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Transcriptome dynamics and molecular cross-talk between bovine oocyte and its companion cumulus cells

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Transcriptome dynamics and molecular cross-talk between bovine oocyte and its companion cumulus cells

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Dedicated to my mother

Transkriptomedynamik und molekulare Kommunikation zwischen Rindereizellen und deren umgebenden Kumuluszellen

Die bidirektionale Kommunikation zwischen der Eizelle und den sie umgebenden Kumuluszellen (CCs) ist ausschlaggebend für die Entwicklung und Funktion beider Zelltypen. Die Ziele dieser Studie waren Transkripte zu identifizieren, die ausschließlich in den Eizellen oder den CCs exprimiert werden, sowie Transkripte, die unterschiedlich während der Maturation der Eizellen mit oder ohne der sie umgebenden Kumuluszellen exprimiert werden und umgekehrt. Zum Schluss sollten funktionelle Änderungen untersucht werden, welche mit Trankripten assoziiert sind, die sich signifikant während der in vitro Maturation (IVM) ändern. Im Experiment 1A wurden die CCs physikalisch von den Eizellen im GV Stadium durch wiederholtes Pipettieren entfernt und die freiliegenden Eizellen (DOs) sowie die CCs eingefroren. Im Versuch 1B wurden die intakten Kumulus-Eizellen-Komplexe (COCs) kultiviert, anschließend die CCs physikalisch entfernt und die freiliegenden MII Eizellen und die CCs eingefroren. Im Experiment 2 wurden die CCs wiederum physikalisch während des GV Stadiums entfernt und sowohl die daraus resultierenden Eizellen ohne CCs (OO-CCs) als auch Pools mit intakten Eizellen (OO+CCs) wurden kultiviert. Danach wurden die CCs von den intakten Eizellen entfernt und alle Eizellen eingefroren. Im Experiment 3 wurde das Ooplasma mikro-chirurgisch im GV Stadium entfernt und die Eizellen-ektomierten Komplexe (CCs-OO) sowie andere intakte Komplexe (CCs+OO) kultiviert. Das Ooplasma wurde im MII Stadium ebenfalls von den CCs+OO entfernt und die MII CCs eingefroren. Im vierten Experiment wurden die CCs sowohl im GV als auch im MII Stadium physikalisch von den Eizellen entfernt und für die nachfolgende totale RNA Isolation eingefroren. In beiden Fällen wurden die Zellen 22h kultiviert und jedes Experiment wurde dreimal wiederholt, indem Pools von biologischen Replikaten (n = 150) verwendet wurden. 15 µg der fragmentierten und Biotin-gelabelten cRNA wurden mit dem Affymetrix GeneChip®Bovine Genome Array hybridisiert und die Daten wurden mittels eines linearen Models für Microarray Daten analysiert. Signifikant veränderte Genontologie (GO), Gennetzwerke und kanonische Pathways wurden mit Hilfe von GO Konsortien beziehungsweise der Ingenuity Pathway Analyse (IPA) untersucht. Im Versuch 1A wurden von 13162 detektierten Proben 1516 ausschließlich in den GV Eizellen und 2727 in den GV CCs exprimiert, wohingegen 8919 Proben

