Impact of the LH surge on granulosa cell transcript levels as markers of oocyte developmental competence in cattle

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Abstract

In the case of *in vitro* embryonic production, it is known that not all oocytes detain the developmental capacity to form an embryo. This capacity appears to be acquired through completion of folliculogenesis, during which the oocyte and follicular cells influence their respective destinies. The differentiation status of granulosa cells (GCs) could therefore offer an indicator of oocyte quality. The aim of this study was to compare mRNA transcript abundance in GCs associated with oocytes that subsequently reach or not the blastocyst stage. GCs were collected from cattle following an ovarian stimulation protocol that did or did not include the administration of LH. GCs were classified according to the developmental stage achieved by the associated oocytes. Transcript abundance was measured by microarray. Follicles ($n=189$) obtained from cows before and after the LH surge were essentially similar and the rates of oocytes reaching the blastocyst stage were not significantly different (52 vs 41%), but blastocyst quality was significantly better in the post-LH-surge group. In GCs from the pre-LH-surge group and associated with developmentally competent oocytes, 18 overexpressed and 22 underexpressed transcripts were found, including novel uncharacterized transcripts, whereas no differentially expressed transcripts were associated with developmentally different oocytes in the post-LH-surge group. The novel transcriptomic response associated with LH appeared to mask the difference. Based on oocyte developmental competence, the period prior to the LH surge appears best suited for studying competence-associated mRNA transcripts in bovine follicle cells.

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Introduction

In the cattle industry, *in vitro* embryo production (IVP) and embryo transfer have become useful for treating infertility in problematic animals or for accelerating genetic gain in fertile animals of high commercial value. These techniques provide effective means of decreasing generation time and thus increasing the speed of genetic progress in large mono-ovulating species. One of the major limitations of IVP comes from the variable quality of oocytes recovered, with or without hormonal treatments. Development to the blastocyst stage, achieved using unprimed animals or slaughterhouse ovaries, has not improved much over the past two decades. Indeed, only 30–40% of oocytes are able to develop to the blastocyst stage *in vitro* (Sirard *et al.* 1988, Wiemer *et al.* 1991, Ward *et al.* 2002), while more than 85% do so *in vivo* (Sirard *et al.* 1985).

The concept of oocyte developmental competence can be summarized as the ability of the oocyte to mature, develop to the blastocyst stage, implant, and generate viable offspring (Duranton & Renard 2001, Sirard *et al.* 2006). This competence is acquired through the

sequential completion of meiotic, cytoplasmic, and molecular maturation steps during folliculogenesis (Sirard *et al.* 2006) and is difficult to ascertain *a priori*. Follicular classification based on morphological characteristics has been used in an attempt to predict the developmental potential of the enclosed oocyte, but has met with mitigated success (Blondin & Sirard 1995). For example, follicular size is strongly correlated with oocyte quality, but is not the only criterion to consider. Indeed, some oocytes that originate from large follicles still fail to produce embryos, while oocytes from medium-sized follicles (5–9 mm) may yield viable embryos (Sirard *et al.* 2006). Commonly used to define oocyte competence in the context of IVP, simply reaching the blastocyst stage is a relatively good indicator of oocyte overall quality.

Evidence accumulated over the past few decades suggests that a bidirectional communication between the oocyte and the surrounding somatic cells takes place during folliculogenesis (Buccione *et al.* 1990a, 1990b, Eppig *et al.* 2002, Gilchrist *et al.* 2004, Makabe *et al.* 2006, Sirard *et al.* 2006, Buratini & Price 2011, Conti *et al.* 2012), confirming a link between oocyte

competence and the follicular environment. This communication can be achieved through paracrine signaling and direct cell-to-cell communication via gap junction channels (reviewed in Kidder & Vanderhyden (2010)). Growth and development, acquisition of meiotic and developmental competence, and regulation of overall transcriptional activity in oocytes rely on granulosa cell (GC) support (Eppig 1991, De La Fuente & Eppig 2001, Eppig *et al.* 2005). However, oocytes in turn guide GC development and metabolic processes (Li *et al.* 2000, Matzuk *et al.* 2002, Gilchrist *et al.* 2008).

GC could be a very convenient source of molecular markers of oocyte developmental potential, since they are simply discarded during oocyte collection in IVP procedures and their recovery does not require destruction of the gamete itself. In cattle and other species, molecular markers of oocyte developmental competence have been identified in surrounding GC and constitute an interesting avenue to investigate (Robert *et al.* 2001, Hamel *et al.* 2008, 2010a, 2010b, Jiang *et al.* 2010).

The aim of this study was thus to use GC obtained at the time of cumulus–oocyte complex (COC) collection to determine gene patterns in follicles containing oocytes capable of sustaining early embryonic development. Moreover, since the LH surge could have the final say on oocyte quality, we investigated GC preovulation transcripts just before and after the LH surge in the hope of finding molecular markers indicative of oocyte quality and thus identifying means of increasing the efficiency of IVP. Our goal was to describe the impact of the LH surge on the GC transcriptome, in addition to finding biomarkers that are associated with the presence of a developmentally competent gamete.

Results

Ovarian stimulation protocol and follicle retrieval

A summary of the number of follicles (i.e. COC) collected, the number of fertilized oocytes that underwent at least one cleavage, and number that reached the blastocyst stage for the protocols 'pre-LH' and 'post-LH' is presented in Table 1. It should be noted that the pre-LH group contained a smaller number of follicles due to a hemorrhagic ovarian cyst forcing the exclusion of one cow from the study. The distribution of the follicles among the various size ranges is shown in Fig. 1. The

different sizes observed were not statistically supported, due to the small number of samples. For both stimulation protocols, mid-sized follicles (6–10 mm) were predominant (60–70%).

