Chapter 3

Isolation and identification of differentially expressed genes involved in meiotic resumption of bovine oocytes in vivo

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(Submitted for publication)

Abstract

Meiotic progression is a tightly controlled cascade of interdependent events controlled by sequential expression of a variety of genes. Here we report the construction of a bovine oocyte DNA microarray using Suppression Subtraction Hybridization (SSH). This microarray was used to analyze the transcriptional activity of bovine oocytes during meiotic resumption. A subtractive library consisting of 945 clones was developed from bovine, presumptive competent oocytes matured in vivo, using 30 pooled oocytes collected before LH surge (-2 h) as driver and 30 oocytes exposed to LH (6 h) as a tester. One hundred fifteen transcripts with known homology and a large number of unknown transcripts were found to be up-regulated during meiotic resumption. Thirty-five genes were clustered into six groups according to their function, and included genes related to signal transduction, cell cycle regulation, transcription and mRNA processing, cytoskeleton and cell adhesion, metabolism and genes related to antioxidant and defense mechanisms. The mRNA expression of 10 genes was further studied using quantitative real-time PCR. Among the mRNAs identified were G protein γ12 subunit which plays an important role in signal transduction, regulators of G-protein signaling 10 (RGS10) which act as GTPs-activating proteins by accelerating GTP hydrolysis, cAMP phosphodiesterase 7A1 mRNA (PDE7A1) encoding for an enzyme that has a high affinity and specificity for cAMP hydrolysis, metabotropic glutamate receptor 5 (mGluR5) that has been shown to induce prolonged Ca^{2+} oscillatory activity upon activation and cytochrome c oxidase subunit VIII (COX8) a nuclear gene, playing a vital role in the cellular energy generation. The expression of most of these genes was not previously associated with oocyte maturation. The use of the SSH and microarray approach combined with in vivo matured oocytes have uncovered novel important genes associated with oocyte maturation in the bovine. The roles of these genes during maturation have not been entirely characterized, but the results presented here will support the establishment of new hypothesis regarding the resumption of meiotic arrest in bovine oocytes.

***Key words*:** meiosis, oocyte maturation, SSH, PDE7A, mGluR5

Introduction

During growth, the mammalian oocyte is characterized by extensive decondensation of chromatin and a high level of transcriptional activity [1]. In bovine, this activity gradually decreases as the oocyte reaches a diameter ≥110 μm and comes to be silent through the completion of meiosis, fertilization, until the 8- to 16- cell stage [2, 3]. Clear evidence in several species supports the concept that with timed activation of the fully grown oocyte stores of presynthesized transcripts are needed for meiotic resumption and early embryogenesis [4, 5].

Inside the ovary, mammalian oocytes are arrested at the G2 prophase of the first meiotic cell cycle. G2 arrest is terminated by specific signals, induced through LH binding to LH receptors on granulosa cells, followed by a decrease in oocyte cAMP which is then translated to a decrease in cAMP dependent protein kinase A (PKA) activity leading to complex signaling transduction pathways and release from cell cycle arrest and progression into the meiotic cell cycles [6, 7].

Resumption of meiosis, represents transition from G2 to M phase and involves condensation of the chromosomes, dissolution of the nuclear membrane referred to as 'germinal vesicle breakdown' (GVBD) and formation of the first metaphase spindle (MI), which is completed by the emission of the first polar body and is immediately followed by the second metaphase (MII), reviewed by Dekel [8]. These nuclear changes are paralleled and supported by certain modification occurring in the cytoplasm, involving, for instance, sister chromatid separation [9], protein degradation [10], and adaptive responses to oxidative stress [11], metabolic processes [12], organelle translocation [13], and membrane fusion. Although many of these main steps in oocyte maturation have been described, our understanding of their mechanistic bases has remained rudimentary.

Precise regulation of nuclear and cytoplasmic events during maturation is crucial to produce developmentally competent oocytes of all mammalian species, and requires cooperative functional interactions between different follicular cells components [14]. Using in vitro culture models to inhibit or to accomplish spontaneous maturation, the mechanisms of oocyte meiotic maturation and developmental competence have been extensively examined by adding gonadotropins, steroid hormones, growth factors, and meiotic inhibitors to the medium. Nevertheless, the insights obtained from spontaneous maturation cannot be extended to the ligand-induced meiotic resumption in vivo. This process demands a strict regulation of oocyte and granulosa cells metabolic activity via the

modulatory action of variety of hormones and growth factors [15, 16]. In vitro models lack the interactions between the oocyte and its follicular components, therefore, the molecular regulatory signals by which the oocyte undergoes intrafollicular activation remain largely unknown [17].

The genome-wide transcriptional activity during oocyte maturation has been investigated. Since no complete microarray is available for bovine, commercially spotted human arrays have been used to analyze the pattern of bovine oocytes gene during maturation. The differential expression of a number of genes demonstrated the correlation of a specific maturation stage with the expression of a different genes subset [18]. Moreover, another study using bovine oocyte microarray and oocytes matured in vitro collected from slaughterhouse ovaries identified several mRNAs involved in developmental competence [19]. Although the use of cell culture systems has contributed to further oocyte mRNAs characterization, and provided insight into changes in the gene expression patterns during maturation and developmental competence, the extent of relevance of these results to the processes in vivo remains unclear. Vice versa, identification of genes that are differentially expressed due to in vivo LH surge may lead to a greater understanding of which processes and signaling pathways are pertinent in meiotic maturation leading to successful fertilization and embryo development.

The aim of this study was to identify and characterize genes that are differentially expressed in bovine oocytes in vivo due to LH surge and therefore are likely to be involved in the initiation and regulation of meiosis and play a role in developmental competence. In this study we examined the differential gene expression in oocytes of bovine 6 h after LH peak by SSH. cDNAs from the forward subtracted library were screened using DNA microarray for differential expression and the up-regulation of 10 transcripts was studied by QPCR. A further 25 genes were clustered based on their function into 6 groups. This study demonstrated that the use of in vivo derived oocytes in powerful molecular techniques can be utilized to identify genes with a potential, thus far unknown, role in developmental competence.

Materials and Methods

Experimental design

Follicle development was stimulated in Holstein-Friesian cows using our standard protocol [20] with oFSH and a Crestar/GnRH-controlled LH surge. Cows were allocated at

random to two experimental groups for ovariectomy (OVX): 1) at onset (2 h before LH), and 2) after initiation (6 h after LH) of maturation to determine changes in mRNA expression related to resumption of meiosis in vivo. Presumptive competent oocytes were selected on the basis of the steroid profile in the enclosing follicle (Table 1), and were assigned to replicates for Suppression Subtractive Hybridization (SSH) and validation by QPCR analysis in such a way that within a group replicates were equivalent with regards to steroid profile and represented maximum numbers of cows. The experiment was carried out as approved by the Ethical Committee of the Veterinary Faculty of Utrecht University.

