Triton X-102 for 3 min at 42 °C.

Microarray data analysis

The slides were scanned using the Tecan PowerScanner microarray scanner (Tecan Group Ltd, Männerdorf, Switzerland)

and features were extracted using ArrayPro 6.4 (Media Cybernetics, Bethesda, MD, USA). Intensity files were analyzed with mean intensities for foreground and median intensities for background with Limma R package. Anova F statistic was used to determine significant gene variation during the coasting period. Specifically, raw data were corrected by background subtraction and then normalized within each array (Loess). Significant differences between treatments were determined with a P value <0.05. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE40916.

Hierarchical cluster analysis (HCA) and PCA analysis were performed with the normalized expression data using Pirouette Lite software (Infometrix, Inc., Bothell, WA, USA).

cDNA preparation and real-time PCR

Validation of microarray results was performed by real-time PCR. A 1 ng sample of extracted RNA from three independent samples (three replicates for each condition) and the three cows used for microarray hybridization were reverse-transcribed using q-Script Flex cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD, USA) with oligo-dT(20) primers following manufacturer's recommendations.

The primers used for real-time RT-PCR were designed using the IDT PrimerQuest tool (available at Integrated DNA technologies website) from sequences obtained using the UMD3.1/bosTau5 assemble version of the bovine genome and results from microarray analysis. To confirm the specificity of each pairs of primers, electrophoresis on a standard 1.2% agarose gel was performed for each amplified fragment. Primer sequences and accession numbers are provided in Supplementary Tables 2 and 3, see section on supplementary data given at the end of this article. Real-time PCR was performed using LightCycler 480 SYBR Green I Master and the LightCycler 480 (Roche Diagnostics). The PCR conditions used for all genes were as follows: denaturing cycle for 10 s at 95 °C; 50 PCR cycles (denaturing, 95 °C for 10 s; annealing for 10 s; extension, 72 °C for 20 s), a melting curve (95 °C for 1 s, 65 °C for 1 s, and a step cycle starting at 72 °C up to 97 °C at 0.11 °C/s), and a final cooling step at 40 °C.

Statistical analysis of real-time PCR results

Data analysis was performed using the LightCycler 480 Software 1.5.0 SP4 (version 1.5.0.39) with the second-derivative maximum analysis method. Analysis of gene expression stability over granulosa cells was performed using the GeNorm VBA applet software (Vandesompele *et al.* 2002). The most stable reference genes were identified by the stepwise exclusion of the least stable gene and recalculating the M values. *ACTB*, *GAPDH*, and *CHUK* were the most stable genes with M values <1.5 as recommended by the software (M value $=1.48$). One-way ANOVA with the Newman–Keuls post-test was performed on normalized biological replicates, using GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA, USA).

Functional analysis of differential gene expression

DAVID software was used to group-overrepresented functions of differentially expressed genes or positive genes into clusters and to identify transcription factors associated with the uploaded list as described (Huang da *et al.* 2009).

Data were analyzed through the use of IPA (Ingenuity Systems, www.ingenuity.com). A data set containing gene identifiers and corresponding differential expression values was uploaded into in the application. Each identifier was mapped to its corresponding object in the Ingenuity Knowledge Base. The functional analysis identified the biological functions that were most significant to the data set. Right-tailed Fisher's exact test was used to calculate a P value determining the probability that each biological function assigned to that data set was due to chance alone. Canonical pathway analysis identified the pathways from the IPA library that were most significant to the data set. The significance of the association between the data set and the canonical pathway was measured in two ways: i) a ratio of the number of molecules from the data set that map to the pathway divided by the total number of molecules that map to the canonical pathway and ii) Fisher's exact test was used to calculate a P value determining the probability that the association between the genes in the data set and the canonical pathway is explained by chance alone.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-13-0032.