sowohl in den Eizellen als auch den CCs exprimiert wurden. Ähnliche Ergebnisse wurden im Experiment 1B erzielt. Hierbei wurden von 13602 detektierten Proben 1423 ausschließlich in MII Eizellen sowie 3100 in MII CCs exprimiert. 9079 Proben wurden in beiden Zelltypen exprimiert. Beim zweiten Versuch wurden 265 Transkripte unterschiedlich exprimiert, wovon 217 in OO+CCs beziehungsweise 48 in OO-CCs überexprimiert wurden. Im Versuch 3 wurden von den 566 Transkripte 320 in CCs+OO beziehungsweise 246 in CCs-OO überexprimiert. Beim vierten Experiment wurden von 12827 detektierten Proben 4689 ausschließlich in CCs des GV Stadiums sowie 834 in den CCs des MII Stadiums exprimiert, wohingegen 7304 in beiden Stadien exprimiert wurden. Eizell-spezifische Transkripte beinhalten Gene, die bei der Transkription (IRF6, POU5F1, MYF5, MED18), der Translation (EIF2AK1, EIF4ENIF1), den biopolymer-metabolischen Prozessen (MOS, ACVR1, ZNF529, MAP3K3), der DNA Replikation (MCM6, NASP, ORC6L) sowie an der Protein-Aminosäuren Phosphorylierung (MAP4K4, PRKCH, MOS) beteiligt sind. Die CCs-spezifischen Transkripte schließen Gene mit ein, die für die makromolekularen, biosynthetischen Prozesse (APOA1, USPL1, APOE; NANS), den Carbohydratmetabolismus (HYAl, PFKL, PYGL, MPI), die Protein-metabolischen Prozesse (IHH, APOA1, PLOD1) und für die Steroid-biosynthetischen Prozesse (APOA1, CYP11A1, HSD3B1, HSD3B7) verantwortlich sind. Während Transkripte, die in den OO+CCs überexprimiert wurden, am Carbohydratmetabolismus (ACO1 und 2), am molekularen Transport (GAPDH, GFPT1) und am Nukleinsäurenmetabolismus (CBS, NOS2) involviert sind, sind Transkripte, die in CCs+OO überexprimiert wurden, für das zelluläre Wachstum und die zelluläre Proliferation (FOS, GADD45A), den Zellzyklus (HAS2, VEGFA), die zelluläre Entwicklung (AMD1, AURKA, DPP4) und die Genexpression (FOSB, TGFB2) verantwortlich. Die Signalübertragung, der Cholesterol-biosynthetische Prozess, die DNA Replikation, das zelluläre Wachstum und die zelluläre Proliferation, die Aktinfilament Polymerisation und die Zelladhäsion gehören zu den, sich am signifikantesten verändernden, biologischen Funktionen, welche mit den Transkripten in Verbindung stehen, die zwischen dem GV und MII Stadium unterschiedlich exprimiert wurden. Zusammenfassend lässt sich sagen, dass diese Studie eine große Anzahl an Genexpressionsdaten von unterschiedlichen Eizellen und CCs Proben generiert hat, die unser Verständnis für die molekularen Mechanismen, welche dem

Eizellen –Kumuluszellen Dialog im Allgemeinen und Maturation der Eizelle im Speziellen unterliegen, verbessern könnten.

Transcriptome dynamics and molecular cross-talk between bovine oocyte and its companion cumulus cells

The bi-directional communication between the oocyte and its companion cumulus cells (CCs) is crucial for development and functions of both cell types. The objectives of this study were to identify transcripts that are exclusively expressed either in oocyte or CCs and those which are differentially expressed when the oocyte matures with or with out their companion CCs and vice versa and to investigate functional changes associated with transcripts that are significantly changed during CCs in vitro maturation (IVM). In experiment 1A, CCs were physically removed from oocytes at GV stage by repeated in and out pipetting and the resulting denuded oocytes (DOs) and their companion CCs were frozen. In experiment 1B, intact COCs were cultured; CCs were physically removed and the resulting denuded MII oocytes and their CCs were frozen. In experiment 2, CCs were physically removed from oocytes at GV stage and the resulting (OO-CCs) and other pools of intact oocytes (OO+CCs) were cultured and their CCs were physically removed and both oocytes were frozen. In experiment 3, the ooplasm were micro surgically removed at GV stage and the resulting oocytectomized complexes (CCs-OO) and other intact complexes (CCs+OO) were cultured. The ooplasm were removed from CCs+OO and the resulting MII CCs frozen. In experiment 4, CCs were physically removed from their enclosed oocytes both at GV and MII stages and frozen for subsequent total RNA isolation. In both cases, cells were cultured for 22 hrs and each experiment was repeated three times using pools of biological replicates (n=150). 15 µg of fragmented and biotin labelled cRNA was hybridized with Affymetrix GeneChip®Bovine Genome Array and data were analyzed using linear model for microarray. Significantly changed gene ontology (GO) terms, gene networks and canonical pathways were analyzed using GO consortium and Ingenuity pathway analysis (IPA) respectively. In experiment 1A, of 13162 detected probe sets, 1516 and 2727 are exclusively expressed in GV oocytes and CCs respectively, and 8919 are expressed in both. Similarly, in experiment 1B, of 13602 detected probe sets, 1423 and 3100 are exclusively expressed in MII oocytes and CCs respectively, and 9079 are expressed in both. In experiment 2, 265 transcripts are differentially expressed of which 217 and 48 are over expressed in OO+CCs and OO-CCs, respectively. In experiment 3, of 566 differentially expressed transcripts, 320 and 246 are over expressed in CCs+OO