Animals and treatments

Normally cyclic Holstein-Friesian cows (n=30) were selected and treated for superovulation using the protocol as described before [20] with oFSH (Ovagen ICP, Auckland, New Zealand), prostaglandin (PG; Prosolvin; Intervet International B.V., The Netherlands) and a timed LH surge controlled by norgestomet/GnRH (Crestar ear implant/Receptal; Intervet International B.V.). Cyclicity was verified by measuring the concentration of progesterone in peripheral plasma during at least 6 weeks before FSH-stimulation [21] and the LH surge was monitored in plasma from 38 h after PG until OVX using a validated RIA with bovine LH (bLH-7981) for iodination and standards, and rabbit anti-bLH (8101) as antiserum [22]. Oocytes and follicular fluids were collected following OVX at 50 and 58 h after PG corresponding with 2 h before and 6 h after the maximum of the LH surge, respectively.

Collection and selection of in-vivo preovulatory oocytes

For every treatment run with a group of 4 cows OVX was performed at 1 h intervals, the time needed to collect all oocytes and follicles from one cow, by laparotomy through flank incision under local infiltration anesthesia [23]. Ovaries were collected in 0.9% (w/v) NaCl at 37^{o}C and immediately transported to the laboratory. The contents of each follicle >9 mm were aspirated using an 18-ga winged infusion set needle attached to 15 ml polystyrene conical tube under pressure by means of a suction pump, and were then immediately stored on ice at 4^{o}C. The size of the follicles was calculated from the volume of follicular fluid after collection. After retrieval of the cumulus oocyte complexes (COCs) under a stereo microscope, the follicular fluids were centrifuged 3,000 *g* for 10 min at 4^{o}C and stored at -25^{o}C until analysis for steroids. Collected COCs were rinsed twice in 700 µl PBS (Sigma-Aldrich, St. Louis, MO, U.S.A.) supplemented with 5% (w/v) polyvinyl alcohol (PVA; Sigma). Cumulus cells were removed by continuous pipetting after

incubation for one minute with 100 µl 5% (w/v) hyaluronidase (Sigma-Aldrich) and denuded oocytes were checked for remaining cumulus cells and washed three times with PBS-PVA and stored individually at -80°°C until RNA extraction.

For each collection group relative to the LH surge, oocytes were selected on the basis of the concentration of steroids in the fluid of the enclosing follicle according to criteria as described previously [24]. Briefly, oocytes from follicles with estradiol 17β > 0.9 µmol/L before LH and > 0.5 µmol/L 6 h after LH were considered to be competent (Table 1). The few oocytes that had heavy atretic features (expanded cumulus scattered in dark clumps in a jelly-like matrix) were excluded.

Table 1. Numbers of follicles and presumptive competent oocytes recovered during maturation in FSH-stimulated cows with a controlled LH surge.

	notes	2 h before LH (Pre)	6 h after LH (Post)
Follicles > 9 mm			
- # total per cow (n cows)		11.2 (n=13)	15.3 (n=12)
- # Normal/total (%)	[1]	104/145 (72)	124/183 (68)
Oocytes collected			
- # total/# follicles > 9 mm (%)		119/145 (82)	135/183 (74)
- # competent/total collected (%)	[2]	81/119 (68)	88/135 (65)
Oocytes used			
- # for SSH	[3]	30	30
- # for QPCR		47	50
- estradiol 17β ± SEM		1.77 ± 0.11	0.84 ± 0.03
- progesterone ± SEM	[4]	0.14 ± 0.01	0.53 ± 0.01
- follicle diameter ± SEM	[5]	13 ± 1	13 ± 0.2

[1] Normal: functional follicles on the basis of steroid profile
[2] Competent: oocytes from functional follicles
[3] SSH: Suppression Subtractive Hybridization; QPCR: quantitative PCR
[4] Mean steroid concentration in µmol/L fluid of follicles from which oocytes were used
[5] Mean diameter in mm as calculated from volume of fluid of follicles from which oocytes were used

RIA of steroids in follicular fluid

Concentrations of the steroid hormones estradiol 17β and progesterone in follicular fluid were determined in aliquots of 1 to 25 µL fluid dependent of the hormone and the size

of the follicle by solid-phase ^{125}I RIA methods (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA, U.S.A; estradiol 17β: TKE2; progesterone: TKPG) as validated for blood plasma of cows [21] with slight modifications such as extraction with diethyl ether (BDH Laboratory Supplies, Poole, England).

Total RNA extraction and precipitation

Total RNA was isolated using microspin column and DNA was digested with Dnase1 to eliminate possible genomic DNA contamination according to manufacturer's instruction (Absolutely RNA Microprep Kit; Stratagen, San Diego, CA, U.S.A.), the RNA was recovered by two subsequent 50-µl elutions with warm (60°C) elution buffer provided in the kit. RNA was then precipitated with 250 µl of 100% ethanol and 10 µl of 3 M sodium acetate pH 5.2, using 1 µl of 1 mg/ml linear acrylamide (Ambion, Austin, TX, U.S.A.) as co-precipitant. The mixture was chilled at -80^{o}C for 30 min, centrifuged for 20 min at 4^{o}C at 16000g. The pellet was then washed with 75% ethanol and resuspended in 3 µl of nuclease-free water for the RNA used for the SSH experiment and in 15 µl of water for the RNA used in real-time PCR analysis.

Generation of a Subtracted Library by Suppression Subtractive Hybridization (SSH)

The SMART^{TM} PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA, U.S.A.) was used to maximize cDNA yields prior to the subtraction. The PCR-Select cDNA Subtraction Kit (Clontech) was used for SSH as described in the manufacturer's recommendations to isolate and enrich for gene sequences differentially-expressed between the two pools of oocytes: 1) collected before LH surge (-2 h, n=30) as driver and 2) exposed to LH (6 h, n=30) as tester.