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

Antczak M & Van Blerkom J 2000 The vascular character of ovarian follicular granulosa cells: phenotypic and functional evidence for an endothelial-like cell population. *Human Reproduction* **15** 2306–2318. (doi:10.1093/humrep/15.11.2306)

Assidi M, Dufort I, Ali A, Hamel M, Algriany O, Dielemann S & Sirard MA 2008 Identification of potential markers of oocyte competence expressed in bovine cumulus cells matured with follicle-stimulating hormone and/or phorbol myristate acetate *in vitro*. *Biology of Reproduction* **79** 209–222. (doi:10.1095/biolreprod.108.067686)

Assidi M, Dieleman SJ & Sirard MA 2010 Cumulus cell gene expression following the LH surge in bovine preovulatory follicles: potential early markers of oocyte competence. *Reproduction* **140** 835–852. (doi:10.1530/REP-10-0248)

Berruyer C, Martin FM, Castellano R, Macone A, Malergue F, Garrido-Urbani S, Millet V, Imbert J, Dupre S, Pitari G *et al.* 2004 Vanin-1−/− mice exhibit a glutathione-mediated tissue resistance to oxidative stress. *Molecular and Cellular Biology* **24** 7214–7224. (doi:10.1128/MCB.24.16.7214-7224.2004)

Blondin P & Sirard MA 1995 Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocytes. *Molecular Reproduction and Development* **41** 54–62. (doi:10.1002/mrd.1080410109)

Blondin P, Coenen K, Guilbault LA & Sirard MA 1996 Superovulation can reduce the developmental competence of bovine embryos. *Theriogenology* **46** 1191–1203. (doi:10.1016/S0093-691X(96)00290-7)

Blondin P, Coenen K, Guilbault LA & Sirard MA 1997 *In vitro* production of bovine embryos: developmental competence is acquired before maturation. *Theriogenology* **47** 1061–1075. (doi:10.1016/S0093-691X(97)00063-0)

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)

Brancaleone V, Dalli J, Bena S, Flower RJ, Cirino G & Perretti M 2011 Evidence for an anti-inflammatory loop centered on polymorphonuclear leukocyte formyl peptide receptor 2/lipoxin A4 receptor and operative in the inflamed microvasculature. *Journal of Immunology* **186** 4905–4914. (doi:10.4049/jimmunol.1003145)

Cariboni A, Davidson K, Dozio E, Memi F, Schwarz Q, Stossi F, Parnavelas JG & Ruhrberg C 2011 VEGF signalling controls GnRH neuron survival via NRP1 independently of KDR and blood vessels. *Development* **138** 3723–3733. (doi:10.1242/dev.063362)

Chandrashekar V, Zaczek D & Bartke A 2004 The consequences of altered somatotropic system on reproduction. *Biology of Reproduction* **71** 17–27. (doi:10.1095/biolreprod.103.027060)

Cherian-Shaw M, Puttabyatappa M, Greason E, Rodriguez A, VandeVoort CA & Chaffin CL 2009 Expression of scavenger receptor-BI and low-density lipoprotein receptor and differential use of lipoproteins to support early steroidogenesis in luteinizing macaque granulosa cells. *Endocrinology* **150** 957–965. (doi:10.1210/en.2008-0619)

Cushman RA, DeSouza JC, Hedgpeth VS & Britt JH 1999 Superovulatory response of one ovary is related to the micro- and macroscopic population of follicles in the contralateral ovary of the cow. *Biology of Reproduction* **60** 349–354. (doi:10.1095/biolreprod60.2.349)

Davies E, Omer S, Buckingham JC, Morris JF & Christian HC 2007*a* Expression and externalization of annexin 1 in the adrenal gland: structure and function of the adrenal gland in annexin 1-null mutant mice. *Endocrinology* **148** 1030–1038. (doi:10.1210/en.2006-0732)

Davies E, Omer S, Morris JF & Christian HC 2007*b* The influence of 17β-estradiol on annexin 1 expression in the anterior pituitary of the female rat and in a folliculo-stellate cell line. *Journal of Endocrinology* **192** 429–442. (doi:10.1677/JOE-06-0132)

Echternkamp SE, Aad PY, Eborn DR & Spicer LJ 2012 Increased abundance of aromatase and follicle stimulating hormone receptor mRNA and decreased insulin-like growth factor-2 receptor mRNA in small ovarian follicles of cattle selected for twin births. *Journal of Animal Science* **90** 2193–2200. (doi:10.2527/jas.2011-4735)

Fayad T, Lefebvre R, Nimpf J, Silversides DW & Lussier JG 2007 Low-density lipoprotein receptor-related protein 8 (LRP8) is upregulated in granulosa cells of bovine dominant follicle: molecular characterization and spatio-temporal expression studies. *Biology of Reproduction* **76** 466–475. (doi:10.1095/biolreprod.106.057216)