Impact of the LH surge on follicular dynamics and embryonic development

To classify GC in terms of embryonic developmental competence status, the COC of each follicle was removed and fertilized *in vitro*. The developmental stage reached in relation to follicular size and the numbers of embryos found at each stage are represented in Fig. 2. No statistical difference was observed between the two stimulation protocols in terms of failure of the fertilized oocytes to cleave (21% in the post-LH group compared to 11% in the pre-LH group). A slightly but not significantly higher number of fertilized oocytes from the pre-LH group reached the blastocyst stage (52 vs 41%). For both cleavage and blastocyst scores, one cow responded differently to treatment, indicative of a normal heterogeneous population.

Impact of the LH surge on blastocyst quality

The developmental and morphological status of each blastocyst was scored on day 9 of incubation. Figure 3 shows the percentages of blastocysts in each status category. A significantly greater number of category 5A blastocysts (i.e. fully hatched, the most advanced blastocyst phase) were found in the post-LH group.

Impact of the LH surge on differential gene expression in GC associated with 'competent' and 'incompetent' oocytes

As a quality assessment step, the reproducibility of the microarray hybridization was evaluated using a signal intensity correlation matrix between replicate experiments for each GC group (pre-LH and post-LH) in a pairwise manner. An excellent correlation (0.95–0.99, data not shown) was found between the biological and technical replicates, indicative of a high homogeneity between treatments.

In the pre-LH group, microarray analyses between 'competent' vs 'incompetent' categories revealed 130 overexpressed and 206 underexpressed transcripts (fold-change ≥ 1.5 and FDR ≤ 0.05), whereas no over- or

Table 1 Summary of bovine embryo development obtained *in vitro*.

Stimulation protocol	Number of cows	Number of follicles (COC)	Underwent at least one cleavage (%)	Reached the blastocyst stage
Pre-LH	5 ^a	84	74 (88.1)	44 (52.4%, 59.5% ^b)
Post-LH	6	105	84 (80.0)	43 (41.0%, 51.1% ^b)

^aOne had a hemorrhagic cyst on an ovary and therefore was excluded. ^bBased on the number that underwent at least one cleavage.

underexpressed signal was found in the post-LH group (Fig. 4), suggesting that the LH surge probably masked all further differences at that stage. After eliminating redundant transcripts, a total of 18 unique transcripts were identified as being upregulated and 22 unique transcripts as being downregulated in the pre-LH GC group and correlated with the developmental competence of the oocyte. Each overexpressed and underexpressed signal found was sequenced and is identified in Table 2. Among the downregulated transcripts, a predominance of genes associated with mitochondrial and ribosomal proteins were found. Some sequences identified in the microarray analyses were not related to any known genes, despite their positioning on the bovine genome, suggesting the presence of novel transcribed regions (NTRs) linked to oocyte developmental competence status.

Molecular marker validation

Validation of the molecular markers found in microarray analyses was performed using qRT-PCR. Five different transcripts and an expressed sequenced tag corresponding to an NTR upregulated and one downregulated transcript were analyzed in the 2 h pre-LH and 6 h post-LH groups (Fig. 5). For the pre-LH protocol, all of the transcripts (apoptotic chromatin condensation inducer 1 (*ACIN1*), inhibin beta A (*INHBA*), serglycin (*SRGN*), tribbles homolog 2 (*TRIB2*), sterol-C4-methyl oxidase (*MSMO1* (*SC4MOL*)), and *NTR3*) were statistically more abundant in the 'competent' group ($P < 0.05$),

whereas *TNFAIP6* mRNA was found to be more expressed ($P < 0.05$) in the incompetent group in concordance with microarray results. These differences were not found in the post-LH case (Fig. 5B).

Pre-LH-surge GC associated with oocyte competence contain an abundance of transcripts of genes known to play a role in lipid metabolism and apoptosis

To evaluate whether the differentially expressed transcripts identified through microarray analysis interact with each other, markers associated with known genes were subjected to pathway analysis. Twelve upregulated markers in GC associated with a competent oocyte were linked together in a network implicated in lipid metabolism, molecular transport, and small molecule biochemistry, reflecting GC physiological functions (Fig. 6A). Fifteen downregulated transcripts were assembled in a network involved in cell death and cell morphology (Fig. 6B). Since follicular atresia results from apoptosis, downregulation of the transcripts in this network may indicate actively growing and proliferating GC.

Discussion

The LH surge plays a critical role in folliculogenesis by inducing extreme reorganization in follicular cells, which in turn results in ovulation and luteinization. In the present study, we sought to investigate whether LH administered in ovarian stimulation protocols can

Figure 1 Effect of the LH surge on follicular size distribution. The number of follicles collected in each size range and their percentage (%) of the total are indicated.

Figure 2 Embryonic development achieved by bovine oocytes obtained from follicles of various sizes. (A) 2 h before and (B) 6 h after LH surge. The box plot charts were displayed with log transformed data. The segments inside the boxes are medians and the bars indicate minimum and maximum sizes. Empty circles denote outliers slightly above the third quartile or $1.5 \times$ the interquartile range. Full circles denote outliers at least $3 \times$ above the interquartile range. n = total number of follicles included in each embryonic stage. Empty box = developmental stage with too few data (pre-LH, $n=1$ at the morula stage; post-LH, $n=3$ at the eight-cell stage). Pie charts show the proportions of the embryos that reached each developmental stage.

improve oocyte developmental competence and whether GC isolated from follicles associated with a competent oocyte carry a distinct gene expression profile representative of oocyte quality.