The subtracted material was then cloned into the pGEM-T and pGEM-T easy vector system Kit (Promega, Madison, WI, U.S.A.) and transformed into DH5α-T1 max efficiency *Escherichia coli* cells (Invitrogen, Burlington, ON, Canada). Selected white colonies were inoculated individually in LB/ampicillin medium in 96 well-plates at 37^{o}C for 6 h with agitation, and screened for inserts by PCR using 1 µl of bacterial stock culture and SSH adaptor-specific nested PCR primer 1 (5'-TCGAGCGGCCGCCCGGGCAGGT-3') and 2R (5'-AGCGTGGTCGCGGCCGAGGT-3') (BD Biosciences,Mississauga, ON, Canada), and 1 U of HotMaster^{TM} Taq DNA polymerase (Eppendorf, Westbury, NY, U.S.A.). Amplified products were visualized on a 2% agarose gel with ethidiumbromide (EtBr).

Microarray preparation

PCR products originating from the subtracted library were purified using the Unifilter 384-well purification plates (Whatman, Clifton, NJ, U.S.A.), speedvac dried (SPD SpeedVac ThermoSavant, Milford, MA, U.S.A.), and resuspended in 5 µl of 3X standard saline citrate (SSC)/dimethylsulfoxide (DMSO; 1:1). Clones (n=945) were spotted twice on GAPS II glass slides (Corning, Acton, MA, U.S.A.) using a VersArray ChipWriter Pro (Bio-Rad, Mississauga, ON, Canada). Various controls were printed on the slide in two replicates: three cDNA products of the SpotReport Alien cDNA Array Validation System (Stratagen) were used as negative controls, a fragment of the Green Fluorescent Protein (*GFP*) as an exogenous positive control, and various housekeeping genes (*actin*, *tubulin*, *H2A*, *and GAPDH*) as internal positive controls. Slides were cross-linked with ultraviolet (UV) rays according to the manufacturer's instructions and quality of the printing was controlled with Terminal Transferase (TdT) (Roche Diagnostics, Laval, QC, Canada) and and Cy3 dye (GE Healthcare Bio-Sciences, Piscataway, NJ, U.S.A.)

Microarray hybridization

Subtracted PCR products were used as probes to hybridize the glass slide in order to select the positive clones for sequencing. Probes were labeled with Alexa Fluor 555 and 647 reactive dye packs (Molecular Probes, Burlington, ON, Canada) using Amino Allyle dUTP (Ambion) according to the manufacturer's instructions. Two dye swap hybridizations were performed with two different protocols. The first protocol is described in detail by Sirard and collaborators [25] and the second using the Solid*Hyb*^{TM} Array Kit (Viridis Biotech, QC, Canada) follows the manufacturers instructions. Loess normalization transformations and background were calculated as described in the *microarray analysis* section below for the second and the third hybridizations.

Microarray analysis

The hybridized slides were scanned using a VersArray ChipReader System (Bio-Rad), the signals were analyzed and normalized with ArrayPro Analyser software (Media Cybernetics, San Diego, CA, U.S.A.). For each hybridized spot, data ratios were calculated and background intensity was subtracted and normalized ($t = m + 2$ x s.d., where t is the calculated threshold, m is the mean of the negative controls raw data and s.d. is the standard deviation of those same negative control raw data). Data points representing the

differentially expressed genes above 1.3 fold were selected as positives. Those clones were sequenced and identified as described previously [25].

Real-time Polymerase Chain Reaction

Reverse Transcription and primers design

Total RNA was reverse-transcribed in a total volume of 20 µl using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.) containing oligo-dT and random hexamer primers. Reactions were incubated for 5 min at 25°C, 30 min at 42°°C, and 5 min at 85°C. Primers (Table 2) used for the real-time PCR analysis of reverse-transcribed mRNAs were designed with the aid of the Beacon Designer 4.0 software (PREMIER Biosoft International, Palo Alto, CA, U.S.A.). The specificity of the primers was confirmed by sequencing the amplified product and visualization on a standard 2% agarose gel with EtBr. The PCR products were sequenced after purification with the QIaquick PCR Purification Kit (Qiagen, Valencia, CA, U.S.A.) and quantified with a spectrophotometer (Nanodrop ND 1000, Isogen, IJsselstein, The Netherlands). PCR products were then diluted from 100 to 0.01 fg as standards to construct the standard curve.

Real-time Polymerase Chain Reaction

Real-time PCR was performed on a Bio-Rad MyiQ system using the 2X iQ SYBR Green Supermix reagents, following the manufacturer's protocols. Reactions were performed using 25 µl duplicate reactions with the quantity of diluted cDNA corresponding to 0.1 to 0.3 of an oocyte, depending on the mRNA abundance as estimated using oocytes from slaughterhouse ovaries matured in vitro, and 0.5 µM of each primer. Each transcript was amplified from four different replicates. Samples and standard curves were amplified on the same run with the same PCR master mix. The thermal cycling program starts with an initial denaturation step at 95°C for 3 min, and is followed by 45 to 50 PCR cycles (dissociation for 5 sec at 95°C, annealing for 5 sec at temperature showed in Table 2, and elongation for 20 sec at 72°C), one melting cycle consisting of 5 sec at 95°C, 30 sec at 72°C, and a step cycle up to 95°C (0.3°C/sec transition rate), and finally a cooling down cycle at 40°C. Amplification of the GAPDH mRNA [26] was performed for each reverse transcribed sample as an endogenous quantification standard. These raw CT values were then analyzed with a modified delta-Ct method with efficiency correction using a PCR data analysis program, qBase (version 1.3.2) (http://medgen.ugent.be/qbase/) to obtain relative quantification values. PCR product was analyzed on a 1.5% agarose gel with EtBr to

confirm amplification. Product sizes and annealing temperatures for each gene are presented in Table 2.

Table 2. Primers used for real-time PCR experiment

Genes	GeneBank accession number	Oligos sequences	Product size (bp)	Annealing temperature (°C)
G-protein γ12	NM_174785	F 5'-agccagtaccaacaacattg-3' R 5'-tccacttcactacttctctataag-3'	222	55
PDE7A1	NM_002604	F 5'-gcttgccaattctgtaactccttg-3' R 5'-attctggcgactgatgtctgtg-3'	286	53
mGluR5	NM_000842	F 5'-gatggctcctcctcttcc-3' F 5'-gcacggctgatacataggtc-3'	262	58
MC1R	NM_174108	F 5'-catcacctactacaaccacaag-3' R 5'-agaggttgaagttcttgaag-3'	311	55
VCP	NM_007126	F 5'-atccgtgaatccatcgagag-3' R 5'-gactctgctgaagggtctgg-3'	202	57
GSTA1	BTU49179	F 5'-cgggaaagacatgaaggaga-3' R 5'-cttgttgcccaccaggtagt-3'	211	58
COX8	NM_174024	F 5'-gctgactccactgctgttg-3' R 5'-catcactcccgcttcttgtag-3'	204	57
β-cat	NM_174637	F 5'-gccgacaagaagattgaag-3' R 5'-atctggtgaaccctctgg-3'	191	56
PPARBP	BC006517	F 5'-atagaccctggagtgaaacc-3' R 5'-agatgttactttgagagccttc-3'	131	56
ARF4	NM_001660	F 5'-ccctcttctcccgactatttgg-3' R 5'-atggtaggaatggtggtgactatc-3'	127	56
GAPDH	NM_001034034	F 5'-ccacgagaagtataacaacacc-3' R 5'-gccagtagaagcagggatg-3'	229	56