Gasperin BG, Ferreira R, Rovani MT, Santos JT, Buratini J, Price CA & Goncalves PB 2012 FGF10 inhibits dominant follicle growth and estradiol secretion *in vivo* in cattle. *Reproduction* **143** 815–823. (doi:10.1530/REP-11-0483)

Gendron L, Perron A, Payet MD, Gallo-Payet N, Sarret P & Beaudet A 2004 Low-affinity neurotensin receptor (NTS2) signaling: internalization-dependent activation of extracellular signal-regulated kinases 1/2. *Molecular Pharmacology* **66** 1421–1430. (doi:10.1124/mol.104.002303)

Gilchrist RB, Ritter LJ & Armstrong DT 2004 Oocyte somatic cell interactions during follicle development in mammals. *Animal Reproduction Science* **82** 431–446. (doi:10.1016/j.anireprosci.2004.05.017)

Glister C, Satchell L & Knight PG 2010 Changes in expression of bone morphogenetic proteins (BMPs), their receptors and inhibin co-receptor βglycan during bovine antral follicle development: inhibin can antagonize the suppressive effect of BMPs on thecal androgen production. *Reproduction* **140** 699–712. (doi:10.1530/REP-10-0216)

Gohin M, Bobe J & Chesnel F 2010 Comparative transcriptomic analysis of follicle-enclosed oocyte maturational and developmental competence acquisition in two non-mammalian vertebrates. *BMC Genomics* **11** 18. (doi:10.1186/1471-2164-11-18)

Hamel M, Dufort I, Robert C, Gravel C, Leveille MC, Leader A & Sirard MA 2008 Identification of differentially expressed markers in human follicular cells associated with competent oocytes. *Human Reproduction* **23** 1118–1127. (doi:10.1093/humrep/den048)

Hamel M, Dufort I, Robert C, Leveille MC, Leader A & Sirard MA 2010 Genomic assessment of follicular marker genes as pregnancy predictors for human IVF. *Molecular Human Reproduction* **16** 87–96. (doi:10.1093/molehr/gap079)

Hembrough TA, Ruiz JF, Papathanassiu AE, Green SJ & Strickland DK 2001 Tissue factor pathway inhibitor inhibits endothelial cell proliferation via association with the very low density lipoprotein receptor. *Journal of Biological Chemistry* **276** 12241–12248. (doi:10.1074/jbc.M010395200)

Hsieh M, Boerboom D, Shimada M, Lo Y, Parlow AF, Luhmann UF, Berger W & Richards JS 2005 Mice null for Frizzled4 (Fzd4−/−) are infertile and exhibit impaired corpora lutea formation and function. *Biology of Reproduction* **73** 1135–1146. (doi:10.1095/biolreprod.105.042739)

Huang H & Tindall DJ 2007 Dynamic FoxO transcription factors. *Journal of Cell Science* **120** 2479–2487. (doi:10.1242/jcs.001222)

Huang Q, Cheung AP, Zhang Y, Huang HF, Auersperg N & Leung PC 2009 Effects of growth differentiation factor 9 on cell cycle regulators and ERK42/44 in human granulosa cell proliferation. *American Journal of Physiology. Endocrinology and Metabolism* **296** E1344–E1353. (doi:10.1152/ajpendo.90929.2008)

Huang da W, Sherman BT & Lempicki RA 2009 Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature Protocols* **4** 44–57. (doi:10.1038/nprot.2008.211)

Ikuta K, Yersin A, Ikai A, Aisen P & Kohgo Y 2010 Characterization of the interaction between diferric transferrin and transferrin receptor 2 by functional assays and atomic force microscopy. *Journal of Molecular Biology* **397** 375–384. (doi:10.1016/j.jmb.2010.01.026)

Jiang JY, Xiong H, Cao M, Xia X, Sirard MA & Tsang BK 2010 Mural granulosa cell gene expression associated with oocyte developmental competence. *Journal of Ovarian Research* **3** 6. (doi:10.1186/1757-2215-3-6)

John CD, Christian HC, Morris JF, Flower RJ, Solito E & Buckingham JC 2004 Annexin 1 and the regulation of endocrine function. *Trends in Endocrinology and Metabolism* **15** 103–109. (doi:10.1016/j.tem.2004.02.001)

Kawamata M 1994 Relationships between the number of small follicles prior to superovulatory treatment and superovulatory response in Holstein cows. *Journal of Veterinary Medical Science* **56** 965–967. (doi:10.1292/jvms.56.965)