Previous studies have shown that administering LH produces competent oocytes that are highly likely to undergo normal embryonic development (Blondin *et al.* 2002, Dieleman *et al.* 2002, Knijn *et al.* 2008). In our study, superstimulation with FSH resulted in 52% of the fertilized oocytes reaching the blastocyst stage, while administering LH provided 41% blastocysts. However, blastocyst yield is not the only criterion to evaluate when determining an ovarian stimulation regimen. Embryo quality, which is more likely to be indicative of embryos that will result in a gestation, should also be taken into account. Despite the larger number of blastocysts obtained without LH, blastocyst quality was better with LH. Since the *in vivo* LH surge is important for follicle dominance and for the oocyte to reach final maturation, the signal triggered by LH might help follicles, as well as oocytes, to acquire important factors necessary for their

terminal differentiation, resulting in blastocysts of better quality.

Molecular marker discovery was achieved using our cDNA boutique array (5.7K) built from several subtractive libraries representative of all GC stages. The major advantage of this array is the possibility of identifying transcripts without any *a priori* selection based on gene function, therefore allowing the discovery of new elements such as novel transcribed regions (NTRs). However, the function of these uncharacterized transcripts remains to be elucidated. Forty differentially expressed transcripts that correlated with oocyte developmental status were found in the pre-LH group. Although the number of total transcripts identified may seem small, a similar study performed in rats using a genome-wide array (30K) identified 13 differentially expressed genes in GC correlated with a competent oocyte (Jiang *et al.* 2010). Since 'competent' and 'incompetent' GCs are at the same growth stages and are compared with each other, it is not surprising that the differences between these two categories are subtle.

Figure 3 Blastocyst scores for the two stimulation protocols, at day 9 of embryonic development. The number indicates blastocyst development and stage status: 1) early blastocyst (blastocoel cavity less than half the volume of the embryo); 2) young blastocyst (blastocoel cavity more than half the volume of the embryo); 3) blastocyst (cavity completely filling the embryo, with thinning of the zona pellucida); 4) prehatching blastocyst (hatching out of the shell); and 5) hatched blastocyst (hatched out of the shell). The letter indicates inner cell mass and trophoctoderm quality: A) very good (many cells tightly packed); B) good (several cells, loosely grouped); C) poor (few cells). *Indicates a statistical difference between the protocols ($P < 0.05$).

The concept of competence refers to the ability of oocytes to develop after fertilization. During folliculogenesis, the oocyte stores the mRNA and proteins that will be required to support subsequent postfertilization development. It has been demonstrated that oocyte transcriptional activity is elevated in the growing follicle until the early antral stages and dramatically decreases afterward (Fair *et al.* 1997, Memili & First 1998, Memili *et al.* 1998). It has thus been proposed that oocyte competence may be acquired before the antral follicle stage (Mermillod *et al.* 2008). In our results, no molecular marker associated with oocyte competence was found in the post-LH group, while a few were discovered in the pre-LH group. We propose two possible explanations for this phenomenon. The first one is that the new GC transcriptomic program induced by the LH (Gilbert *et al.* 2011) surge masks differences and variability that might exist between GC associated with good or poor developmental competence in oocytes. The second possibility is that the bidirectional communication between the two cell types is no longer synchronized 6 h after the LH surge. At this stage, the message sent by the LH surge induces resumption of meiosis in the oocyte and the expansion of cumulus cells, while GC are preparing for luteinization. The follicular cells and the gamete have undertaken separate differentiation programs to achieve ovulation, suggesting that oocyte competence at this stage is no longer reflected in the GC transcriptional profile.

In this study, GC SERPINE2 was found upregulated in association with competent oocytes. SERPINE2 belongs to the serine protease inhibitor superfamily, modulates the activity of plasminogen activators and is involved in tissue remodeling (Silverman *et al.* 2001). In cattle, expression of SERPINE2 has been found elevated in

dominant follicles and to decrease after the LH surge (Bedard *et al.* 2003).

For the other markers, no function has been ascribed in the ovary, however their role in other tissues is interesting. For example, SRGN is a proteoglycan stored primarily in the secretory granules of many hematopoietic cells (Abrink *et al.* 2004). TRIB2 and other members of this family are involved in many physiological processes, such as induction of apoptosis in hematopoietic cells, regulation of inflammatory activation of monocytes, and modulation of gene expression (Hegedus *et al.* 2007, Eder *et al.* 2008). Although no precise role in ovarian physiology has been assigned to SRGN and TRIB2, cattle studies indicate upregulation of their mRNA in association with the dominant follicle, decreasing after the LH surge (Ndiaye *et al.* 2005).

The increased level of *INHBA* mRNA in GC associated with competent oocytes does not allow us to determine whether the production of inhibin A, activin A, or AB proteins is altered. Indeed, the *INHBA* gene nomenclature represents the βA subunit and not the dimeric protein. However, during folliculogenesis, activin increases FSH secretion and promotes GC proliferation, while inhibin antagonizes activin by decreasing FSH secretion. Inhibin can also increase LH-induced thecal androgen synthesis (Hillier 1991, Wrathall & Knight 1995). *In vitro*, the presence of activin A in COCs is linked to oocyte developmental competence (Silva & Knight 1998). These results reflect a pivotal role for the inhibin βA (or activin βA) subunit in GC growth and differentiation and in the acquisition of competence by oocytes.