G-protein γ12, *Homo sapiens* G protein gamma 12 subunit; PDE7A1, *Homo sapiens* cAMP phosphodiesterase PDE7A1; mGluR5, *Homo sapiens* metabotropic glutamate receptor 5; MC1R, *Bos taurus* melanocortin 1 receptor; VCP, *Homo sapiens* valosin-containing protein; GSTA1, *Bos taurus* glutathione S-transferase subunit A isoform I; COX8, *Bos taurus* cytochrome c oxidase subunit VIII; *Bos taurus* β-cat, beta catenin like 1; PPARBP, *Homo sapiens* PPAR binding protein; ARF4, *Homo sapiens* ADP-ribosylation factor 4; GAPDH, *Bos taurus* glyceraldehyde-3-phosphate dehydrogenase.

Statistical analysis

Data are presented as the mean ± SEM. Statistical differences between the two groups were determined by means of the unpaired Student's t-test. Differences were considered statistically significant at the 95% confidence level ($P < 0.05$).

Results

Collection and selection of oocytes

Two cows of the thirty FSH-stimulated cows were excluded: in one the concentration of LH was elevated during the norgestomet treatment and another cow showed < 3 preovulatory-sized follicles. The 52 oocytes collected from 96 follicles > 9 mm of a further 3 cows were not used for SSH and QPCR since all follicles showed an estradiol 17β concentration that was 5 times lower than the threshold value for selecting competent oocytes. Of the remaining 25 cows on average 6.2 and 7.3 oocytes were collected per cow at 2 h before and 6 h after the maximum of the LH surge, respectively (Table 1). The proportion of functional follicles varied noticeably between cows. Six of the 28 cows (21.4%) had a population with > 90 to 100% of the follicles being functional, another 6/28 cows (21.4%) had < 20 to 0%, and the remaining 16 cows (57.2%) showed a population with 73% (median; range 42 to 89%) of the follicles being functional.

Suppressive Subtractive Hybridization

We used SSH to isolate genes that are differentially expressed in oocytes that were exposed to LH in vivo. The subtraction efficiency was evaluated by the GAPDH housekeeping genes expression, which was dramatically reduced in the subtracted library. Among the 1092 clones screened, 945 showed single insert-containing clones from the subtracted library enriched for LH up-regulated sequences. The insert size range was between 250 and 800 bp. The cDNA fragments were amplified, and then blotted onto glass slides together with control cDNAs and probed with the subtracted cDNA pools.

Table 3. Genes identified in the SSH library with known identity and function

GeneBank name	Accession No.
Genes related to signal transduction and calcium	
1. Guanine nucleotide binding protein (G-protein) gamma 12	NM_174785
2. Heat shock protein 90 alpha (HSP90 alpha)	AB072368
3. Regulator of G-protein signaling 10 (RGS10)	AF493934
4. metabotropic Glutamate receptor 5 (GRM5)	NM_000842
5. Calcium binding protein P22 (CHP)	NM_007236
6. cAMP phosphodiesterase PDE7 (PDE7A1)	NM_002604
7. Melanocortin 1 receptor (MC1R)	NM_174108
8. Rod outer segment guanylate cyclase precursor (ROS-GC1)	AF027201
Genes related to transcription and mRNA processing	
1. SWI/SNF chromatin remodeling complex subunit OSA1 (OSA1)	AF521670
2. PRP8 pre-mRNA processing factor 8 homolog	NM_006445
3. Homeobox-containing protein PKNOX1	AY196965
4. polymerase (DNA-directed), delta 3, accessory subunit	NM_006591
Genes related to cytoskeleton and cell adhesion	
1. Thymosin β4	AY192438
2. β-Actin	AY141970
3. Catenin alpha like 1	AY523969
4. Catenin Beta like	NM_174637
5. Myosin regulatory light chain (MRLC)	AF513721
6. Putative rab5 GDP/GTP exchange factor homologue (RABEX5)	NM_174591
7. Syntaxin 8	BT007319
8. ADP-ribosylation factor 4 (ARF4)	NM_001660
9. Dishevelled associated activator of morphogenesis 1 (Daam1)	NM_014992
Genes related to metabolism	
1. AMP-activated protein kinase subunit gamma 1 (AMPK)	NM_174586

2. Cytochrome c oxidase subunit VIII (COX8)	NM_174024
3. PPAR binding protein (PPARBP)	BC006517
4. ornithine decarboxylase (ODC)	U36394
Genes related to antioxidant	
1. Peroxiredoxin 1 (PRDX1)	NM_174431
2. Glutathione S-transferase subunit isoform I (bGSTA1)	U49179

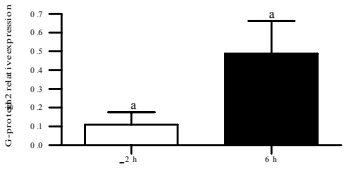
Microarray hybridization

Determination of the background hybridization signal threshold was performed by considering all of the *GFP* negative controls and SpotReport Alien cDNA. All of the poor quality spots were removed from further analysis. Fluorescence signal was calculated using each replicate for each clone. If one of the replicates was lower than the corrected background, then the corresponding clone was completely eliminated from the analysis.

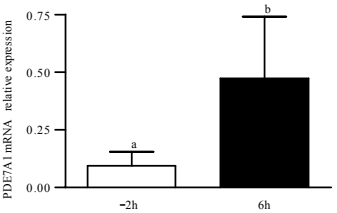
Following the hybridizations, three hundred and thirty transcripts showed more than 1.3 fold increases and were selected for sequencing analysis. The output sequences were analyzed using nucleotide-nucleotide BLAST (blastn) program on the NCBI database in order to find homologies with already known genes. When clones were examined, 115/350 showed identity to known sequences, while 36/350 matched uncharacterized sequences (clone, BAC, RIKEN, or hypothetical protein), 167/330 had no significant match to any known sequence. In the known sequences, 11 were represented twice, 2 represented 3 times, 1 represented 4 times and 1 represented 6 times, and 1 represented 7 times in the sequenced clones. Of the 115 genes whose expression showed similarity to known genes, 79 had homology to human, 27 to bovine and 9 to other species.
Thirty-five genes presented in Table 3 represent a proportion of the genes that have well-known identifiable molecular functions.