Kyng KJ, May A, Brosh RM Jr, Cheng WH, Chen C, Becker KG & Bohr VA 2003 The transcriptional response after oxidative stress is defective in Cockayne syndrome group B cells. *Oncogene* **22** 1135–1149. (doi:10.1038/sj.onc.1206187)

Lee D, Kim JW, Seo T, Hwang SG, Choi EJ & Choe J 2002 SWI/SNF complex interacts with tumor suppressor p53 and is necessary for the activation of p53-mediated transcription. *Journal of Biological Chemistry* **277** 22330–22337. (doi:10.1074/jbc.M111987200)

Li HJ, Liu DJ, Cang M, Wang LM, Jin MZ, Ma YZ & Shorgan B 2009 Early apoptosis is associated with improved developmental potential in bovine oocytes. *Animal Reproduction Science* **114** 89–98. (doi:10.1016/j.anireprosci.2008.09.018)

Lucy MC 2011 Growth hormone regulation of follicular growth. *Reproduction, Fertility, and Development* **24** 19–28. (doi:10.1071/RD11903)

Luo W, Diaz FJ & Wiltbank MC 2011 Induction of mRNA for chemokines and chemokine receptors by prostaglandin $F_{2\alpha}$ is dependent upon stage of the porcine corpus luteum and intraluteal progesterone. *Endocrinology* **152** 2797–2805. (doi:10.1210/en.2010-1247)

Mack EM, Smith JE, Kurz SG & Wood JR 2012 cAMP-dependent regulation of ovulatory response genes is amplified by IGF1 due to synergistic effects on Akt phosphorylation and NF-kappaB transcription factors. *Reproduction* **144** 595–602. (doi:10.1530/REP-12-0225)

Matsuda F, Inoue N, Manabe N & Ohkura S 2012 Follicular growth and atresia in mammalian ovaries: regulation by survival and death of granulosa cells. *Journal of Reproduction and Development* **58** 44–50. (doi:10.1262/jrd.2011-012)

Miyoshi T, Otsuka F, Suzuki J, Takeda M, Inagaki K, Kano Y, Otani H, Mimura Y, Ogura T & Makino H 2006 Mutual regulation of follicle-stimulating hormone signaling and bone morphogenetic protein system in human granulosa cells. *Biology of Reproduction* **74** 1073–1082. (doi:10.1095/biolreprod.105.047969)

Monniaux D, Huet-Calderwood C, Le Bellego F, Fabre S, Monget P & Calderwood DA 2006 Integrins in the ovary. *Seminars in Reproductive Medicine* **24** 251–261. (doi:10.1055/s-2006-948554)

Neaud V, Duplantier JG, Mazzocco C, Kisiel W & Rosenbaum J 2004 Thrombin up-regulates tissue factor pathway inhibitor-2 synthesis through a cyclooxygenase-2-dependent, epidermal growth factor receptor-independent mechanism. *Journal of Biological Chemistry* **279** 5200–5206. (doi:10.1074/jbc.M306679200)

Nilsson EE, Doraiswamy V & Skinner MK 2003 Transforming growth factor-β isoform expression during bovine ovarian antral follicle development. *Molecular Reproduction and Development* **66** 237–246. (doi:10.1002/mrd.10350)

Nivet AL, Bunel A, Labrecque R, Belanger J, Vigneault C, Blondin P & Sirard MA 2011 FSH withdrawal improves developmental competence of oocytes in the bovine model. *Reproduction* **143** 165–171. (doi:10.1530/REP-11-0391)

Parrott JA & Skinner MK 1998 Developmental and hormonal regulation of hepatocyte growth factor expression and action in the bovine ovarian follicle. *Biology of Reproduction* **59** 553–560. (doi:10.1095/biolreprod59.3.553)

Perez GI, Maravei DV, Trbovich AM, Cidlowski JA, Tilly JL & Hughes FM Jr 2000 Identification of potassium-dependent and -independent components of the apoptotic machinery in mouse ovarian germ cells and granulosa cells. *Biology of Reproduction* **63** 1358–1369. (doi:10.1095/biolreprod63.5.1358)

Reinecke M 2010 Insulin-like growth factors and fish reproduction. *Biology of Reproduction* **82** 656–661. (doi:10.1095/biolreprod.109.080093)

Richards JS & Pangas SA 2010 The ovary: basic biology and clinical implications. *Journal of Clinical Investigation* **120** 963–972. (doi:10.1172/JCI41350)