Many of the upregulated markers are associated with chromatin, cell cycle, and DNA stability. For example, ACIN1 was initially found to be activated by caspases and to activate chromatin condensation during apoptosis

Figure 4 Pairwise comparison of gene expression log mean ratio of competent to incompetent groups of oocytes, obtained by microarray analysis. (A) 2 h-pre-LH protocol and (B) 6 h-post-LH protocol. Horizontal axis represents log average intensity and the vertical axis represents log signal intensity ratio of competent to incompetent groups for each clone. The red spots represent overexpressed clones and green spots represent underexpressed clones, using a 1.5-fold-change filter with $FDR \leq 0.05$.

(Joselin *et al.* 2006). Other roles in mRNA processing and spliceosomes have also been attributed to this factor and its knockdown has been shown to lead to a decrease in cell growth (Rappsilber *et al.* 2002, Zhou *et al.* 2002, Joselin *et al.* 2006). The mitotic checkpoint component Mad2 (MAD2) is a component of the spindle checkpoint (Musacchio & Hardwick 2002) for mitotic cells, oocytes need MAD2 in order to bring about meiosis 1 arrest (Homer *et al.* 2005). So far, little is known about calmodulin-binding transcription activator 1 (CAMTA1), but it has been demonstrated that its mRNA levels in neuroblastoma cells can be high during the S and M phases and decrease after mitosis (Nakatani *et al.* 2004). Single-stranded DNA binding protein 1 (SSCBP1) is essential for the response to DNA damage, and with the help of Integrator3 protein, it controls G(2)/M checkpoint activation (Zhang *et al.* 2009). Replication protein A3 (RPA3), like SSCBP1, is also a single-stranded DNA-binding protein and plays a central role in many aspects of nucleic acid metabolism, including DNA replication, repair, and recombination (Salas *et al.* 2009, Mason *et al.* 2010). Proliferating cells are challenged with maintenance of genome integrity and chromatin organization in order to avoid aberrant gene expression. Since chromatin is involved in many events, including transcription, replication, repair, and recombination, cells have developed mechanisms such as cell cycle

checkpoints to avoid defects in replication or in mitotic spindle assembly (reviewed by Koundrioukoff *et al.* (2004)). In the present study, the implication of markers involved in genome stability and in cell cycle checkpoints is interesting since GC at this stage (i.e. before the LH surge) seems to be growing and proliferating actively due to FSH administration. The majority of the molecular markers found in this study seem to be involved in chromatin integrity and cell cycle checkpoints, which are essential for GC proliferation and follicular differentiation (through dominance acquisition) in order to achieve preovulation status and ultimately ovulation.

From pathway analyses, two networks were identified using upregulated and downregulated molecular markers associated with oocyte developmental competence. The network linking the overexpressed transcripts is involved in lipid metabolism, molecular transport, and small molecule biochemistry. The transcripts of two enzymes involved in lipid metabolism were thus identified, namely stearoyl-CoA desaturase (SCD) and MSMO1. SCD is involved in unsaturated fatty acid synthesis and therefore influences cell membrane fluidity (Zhang *et al.* 1999). It has also been involved in membrane organization and depletion of a specific pool of membrane cholesterol, thereby increasing intracellular cholesterol bioavailability (Sun *et al.* 2003). The MSMO1 enzyme also plays a role in cholesterol biosynthesis. In BMP15 GDF9 double-mutant mice, cumulus cell transcripts involved in cholesterol synthesis, including MSMO1 are reduced before the LH surge (Su *et al.* 2008). These results show that the oocyte cannot synthesize cholesterol and relies on follicular cells to provide the enzyme required for cholesterol synthesis (Su *et al.* 2008). GC acquires a high level of steroidogenic potential after the LH surge and it therefore appears that the enzymes required for cholesterol biosynthesis must be present.

In summary, the data presented in this study show that GC transcripts may be markers of the ability of oocytes to proceed through the early stages of embryonic development. Cattle are highly suitable for this type of study, since it is possible to examine the preovulation period, the moment at which differences in oocyte competence may be decisive. It thus appears that the right time to solve the competence puzzle is before the LH surge. Better knowledge of the molecular markers involved in oocyte competence will increase the success of ovarian stimulation and subsequent embryo production and will allow the identification of important metabolic pathways for functions that remain to be resolved.

Materials and Methods

Ovarian stimulation and GC collection

Twelve cows at mid-cycle were treated using an intravaginal progesterone-releasing device to prevent premature LH surge

Table 2 Differential expression of RNA transcripts by GCs associated with competent vs incompetent oocytes under the 2 h-pre-LH stimulation protocol.