Quantitative RT-PCR Analysis of Target Genes

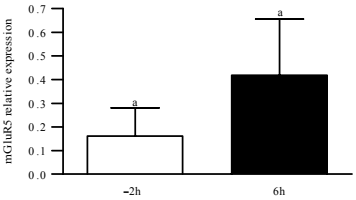
Targets of particular interest found with the arrays were selected based on their reported function in somatic cells, (Table 2) and screened to validate the SSH finding by real-time RT-PCR using RNA extracted from 4 different oocytes pools. The primer sequences used are summarized in Table 2.



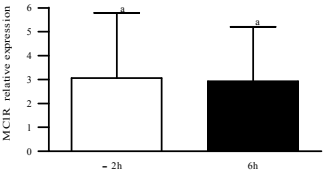
A. G-protein γ 12



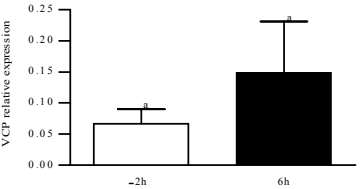
B. cAMP phosphodiesterase PDE7A1



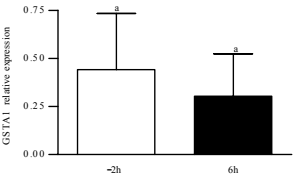
C. metabotropic Glutamate receptor 5



D. Melanocortin 1 receptor



E. Valosin containing protein



F. Glutathione S-transferase subunit A isoform I

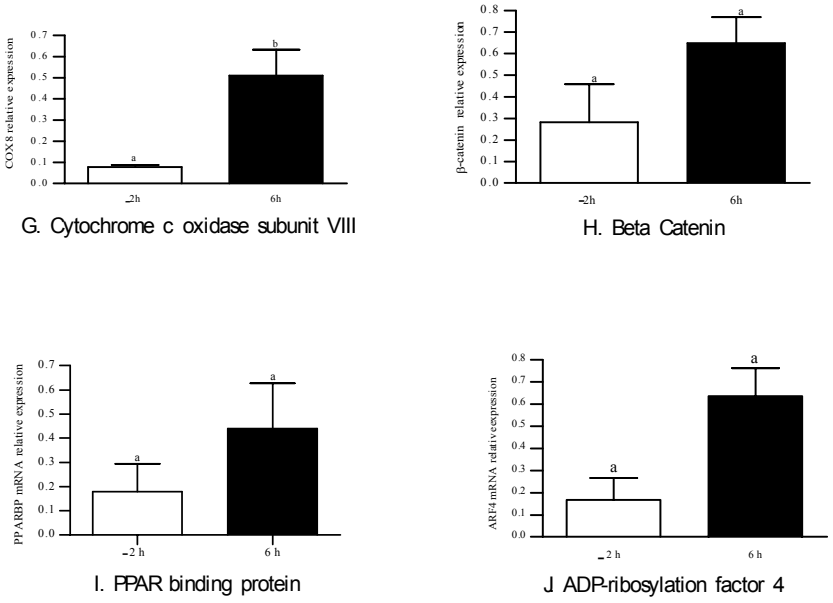


Figure 1. Real-time PCR analysis of mRNA expression of the ten genes (A to J) selected from microarray results; data are shown as mean ±± SEM of the mRNA level of the oocytes collected 2 h pre LH surge and the oocytes collected 6 h following the LH surge. Different letters indicate statistical significance between the two groups as determined by unpaired Student's *t*-test. For all mRNAs, $P < 0.05$ was considered statistically significant.

Real-time PCR experiments revealed the presence of all the genes tested. Two candidates (COX8 and PDE7A1) showed significant differential mRNA levels. We did not detect the statistically significant differences in the amount of other genes by QPCR although 6 of these genes tended ($P < 0.1$) to show increase at 6 h post LH (ARF4, g-protein γ12, PPARBP, ß-catenin, VCP and mGluR5). Figure 1 (A to J) presents the quantification of mRNA levels in oocytes collected 2 h before or 6 h after LH surge.

Discussion

The current study demonstrates the value of using in vivo matured oocytes with SSH and microarray technique to unravel the transcriptional changes occurring during the onset of meiotic resumption within the follicle. The most significant aspects of this study were to examine the effect of in vivo LH surge on gene expression in the bovine oocytes. These data further extend our understanding of the mechanisms of meiotic resumption in the mammalian oocytes. Compared to the first application of microarray analysis with bovine slaughterhouse ovaries oocytes matured in vitro and hybridized to a human cDNA array [18], very few genes are represented in our library, indicating potentially different mechanism regulating meiotic resumption in vitro compared to in vivo. Moreover, comparison of the present results to our recently published results [27] using the same tester cDNA (6 h post LH surge oocytes) and 6 h in vitro matured oocyte as driver led to large proportion of different genes. However, the functional distinctions between the two culture systems do not seem to exist only because of alterations of the culture medium, but we need to consider also the role of cumulus and granulose cells to fully understand the difference between the two systems.

Several of the transcripts identified in our study represent genes well known to participate in oocyte maturation pathways, modulating cAMP level (such as cAMP phosphodiesterase), cell cycle regulation (such as CDC28) and chromatid separation (PTTG1) which provides validation of the SSH approach as an effective high-throughput technology to derive a profile of factors involved in meiotic resumption. Additionally, much of the expression data is consistent, or at least compatible, with current notions of how meiotic resumption might occur, and it is not difficult to construct a tentative hypothesis about how several identified genes might be involved is some aspects of oocyte maturation. However, quantitative real time PCR analysis revealed a significant difference for only tow genes amongst the genes tested. Many of the tested genes show a different level but did not reach the significant level with the number of replicates used in this study. Since these factors are believed to be polyadenylated early during the maturation process [28], the QPCR may detect only a portion of the RNA while the micro array might have shown all possible transcripts since PCR was used to generate the library and the polyadenylation status does not limit the messenger abundance.

Half of the sequenced clones identified have no known matches in the NCBI database (23-3-2004). Bovine genome has now been completely sequenced and it is possible that many of the unidentified sequences will find matches as the database growing. It is also

possible that these orphan genes may be the results of rapid evolution such that the sequence similarity is lost even within relatively short evolutionary time [29].