Rico C, Fabre S, Medigue C, di Clemente N, Clement F, Bontoux M, Touze JL, Dupont M, Briant E, Remy B *et al.* 2009 Anti-Müllerian hormone is an endocrine marker of ovarian gonadotropin-responsive follicles and can help to predict superovulatory responses in the cow. *Biology of Reproduction* **80** 50–59. (doi:10.1095/biolreprod.108.072157)

Robert C, Gagne D, Bousquet D, Barnes FL & Sirard MA 2001 Differential display and suppressive subtractive hybridization used to identify granulosa cell messenger RNA associated with bovine oocyte developmental competence. *Biology of Reproduction* **64** 1812–1820. (doi:10.1095/biolreprod64.6.1812)

Robert C, Nieminen J, Dufort I, Gagné D, Grant JR, Cagnone G, Plourde D, Nivet AL, Fournier E, Paquet E, Blazejczyk M, Rigault P, Juge N & Sirard MA 2011 Combining resources to obtain a comprehensive survey of the bovine embryo transcriptome through deep sequencing and microarrays. *Molecular Reproduction and Development* **78** 651–664. (doi:10.1002/mrd.21364)

Sirotkin AV 2011 Transcription factors in control of ovarian functions. In *Regulators of Ovarian Functions*, ch 6, pp 101–123. AV Sirotkin. New York: Nova Science Publishers, Inc.

Sirotkin AV, Ovcharenko D, Benco A & Mlyncek M 2009 Protein kinases controlling PCNA and p53 expression in human ovarian cells. *Functional & Integrative Genomics* **9** 185–195. (doi:10.1007/s10142-008-0102-y)

Sugiyama R, Fuzitou A, Takahashi C, Akutagawa O, Ito H, Nakagawa K & Isaka K 2010 Bone morphogenetic protein 2 may be a good predictor of success in oocyte fertilization during assisted reproductive technology. *Human Cell* **23** 83–88. (doi:10.1111/j.1749-0774.2010.00088.x)

Tanwar PS & McFarlane JR 2011 Dynamic expression of bone morphogenetic protein 4 in reproductive organs of female mice. *Reproduction* **142** 573–579. (doi:10.1530/REP-10-0299)

van Tol HT, van Eerdenburg FJ, Colenbrander B & Roelen BA 2008 Enhancement of bovine oocyte maturation by leptin is accompanied by an upregulation in mRNA expression of leptin receptor isoforms in cumulus cells. *Molecular Reproduction and Development* **75** 578–587. (doi:10.1002/mrd.20801)

Trombly DJ, Woodruff TK & Mayo KE 2009 Suppression of Notch signaling in the neonatal mouse ovary decreases primordial follicle formation. *Endocrinology* **150** 1014–1024. (doi:10.1210/en.2008-0213)

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A & Speleman F 2002 Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* **3** RESEARCH0034. (doi:10.1186/gb-2002-3-7-research0034)

Wang Y & Ge W 2004 Cloning of epidermal growth factor (EGF) and EGF receptor from the zebrafish ovary: evidence for EGF as a potential paracrine factor from the oocyte to regulate activin/follistatin system in the follicle cells. *Biology of Reproduction* **71** 749–760. (doi:10.1095/biolreprod.104.028399)

Xia Z, Luo T, Liu HM, Wang F, Xia ZY, Irwin MG & Vanhoutte PM 2010 L-arginine enhances nitrative stress and exacerbates tumor necrosis factor-α toxicity to human endothelial cells in culture: prevention by propofol. *Journal of Cardiovascular Pharmacology* **55** 358–367. (doi:10.1097/FJC.0b013e3181d265a3)

Yamamizu K, Furuta S, Katayama S, Narita M, Kuzumaki N, Imai S, Nagase H, Suzuki T & Yamashita JK 2011 The kappa opioid system regulates endothelial cell differentiation and pathfinding in vascular development. *Blood* **118** 775–785. (doi:10.1182/blood-2010-09-306001)

Zitzler J, Link D, Schafer R, Liebetrau W, Kazinski M, Bonin-Debs A, Behl C, Buckel P & Brinkmann U 2004 High-throughput functional genomics identifies genes that ameliorate toxicity due to oxidative stress in neuronal HT-22 cells: GFPT2 protects cells against peroxide. *Molecular and Cellular Proteomics* **3** 834–840. (doi:10.1074/mcp.M400054-MCP200)

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