Gene	Symbol	Accession no.	Relative expression	Redundancy factor
Overexpressed transcripts in the 2 h pre-LH group (more expressed in GC associated with competent oocyte)				
Apoptotic chromatin condensation inducer 1	<i>ACIN1</i>	NM_001105410	1.78	1
ATP synthase, H ⁺ transporting, mitochondrial F1 complex	<i>ATP5A1</i>	BC116059	1.58	2
Inhibin, beta A	<i>INHBA</i>	NM_174363	1.78	4
Mitotic checkpoint component Mad2	<i>MAD2</i>	NM_001079796	1.52	1
Phosphatidylethanolamine binding protein	<i>PEBP1</i>	NM_001033623	1.60	1
Replication protein A3	<i>RPA3</i>	NM_001034427	1.63	4
Serglycin	<i>SRGN</i>	NM_001025326	2.05	35
Serpin peptidase inhibitor, clade E member 2	<i>SERPINE2</i>	NM_174669	1.55	5
Single-stranded DNA binding protein	<i>SSBP1</i>	NM_001037466	1.68	1
Stearoyl-CoA desaturase	<i>SCD</i>	NM_173959	1.52	2
Sterol-C4-methyl oxidase-like	<i>MSMO1</i>	NM_001098863	1.83	2
Tribbles homolog 2	<i>TRIB2</i>	NM_178317	1.81	6
Predicted: calmodulin-binding transcription activator 1	<i>CAMTA1</i>	XM_610833	1.65	2
Predicted: mCG1035404-like	<i>HNRNPA3</i>	XM_002685299	1.64	1
Novel transcribed region 1	Unassigned		1.68	3
Novel transcribed region 2	<i>Chr4</i>		1.99	53
Novel transcribed region 3	<i>Chr19</i>		1.55	1
Novel transcribed region 4	<i>Chr3</i>	AB047862	1.70	6
Underexpressed transcripts in the 2 h pre-LH group (more expressed in GC associated with incompetent oocyte)				
Annexin A4	<i>ANXA4</i>	NM_001001440	−1.54	1
Cytochrome c, somatic nuclear gene encoding mitochondrial protein	<i>CYCS</i>	NM_001046061	−1.78	2
Mediator complex subunit 21	<i>MED21</i>	NM_001038566	−1.64	1
Mitochondrial DNA		HM045018	−2.03	146
Ribosomal protein L13a	<i>RPL13A</i>	NM_001076998	−2.10	1
Ribosomal protein L18	<i>RPL18</i>	NM_001015556	−1.50	1
Ribosomal protein L36	<i>RPL36</i>	NM_001078139	−1.58	2
Ribosomal protein L7a	<i>RPL7A</i>	NM_001040520	−2.01	2
Ribosomal protein S13	<i>RPS13</i>	NM_001025342	−1.51	1
Ribosomal protein S20	<i>RPS20</i>	NM_001034438	−1.53	1
Secreted protein, acidic, cysteine-rich	<i>SPARC</i>	NM_174464	−1.85	7
Selenoprotein K	<i>SELK</i>	NM_001037489	−1.52	1
Solute carrier family 38, member 2	<i>SLC38A2</i>	NM_001082424	−2.18	1
Steroidogenic acute regulatory protein	<i>STAR</i>	NM_174189	−2.26	1
Thymosin beta 4, X-linked	<i>TMSB4X</i>	NM_001002885	−1.59	2
Tumor necrosis factor, alpha-induced prot 6	<i>TNFAIP6</i>	NM_001007813	−2.57	2
Tumor protein, translationally-controlled 1	<i>TPT1</i>	NM_001014388	−1.51	1
Novel transcribed region 5	Unassigned		−1.78	26
Novel transcribed region 6	<i>Chr17</i>		−1.66	1
Novel transcribed region 7	<i>Chr18</i>		−1.82	1
Novel transcribed region 8	<i>Chr26</i>		−1.83	1
Novel transcribed region 9	<i>Chr5</i>		−2.10	4

(CIDR; Pfizer Canada Inc., Saint-Laurent, QC, Canada) for 7 days. On day 1 AM, dominant follicle was punctured using transvaginal ultrasonography (3 days after insertion of the device). Follicular growth was initiated by injection of FSH (Follitropin-V; Bioniche Animal Health, Belleville, ON, Canada) in decreasing doses at 12 h intervals over the next 5 days (day 2 PM: 70 mg; day 3 AM/PM: 60 mg; day 4 AM/PM: 50 mg; day 5 AM/PM: 40 mg; and day 6 AM: 30 mg; for a total of 400 mg of NIH-FSH-PI). Two milliliters of prostaglandin F2a (Estrumate; Intervet Canada Corp., Kirkland, QC, Canada) containing 500 µg of cloprostenol were injected 12 h before (day 4 PM) and after (day 5 AM) the removal of the progesterone device (day 5 AM). LH (25 mg of Lutropin-V; Bioniche Animal Health) was administered 12 h after the final FSH injection (day 6 PM). Ovariectomies were done either 2 h

before this (pre-LH group) or 6 h after (post-LH group). Ovaries were transported without delay to the laboratory in 0.9% NaCl at 37 °C. COC from all follicles >4 mm were removed by aspiration and utilized for the IVP. Then, follicles were cut and turned inside out and GCs were scrapped off in a small volume of PBS and centrifuged. The supernatant was removed and the GC pellet was placed in RNeasy lysis solution (Applied Biosystems, Streetville, ON, Canada) and frozen at −20 °C until RNA extraction.

COC pooling

Previous experiments have indicated that cultured bovine oocytes develop better in groups than individually (data not shown). We therefore pooled oocytes, of the same cow, from

Figure 5 Quantitative RT-PCR. Reactions were performed in triplicate with nonamplified cDNA in the (A) 2 h pre-LH and (B) 6 h post-LH. Differing letters indicate significant differences. Overexpressed markers: *ACIN1*, apoptotic chromatin condensation inducer 1; *INHBA*, inhibin beta A; *MSMO1*, sterol-C4-methyl oxidase-like; *SRGN*, serglycin; *TRIB2*, tribbles homolog 2; *NTR3*, novel transcribed region 3, and underexpressed marker: *TNFAIP6*, tumor necrosis factor alpha-inducible protein 6.

follicles of similar size into groups of three and monitored their development up to the blastocyst stage. This pooling also decreases the impact of individual variations, which are not of interest, and therefore increases the chance of identifying reliable markers of competence by group contrast.