One of the interesting findings in the current study is the expression of G protein γ12 subunit. During meiotic arrest, the conversion of intracellular ATP to cyclic AMP (cAMP), is catalyzed principally by the precise regulation of adenylyl cyclase activity [30]. Adenylyl cyclases are either activated or inhibited by interactions with Gαα or βγ subunits liberated from heterotrimeric G proteins in response to ligand-activated G protein- coupled receptors like hormones, neurotransmitters and growth factors. Different Gα subunits have different sets of intracellular targets and functions. For example, Gααs is known as adenylate cyclase activator while Gααi as inhibitor. Similarly, there are multiple forms of β and γ subunits, the amino acids sequences of different β subunits are 53-90% identical to one another, for review see [31, 32], whereas different kinds of mammalian γ subunits are present as different isomers, the biological properties of the βγ subunit have been attributed to the γ rather than β subunit. The preferential Gβ/Gγ interaction determines which G αβγ heterotrimers will be available and thus determine the action and direct certain routes of signaling [33]. It has been suggested that meiotic arrest in mouse follicle-enclose oocytes needs functional Gαs. Targeting of Gαs using anti-Gαs inhibitory antibody induced oocyte maturation in follicle-enclosed oocytes, while injection of anti-Gαi inhibitory antibody did not have any effect [34]. However, the finding that γ12 has a function through specific interaction with Gαo and Gαi, and that βγ12 by itself was able to inhibit adenylyl cyclase in rat retinal membranes and stimulate the activity of phospholipase C in the cytosol of HL60 cells [35] may suggest an important role during oocyte meiotic resumption. Moreover, in *C. elegans* and *Drosophila,* G proteins play a direct role in position and orientation of mitotic spindle, but are not required for spindle assembly indicating a direct role in meiosis [36]. Furthermore, to exert their role, G-proteins attach to a family of regulators of G-protein signaling (RGS) which act as GTPs-activating proteins by accelerating GTP hydrolysis because Gα-bound nucleotide has a very slow dissociation rate [37], our results revealed the presence of RGS10 mRNA which has been identified to interact specifically with several activated forms of Gααi [38]. In mitosis, it has been shown that GTP hydrolysis is important for the mitotic function of G proteins. GTP hydrolysis terminates the signal and in RGS mutants, signaling is enhanced and prolonged. Therefore, spindle forces are reduced in RGS-7 mutants of the *C. elegans* embryo. The mitotic role of RGS proteins is conserved in mammals [39], In early embryos of RGS14 knockout mice microtubules had completely disappeared, and in RNAi experiments of RGS14 with HeLa cells severe spindle defects occurred upon complete depletion [39]. Although these findings might help to explain the role of G protein and RGS in mammalian oocytes meiotic resumption, the

mechanism by which these proteins regulate the adenylyl cyclases, cAMP and chromosomal segregation in mammalian oocytes remain unknown. The identification of G protein subunits and effectors that couple GPCR will provide additional clues to the role of G proteins pathways in oocyte maturation.

The cAMP phosphodiesterase (PDE) superfamily is large, showing at least 12 families, These enzymes catalyze the hydrolysis of cAMP and cGMP or both [40]. PDE7 enzymes have a high affinity and specificity for cAMP hydrolysis and are encoded by two genes designated PDE7A and PDE7B [41]. PDE7A occurs as three alternative splice variants, PDE7A1, PDE7A2, and PDE7A3 [42]. The results of the present study demonstrate that bovine oocytes show PDE7 isozymes expression, namely PDE7A1, thereby providing the first characterization of different PDE isozymes in mammalian oocytes. Within the mouse ovarian follicle, PDE3 has been shown to localize mainly in the oocyte while PDE4D localizes to granulosa cells. Inhibitors of PDE3 have been shown to block meiotic resumption of 90 to 100% of rodent oocytes [43, 44], while only 20% of bovine COCs remain indefinitely arrested using the same inhibitor [45, 46]. Interestingly, the expression of PDE7 in the bovine oocyte may explain this variation; the pattern of PDE7 inhibition is quite different from inhibition of PDE3. This enzyme is resistant to all standard PDE inhibitors, including nonselective compounds such as isobutylmethylxanthine (IBMX) [47]. Because no PDE7 selective inhibitors are available yet [48], the role PDE7 may play in cAMP metabolism remains to be determined. Expression of PDE7A in the bovine oocyte may indicate that the mechanism of meiotic maturation differs according to species. Understanding the mechanisms of meiotic arrest and resumption require the characterization the multiple phosphodiesterases activities in various follicular cells compartments which will provide crucial insights into the control of meiotic resumption mechanism, and ultimately, the improvement of the oocyte in vitro culture conditions.

The melanocortin 1 receptor (MC1R) is a G protein coupled receptor (GPCR) expressed preferentially in epidermal melanocytes coupled positively to adenylyl cyclase via the Gs protein and is a key regulator of mammalian pigmentation. MC1R is activated by α-melanocyte stimulating hormone (α-MSH) and its action has been proposed to be mediated by cAMP-dependent activation of the kinases PKA, PKC and MAPKs has been proposed, for review see [49]. However, recent findings showed that specific serine/threonine kinases (GRK2 and GRK6) expressed in melanoma cells of human and mice can recognize and occupy MC1R receptors, transiently inhibiting its responsiveness to its agonist (desensitization) and significantly impairing agonist-dependent cAMP production [50], these results may indicate the existence of other unidentified kinases able

to target MC1R. Moreover, it is possible that MC1R may initiate the meiotic resumption through a distinct receptor(s) on a different signaling route. It has been proposed that the phosphorylated receptors interact with proteins of the arrestin family, which uncouple the complex from the G protein effectors often leading to activation of new signaling events [51]. Collectively, this finding suggests that the process of meiotic resumption via cAMP degradation may be more complex, than originally proposed.

In the present study as in other reports [18, 52], we identified the yeast Cdc2 homolog CDC28 a well known cyclin dependent kinase (CDK) recognized as the central component controlling the timing of events in the *Saccharomyces Cerevisiae* cell cycle [53, 54]. Cdc28 mutants arrest cell cycle progression at the pachytene stage of meiotic G2 [55] and show a defect in mitotic chromosome stability [56], when in complex with cyclins, Cdc28 promote entry into mitosis and can trigger cytoskeletal reorganization [57]. Beside that, it is known that formation of at least some CDC28-cyclin complexes require one or more assembly factors. Interestingly, the molecular chaperone of the heat shock protein 90 (HSP 90), which is required for correct folding of proteins limited to cellular signal transduction, such as kinases and transcription factors [58], has been suggested to participate in CDC28-cyclin complex formation [59, 60]. These data may indicate that CDC28 helps to constitute the active bovine MPF and is essential for progression through meiosis. Co-expression of HSP90 with CDC28 and other genes indicates that it is important for the correct folding of newly synthesized proteins into their final conformation in order to perform their assumed function. Moreover, the expression of pre-mRNA processing factor 8 homolog (*PRP8*) is another interesting finding, fission-yeast pre-mRNA splicing *prp8* and *cdc28* are identical loci. Thus, a mutation in a single gene can cause a specific arrest in progression through the cell cycle and a block in pre-mRNA splicing [61]. This point to a link between RNA metabolism and meiotic progression and, similar action may occur in the oocyte, but further functional experiments will be required to confirm this hypothesis.