Oocyte maturation and in vitro production

COCs were placed in HEPES-buffered Tyrode's medium (THL) supplemented with 10% bovine serum, 200 µM pyruvate and 50 µg/ml gentamicin. Group of three COC was placed in 50 µl droplets of medium under 9 ml of filtered mineral oil. Maturation medium was composed of TCM199 (Gibco 11150-059; Invitrogen), 10% FCS (Sterile Foetal Bovine Serum for cell Culture; Mediatech, Montreal, QC, Canada), 200 µM pyruvate, 50 µg/ml gentamicin with 50 µg/ml of porcine LH (pLH; Lutropin-V, Bioniche Animal Health), 0.5 µg/ml porcine FSH (pFSH; Folltropin-V), and 1 mg/ml of estradiol-17b (Sigma-Aldrich Canada Ltd). Droplets containing COCs were incubated for 24 h at 38.5 °C in a humidified 5% CO₂, 20% O₂ atmosphere.

Fertilization was then carried out using thawed bovine semen (Centre d'insemination artificielle du Quebec) and fertilization medium consisting of modified Tyrode-lactate medium with fatty acid-free BSA, pyruvic acid, gentamicin, heparin (final concentration, 2 µg/ml), and penicillamine, hypotaurine, and epinephrine. Fertilization took place during 15–18 h of incubation at 38.5 °C in a humidified 5% CO₂, 20% O₂ atmosphere. The fertilized oocytes were then cocultured in group of three in 50-µl drops with Buffalo Rat Liver (BRL) cells in Ménézo's B2 medium (Mediatech Canada, Inc., Montreal, QC, Canada) with 0.4% fatty acid-free BSA and then transferred to other droplets containing B2-BRL and 10% estrus cow serum in humidified atmosphere containing 5% CO₂. Embryos were transferred 96 h postfertilization to fresh droplets of B2-BRL with 20% estrus cow serum. On day 9 of culture, embryos were graded according to the criteria of the International Embryo Transfer Society (Robert & Nelson 1998). To be assigned to the competent group, the three oocytes of the same droplets had to reach the blastocyst stage. To be designated to the incompetent group, the three oocytes of the same droplets had led to three developmental arrests.

Figure 6 Network of interactions among markers found in the 2 h-pre-LH group, based on microarray analysis. (A) Genes found upregulated: *ACIN1*, apoptotic chromatin condensation inducer 1; *ANKRD1*, ankyrin repeat domain 1; *ATP5J*, ATP synthase; *CEBPB*, CCAAT/enhancer binding protein (C/EBP) beta; *CYP26A1*, cytochrome P450 family 26 subfamily A polypeptide 1; *DGAT2*, diacylglycerol O-acyltransferase 2; *ENG*, endoglin; *MGP*, matrix Gla protein; *ERCC6L*, excision repair cross-complementing rodent repair deficiency complementation group 6-like; *HNRNP3A3*, heterogeneous nuclear ribonucleoprotein A3; *IFNG*, interferon gamma; *INHBA*, inhibin beta A; F2, coagulation factor II (thrombin); *INSIG1*, insulin induced gene 1; *CIITA*, major histocompatibility complex class 2; *PEBP1*, phosphatidylethanolamine binding protein 1; *PPY*, pancreatic polypeptide; *RPA3*, replication protein A3; *RXRA*, retinoid X receptor alpha; *SCD*, stearoyl-CoA desaturase; *MSMO1*, sterol-C4-methyl oxidase-like; *SLC2A2*, solute carrier family 2 member 2; *SRGN*, serglycin; *SSBP1*, single-stranded DNA binding protein; *THBD*, thrombomodulin; *TNF*, tumor necrosis factor; *TRIB2*, tribbles homolog 2; and *VLDLR*, very low density lipoprotein receptor. (B) Genes found downregulated: *ANXA13*, annexin A13; *ARRB2*, arrestin beta 2; *CES3*, carboxylesterase 3; *CYCS*, cytochrome c somatic; *ETS1*, v-ets erythroblastosis virus E26 oncogene homolog 1; *GNLY*, granulysin; *SLC38A2*, solute carrier family 38 member 2, *IFNB1*, interferon, beta 1 fibroblast; *MAP3K1*, mitogen-activated protein kinase 1; *MED21*, mediator complex subunit 21; *NFKB*, nuclear factor of kappa light polypeptide gene enhancer in B-cells; *NUCB2*, nucleobindin 2; *MED10*, mediator complex subunit 10 (MED10); *OSGIN1*, oxidative stress induced growth inhibitor 1; *RPL7A*, ribosomal protein L7a; *RPL13A*, ribosomal protein L13a; *RPL18*, ribosomal protein L18; *RPS13*, ribosomal protein S13; *RPS20*, ribosomal protein S20; *RPL36*, ribosomal protein L36, *SPARC*, secreted protein acidic cysteine-rich; *STAR*, steroidogenic acute regulatory protein; *STEAP3*, STEAP family member 3 metalloredutase; *TDPX2*, thioredoxin-dependent peroxide reductase 2; *THBS2*, thrombospondin 2; *TMSB4X*, thymosin beta 4 X-linked; *TNFAIP6*, tumor necrosis factor alpha-induced protein 6; *TP53*, tumor protein p53; *TP53AIP1*, tumor protein p53 regulated apoptosis inducing protein 1; *TPT1*, tumor protein translationally-controlled 1; *TRAF6*, TNF receptor-associated factor 6; *TNFRSF10B*, tumor necrosis factor receptor superfamily member 10b; and *TXNRD1*, thioredoxin reductase 1. Uncolored nodes represent eligible molecules provided by the Ingenuity knowledge base to maximize connectivity.