We have demonstrated several subunits of anaphase-promoting complex (APC), known to play an essential meiotic role in other organisms by destroying cyclins, in order to produce an alternation between interphase and mitosis [62], like Ubiquitin-specific protease, Polyubiquitin, UBXD2 and valosin containing protein (VCP). VCP is a mammalian homolog of the yeast cell cycle division protein Cdc48 in budding yeast and of p97 in *Xenopus laevis*, acts as a molecular chaperone in many diverse cellular activities, such as membrane fusion, cell cycle regulation, vesicle-mediated transport, stress response, programmed cell death. Almost all these activities are directly or indirectly regulated by the ubiquitin–proteasome system, (for review see ref. [63]). Loss of VCP function has been

shown to inhibit the degradation of many Ub-Pr substrates including cyclin B1 molecules [64]. Moreover, in budding yeast, Cdc48 is essential for cell cycle progression through the degradation of the CDK inhibitor Far1[65]. Our study revealed also Ubx cofactors subunit 2 that has previously been shown to interact and direct the proteolytic activity of Cdc48. In budding yeast, Ubx2 mutant cells have been shown to accumulate CDK inhibitors due to compromised the ubiquitin-proteosome system [66].

Sister chromatid separation which takes place at the metaphase-to-anaphase transition is triggered by the ubiquitin-dependent degradation of an anaphase inhibitor known as securin, which associates with and inhibits an anaphase activator known as separase, a protease that cleaves a subunit of the cohesion complex that mediates the association between the two sister chromatids. The budding yeasts lacking securin (Pds1) are unable to arrest mitotic progression in response to DNA or spindle damage [67]. In *Drosophila*, cells defective in securin function fail to separate sister chromatids [68], and the absence of the human securin, known as pituitary tumor transforming gene (PTTG), results in reduced levels of separase [69]. Protein sequence and immunoprecipitation studies have shown that securin-separase interaction is dependent on Cdc28-mediated phosporylation. Recently, Mourot et al. [27] showed increased accumulation of oocyte PTTG1 mRNA in oocytes from large follicles compared to the small ones, suggesting a prominent role to permit normal and efficient chromosome separations. Taken together, these data demonstrate that despite the cell type differences many of the molecular components involved in chromatids separation are conserved between meiosis and mitosis, which may suggests that some of the mechanisms may also be conserved.

Many types of stimuli function to release Ca^{2+} from an internal stores through generation of Ins (1, 4, 5)P3 (IP3) [70]. The dynamics of IP3 production can be very different depending on the receptor type being activated. Metabotropic glutamate receptor 5 (mGluR5) is a GPCR widely expressed in neurons and is activated by its natural ligand glutamate. Activation of mGluR5 has been shown to induce prolonged Ca^{2+} oscillatory activity when activated by endogenously released glutamate [71], whereas stimulation of other receptors give rapid and short Ca^{2+} transients. Oscillations in cytoplasmic Ca^{2+} levels have been implicated in the control of many different processes in different mammalian cells such as increasing the efficiency and specificity of gene expression [72], exocytosis [73], mitochondrial redox state [74], and moreover, oocyte maturation [75-77]. These receptors stimulate IP3 synthesis and the mobilization of Ca^{2+} from IP3-sensitive intracellular Ca^{2+} stores and also more efficiently through ryanodine-sensitive Ca^{2+} stores [78]. Phospholipase C activation is coupled to the activation of mGluR5 and can induce Ca^{2+} release from intracellular stores through the IP3 pathway [79]. It has also been shown

that phosphorylation and dephosphorylation of mGluR5 by protein kinase C are responsible for Ca^{2+} oscillations [80]. Although Ca^{2+} oscillations have been reported to occur during maturation of bovine oocytes in vitro, [81] and both receptors have been identified in the bovine oocyte [82], the molecular mechanisms responsible for the generation of these oscillations have not been thoroughly investigated. The stimulation of PKC by the agonist PMA can substitute for FSH induced competence in immature bovine oocytes supporting the activation of this pathway in vivo [83]. The critical role of mGluR5 in the generation of Ca^{2+} oscillations using Ca^{2+} influx from extracellular media and from intracellular stores is well documented in several mammalian species, and the same physiological role in mammalian oocyte is possible. Moreover, the presence of multiple messengers for Ca^{2+} mobilization has added further complexity to our understanding of Ca^{2+} signaling, and it is clear that different patterns of Ca^{2+} signals are generated by complex interactions between multiple Ca^{2+} release mechanisms. The identification of calcium binding protein mRNA in the present study indicates that Ca^{2+} can also act indirectly through binding to these proteins.

Our study identified a member of guanylate cyclases involved in photo transduction known as rod outer segment guanylate cyclase (ROS-GC1). Guanylate cyclase regulates cellular physiology by activating protein kinases, directly gating specific ion channels, or altering intracellular cyclic nucleotide concentrations through regulation of phosphodiesterases (PDEs) via the synthesis of cyclic GMP [84]. Synthesis of cyclic GMP in rod outer segments is catalyzed by two isoforms of this enzyme, ROS-GC1 and ROS-GC2 [85]. However, in contrast to the surface receptors guanylate cyclase family, they are not activated by any of the peptide hormones [86]. Alternatively, ROS-GC1 is modulated by the intracellular Ca^{2+} signals via Ca^{2+} binding proteins. Lower Ca^{2+} concentrations stimulated ROS-GC1 and hence guanylate cyclase while the higher Ca^{2+} concentrations inhibited the cyclase [87], consistent with a presumptive role in oocyte maturation.