Statistical analysis

To assess the effect of the LH surge on follicle size, a linear mixed model was used. To assess the effect of the LH surge on blastocyst production, a generalized estimating equations (GEE) model was used with a logit link. To assess the effect of the LH surge on blastocyst scores at day 9, GEE models with a generalized logit link were used. In every case, correlation between observations for the same cow was taken into account, using a random effect in the linear mixed model and using a sandwich estimator in the GEE models. The normality criterion of the linear mixed model was not met. A log transformation was applied to follicle size in order to meet the normality criterion. Since both the model applied to transformed data and the one applied to untransformed data yielded the same conclusions, only the results obtained using the untransformed data were interpreted.

GC selection

The GC molecular marker investigation was done using comparison within ovarian stimulation protocols (i.e. pre-LH

or post-LH). Microarray comparisons were performed with three biological replicates, from three different cows, for each GC categories, e.g. three 'competents' vs three 'incompetents' for the pre-LH group and three 'competents' vs three 'incompetents' for the post-LH groups (for a total of 12 groups). For each stimulation protocol, GCs associated with 'competent' oocytes were compared with those associated with 'incompetent' oocytes. GCs were deemed 'competent' only when all three oocytes in a droplet yielded a blastocyst. GCs were deemed 'incompetent' when three developmental arrests were noted (Table 3).

Total RNA extraction

Total RNA extracted from each pool (three follicles) of GC using the Absolutely RNA Miniprep Kit (Stratagene, LaJolla, CA, USA) was recovered in a 30- μ l 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).

Table 3 Description of the samples used for microarray hybridizations.

Treatment	Group	Follicle size (mm)	Stage reached	Blastocyst score
2 h-pre-LH	Competent	6	Blast	3B
		6	Blast	3B
		6	Blast	3B
		8	Blast	5A
		8	Blast	4A
		8	Blast	2B
		8	Blast	5A
		8	Blast	5A
	Incompetent	8	Blast	5A
		12	16-cell	NA
		12	8-cell	NA
		12	2-cell	NA
		10	16-cell	NA
		10	16-cell	NA
		10	2-cell	NA
		8	4-cell	NA
6 h-post-LH	Competent	8	4-cell	NA
		8	No cleavage	NA
		10	Blast	5A
		10	Blast	4A
		10	Blast	3B
		10	Blast	5A
		10	Blast	5A
		10	Blast	5B
	Incompetent	10	Blast	5A
		10	Blast	3B
		15	16-cell	NA
		15	16-cell	NA
		15	No cleavage	NA
		8	16-cell	NA
		8	4-cell	NA
		8	2-cell	NA
		10	4-cell	NA
		10	2-cell	NA
		10	No cleavage	NA

NA, not applicable.

Custom-made cDNA microarray slide conception

Results obtained by many researchers have demonstrated the value of combining cDNA arrays with subtractive suppressive hybridization (SSH) to increase the probability of identifying genes of interest (Hamel *et al.* 2008). A boutique microarray representative of all GC stages in bovine follicles was therefore generated using the SSH technique. Five ovarian combinations, namely 6 h-post-LH vs <3-mm follicles and its reverse (<3 mm vs 6 h-post-LH), 2 h-pre-LH vs preovulatory follicle (e.g. 22 h-post-LH) and its reverse (22 h-post-LH vs 2 h-pre-LH), and finally 6 h-post-LH 'competent' vs 6 h-post-LH 'incompetent' follicles thus provided cDNA. Briefly, the SSH was performed using pools of 1 µg of total RNA. The mRNAs were reverse transcribed and the cDNAs were amplified using the BD SMART PCR cDNA Synthesis Kit (BD Biosciences, Mississauga, ON, Canada) according to the manufacturer's instructions. The SSH was performed with the PCR Select cDNA Subtraction Kit (BD Biosciences) as described by the manufacturer. The subtracted PCR products generated by SSH were cloned into the pGEM-T easy vector systems (Promega) and then transformed into One-shot max efficiency DH5α-T1 competent cells (Invitrogen). For each library, 1152 clones

were randomly selected for PCR amplification. The PCR products of 5760 randomly selected clones were purified using UNIFILTER 384-well purification plates (Whatman Inc., Clifton, NJ, USA) according to the manufacturer's instructions. Purified PCR products were speedvac-evaporated (SPD SpeedVac ThermoSavant, Fisher Scientific Limited, Nepean, ON, Canada), resuspended in a mixture of equal parts of 3 × SSC (1 × SSC: 0.15 M sodium chloride and 0.015 M sodium citrate with dimethyl sulfoxide), and spotted in two replicates on GAPS II glass slides (Corning, Corning, NY, USA) using a VersArray Chip Writer Pro Robot (Bio-Rad). Slides were then cross-linked with u.v. according to the manufacturer's instructions. Quality control experiments were performed after each set of slides with TOTO-1 dye (Molecular Probe, Burlington, ON, Canada) and Terminal Transferase dye (Roche Diagnostics).