The translocation of organelles in association with elements of the cytoskeleton is essential for a variety of cellular processes including cortical granules exocytosis and secretion. In the present study we identified several transcripts involved in a late stage of exocytosis. Cortical granules are targeted to designated areas of the plasma membrane via actin cables using myosin motor proteins, in the present study we demonstrated myosin regulatory light chain mRNA. Inhibition of myosin light chain kinase (MYLK2) the enzyme that specifically phosphorylates and inhibits myosin regulatory light chain [88], reduces cortical granules exocytosis in mouse oocytes [89]. The interaction between vesicle and plasma membrane integral proteins (v-SNAREs and t-SNAREs, respectively) (SNARE,

soluble *N*-ethylmaleimide-sensitive fusion attachment protein receptors), lead to the fusion of the bilayers of the secretory vesicles and the plasma membrane [90], which allow the secretion of vesicle contents and the incorporation of membrane proteins at specific plasma membrane domains. We identified Syntaxin 8 which belongs to a subfamily of the t-SNAREs that has been shown specifically required for the fusion of early endosome [91]. Our study also identifies Rab5 a member of rab family of small GTPases function as a master regulator of exocytosis by interacting with vesicle cargo, organelle fusion, organelle transport along microtubules and signal transduction (for review, see [92]). The known functions of most of these genes in other mammalian cells, and their presence in the oocyte strongly suggest roles for these proteins in the control of cortical granules exocytosis, membrane fusion and protein secretion and consequently oocyte developmental competence. However, a better understanding of this overall process will require more detailed characterization of the proteins that mediate this specific exocytosis and fusion events.

The regulation of cell-cell adhesion is a well understood function of beta and alpha catenin, nevertheless, during maturation the contact between oocyte and cumulus cells decreases and it is unlikely that the oocyte may employ the catenin complex to regulate adherent junction between the oocyte and cumulus cells. A potential role for catenin in the Wnt/wingless signaling pathway has been emerged, this pathway plays a key role in control of differentiation and development [93]. The elements of this signaling pathway are associated with cytoskeletal components and activates the cytoskeletal regulator Rho through activation of Dishevelled, and a direct interaction of Dishevelled with dishevelled-associated activator of morphogenesis (Daam1) [94]. The expression of α-catenin, β-catenin and Daam1 in the present study indicates that bovine oocytes may indeed use this complex as one of the functional units to regulate signal transduction.

During maturation, chromosomal DNA is packaged into a highly condensed chromatin structure, which inhibits transcription presumably by preventing the loading of transcription factors onto the target DNA sites. Therefore, the chromatin structure around DNA should be converted into an open configuration before the initiation of transcription processes [95]. Among the various chromatin modifying enzymes, there are two structurally distinct categories, histone-modifying enzymes which covalently acetylate, phosphorylate, ubiquitinate, or methylate histones, and chromatin remodeling complexes that use the energy of ATP hydrolysis to alter nucleosome conformation and/or position [96]. In the present study we identified a member of the ATP-dependent chromatin remodeling complexes (SWI/SNF chromatin remodeling complex subunit OSA1) which are highly

related to their *Drosophila* counterparts and display similar biochemical activities and share several conserved domains (for review, [97]). The SWI/SNF complex is required for the transcription of a subset of yeast genes and also for the function of several heterologous transcription activators in yeast [98, 99]. Different studies have revealed a cross talk between ATP-dependent chromatin remodeling complexes, histone-acetyltransferase (HATs) and the Transcription Machinery in the regulation of gene expression [96, 100]. Direct interactions between the ATP-dependent remodelers and chromatin modifiers could increase their affinity for the chromatin template, which could also affect the activities of each complex. Moreover, alteration of the chromatin template by one complex could make it a better substrate for the other complex. It has been shown that SWI/SNF is preferentially bound on an acetylated template, implying that acetylation stabilizes SWI/SNF association [100, 101].

One of the most significant findings in the present study was the up-regulation of several transcripts playing a role in the cellular energy generation. During maturation, the oocyte undergoes a series of modifications like GVBD, chromosome separations, organelle translocation, receptor phosphorylation and de-phosphorylation. All these modifications are ATP-consuming process. Based on the identified transcripts, it appears that the oocytes evolve adaptive responses to compensate for the energy requirements caused by meiotic resumption, and one of these regulatory systems could be through cytchrome c oxidase (COX), which is a terminal component of the mitochondrial respiratory chain, encoded by mitochondrial and nuclear genes, playing a vital role in the cellular energy generation (mainly heat and adenosine triphosphate, ATP) [102]. However, due to limited coding capacity of mtDNA, nuclear genes make a major contribution to mitochondrial metabolic system [103]. In our study, we have identified nuclear transcript COX8. In yeast, it has been shown that the level of COX8 transcripts expression increases with increasing oxygen concentration and falls off rapidly at lower oxygen concentrations [104]. Therefore, their activity is proportional to oxygen availability. Furthermore, AMP-activated protein kinase (AMPK) a metabolic sensing protein kinase is another interesting transcript identified in our study and also reported in mouse oocytes [105]. In various cell types, AMPK plays a fundamental role in cellular homeostasis by monitoring the availability of energy stores and is activated by the factors that cause energy depletion, AMPK activation shuts down the energy consuming processes like glucose and fatty acid synthesis and facilitates energy producing processes such as fatty acid breakdown and glucose uptake [106]. Most cell types, under normal aerobic conditions, 50 to 70% of the total energy is obtained from fatty acids, while the majority of the rest is obtained from carbohydrates (mainly glucose and lactate). All of the ATP produced from fatty acid oxidation is dependent on the presence of

oxygen. In contrast, ATP production from glucose originates from both glycolysis (which is not dependent on oxygen) and glucose oxidation (which is dependent on the presence of oxygen). It is clear that during oocyte maturation higher energy and oxygen supply are needed and fatty acids oxidation probably constitutes a large portion of energy supply.

In conclusion, by using SSH we have been able to enrich and normalize rare mRNAs using the microarray technique, which detects a greater number of genes, by which we were able to identify a set of genes previously not known to be involved in oocyte maturation. The physiological significance of these genes during maturation has not been entirely characterized.

Acknowledgements

Mrs. D.M. Blankenstein and Mrs. C.H.Y. Oei for their excellent technical assistance with all the endocrine monitoring of cows and follicles; post-graduate students H. Groenendaal, A.C.T.M. van Gastel and Mrs. E. van Reenen for round-the-clock assistance with managing the experiments; J.H.M. Lutz and animal caretakers for their dedication with treating the animals; F.J. van Kooi and assistants for help with surgery; for skilled help with collecting the oocytes Mrs. H.T.M. van Tol, Mrs. E.C. Zeinstra and E.J. Schoevers, and the PhD-students Mrs. A. Kidson and F.J. Rubio-Pomar.

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