Sample labeling, hybridization, and microarray scanning

Five hundred nanograms of total RNA from 12 pools of 2 h-pre-LH and 6 h-post-LH were amplified using T7 RNA polymerase (Arcturus RiboAmp PLUS; Life Technologies Inc., Burlington, ON, Canada) according to the manufacturer's indications. A fixed amount of 2.5 µg aRNA was labeled indirectly using ULS aRNA Fluorescent Labeling (Kreatech 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. Six hybridizations for each treatment (total of 12 hybridizations) were performed in a dye swap design and hybridized on the cDNA boutique arrays.

Hybridizations were performed in SlideHyb buffer #1 (Ambion, Austin, TX, USA) at 55 °C for 18 h in the SlideBooster hybridization station (Advantix, San Francisco, CA, USA). Slides were then washed twice with 2 × SSC-0.5% SDS at 55 °C for 15 min and twice with 0.5 × SSC-0.5% SDS at 55 °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 1200 g at room temperature for 5 min.

Slides were scanned using the VersArray ChipReader System (Bio-Rad) and visualized with the ChipReader software (Media Cybernetics, San Diego, CA, USA). After acquisition, scanned images were analyzed using ArrayPro Analyzer software (Media Cybernetics).

Microarray data normalization and statistical analysis

Signal intensity data files were normalized using ArrayPro software (Media Cybernetics). 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.

To identify upregulated and downregulated genes, normalized data were assessed using NIA array analysis software (<http://lgsun.grc.nia.nih.gov/ANOVA>). A cutoff threshold on minimum log intensity was first applied to the data and this threshold was determined using the average log

Table 4 Primer sequences used for real-time PCR validation.

Gene	Forward sequence	Reverse sequence	Annealing temp (°C)	Fluorescence acquisition temp (°C)	Product size (bp)	Accession number
ACIN1	5'-ctcagtagaggtggccttgc-3'	5'-cctaaagtgaaggacgaacc-3'	57	85	216	NM_001105410
HPRT1 ^a	5'-ggctcgagatgtgatgaagg-3'	5'-gcaaagtctgcatgtcttcc-3'	57	83	293	NM_001034035
INHBA	5'-gaagggaagaagaggatgg-3'	5'-agtcattccagccaatgtcc-3'	57	89	208	NM_174363
SRGN	5'-cagtgcaatcctgacagtcc-3'	5'-tctgtccccaacacgtatcc-3'	57	82	197	NM_001025326
MSMO1	5'-agctttaaggccatttgc-3'	5'-ttcaataggaggccaacacc-3'	57	78	176	NM_001098863
NTR3	5'-tctctgctctccctcaatcc-3'	5'-cctccacctcaaagttgtcc-3'	57	79	181	Unknown
TNFAIP6	5'-caagggcagagttggatacc-3'	5'-tgtgccagtagcagattgg-3'	57	81	230	BC151789
TRIB2	5'-gtggcatgtatgtgcagacc-3'	5'-acaggacaaagcaccagagg-3'	57	84	300	NM_178317
YWHAZ ^a	5'-ccagtcacagcaagcatacc-3'	5'-cttcagctcgtctccttgg-3'	57	82	287	NM_174814

ACIN1, apoptotic chromatin condensation inducer 1; HPRT1, hypoxanthine phosphoribosyltransferase 1; INHBA, inhibin beta A; SRGN, serglycin; MSMO1, sterol-C4-methyl oxidase-like; NTR3, novel transcribed region 3; TRIB2, TRB-2 protein; and YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase.

^aHousekeeping genes used for data normalization.

intensity of the negative controls present on the slide. All probes with an increase > 1.5 and false-discovery rate (FDR) value < 0.05 were deemed differentially expressed.

DNA sequencing and clone identification

After microarray analysis, PCR products of all positive signal clones were sent for DNA sequencing (CRCHUL sequencing service, Laval University, PQ, Canada) as previously described (Sirard *et al.* 2005). Briefly, the resulting sequence traces were uploaded into a cDNA Library Manager program (coded by Genome Canada Bioinformatics) that automates and facilitates sequence analysis and clone identification (Vallee *et al.* 2005). For clone identification, sequence traces were uploaded into the cDNA Library Manager, trimmed (Phred software, www.phrap.org) and compared against a locally installed GenBank database (Basic Local Alignment Search Tool (BLAST); <http://www.ncbi.nlm.nih.gov/blast/>).

Data mining: functional annotation and pathway analysis

Differentially expressed genes were also used for pathway analysis and were imported into the Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems; <http://www.ingenuity.com>, Mountain View, CA, USA). IPA was used to generate networks of genes and to perform a functional analysis associated with biological functions and molecular processes.

Real-time PCR

Validation of the microarray results was performed by the real-time PCR technique using the same RNA samples (nonamplified) as the microarrays. Five hundred nanograms of total RNA were reverse transcribed using the Transcriptor reverse transcriptase (Roche) with a mix of oligo dt (Applied Biosystems) and decamer (Applied Biosystems) according to manufacturer's instructions. All reactions were performed in triplicate with nonamplified cDNA in a final volume of 20 µl. The primers for each gene were designed using the Primer3 web interface (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Sequences, size of amplified product, and

GenBank accession numbers, as well as annealing temperatures, are presented in Table 4. For each candidate gene tested, a standard curve based on 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 µl (Roche Diagnostics). Target transcripts were normalized with tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ), hypoxanthine phosphoribosyltransferase 1 (HPRT1; Table 4) using Geometric averaging normalization (GeNorm, <http://medgen.ugent.be/~jvdesomp/genorm/>). A 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.

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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