and CCs-OO, respectively. In experiment 4, of 12827 detected probe sets, 4689 and 834 are exclusively expressed in GV and MII CCs respectively, while 7304 are expressed in both. Oocyte specific transcripts include those involved in transcription (IRF6, POU5F1, MYF5, MED18), translation (EIF2AK1, EIF4ENIF1), biopolymer metabolic process (MOS, ACVR1, ZNF529, MAP3K3), DNA replication (MCM6, NASP, ORC6L), protein amino acid phosphorylation (MAP4K2, PRKCH, MOS) and CC specific ones include those involved in macromolecule biosynthetic process (APOA1, USPL1, APOE, NANS), carbohydrate metabolism (HYAL1, PFKL, PYGL, MPI), protein metabolic processes (IHH, APOA1, PLOD1), steroid biosynthetic process (APOA1, CYP11A1, HSD3B1, HSD3B7). While transcripts over expressed in OO+CCs are involved in carbohydrate metabolism (ACO1, 2), molecular transport (GAPDH, GFPT1) and nucleic acid metabolism (CBS, NOS2), those over expressed in CCs+OO are involved in cellular growth and proliferation (FOS, GADD45A), cell cycle (HAS2, VEGFA), cellular development (AMD1, AURKA, DPP4) and gene expression (FOSB, TGFB2). Signal transduction, cholesterol biosynthetic processes, DNA replication, cellular growth and proliferation, actin filament polymerisation and cell adhesion are among the top significantly changed biological functions associated with transcripts that are differentially expressed between GV and MII CCs. In conclusion, this study generated large scale gene expression data from different oocyte and CCs samples that would enhance our understanding of the molecular mechanisms underlying oocyte-CCs dialogue in general and oocyte maturation in particular.

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List of abbreviations

A:	Adenine
A260:	UV light absorbance at 260 nm wavelength
aRNA:	Amplified RNA
ATP:	Adenosine triphosphate
Ann. Tem:	Annealing temperature
BCB^{+}:	Brilliant cresyl blue positive oocytes
BCB^{-}:	Brilliant cresyl blue negative oocytes
BP:	Base pairs
BSA:	Bovine serum albumin
C:	Cytosine
cDNA:	Complementary DNA
Cl:	Chlorine
COC:	Cumulus oocyte complex
cRNA:	complementary RNA
DEPC:	Diethylpyrocarbonate
DF:	Dominant follicle
DNA:	Deoxynucleic acid
dNTP:	Deoxyribonucleoside triphosphate
dt:	Dinucleotide
DTT:	Dithiothreitol
EB:	Elution buffer
E. coli:	Escherichia coli
EDTA:	Ethylenediaminetetra acetic acid
FITC:	Fluorescein isothiocyanate
G:	Guanine
GC:	Granulosa cell
GSH:	Glutathione
GV:	Germinal vesicle
GVBD:	Germinal vesicle breakdown
hr:	Hour
IGF:	Insulin like growth factor

IPTG:	Isopropyl ß-D-thiogalactopyranoside
IVC:	In vitro culture
IVF:	In vitro fertilization
IVM:	In vitro maturation
IVP:	In vitro production
MII:	Metaphase II
µg:	Microgram
µl:	Micro litre
µM:	Micro molar
ml:	Millilitre
min:	Minute
mRNA:	Messenger RNA
NACl:	Sodium chloride
NAD:	Nicotinamide adenine dinucleotide
NADPH:	Nicotinamide adenine dinucleotide phosphate
NaOAc:	Sodium oxaloacetate
n:	Number
ng:	Nanogram
PBS:	Phosphate buffered saline
PCR:	Polymerase chain reaction
PE:	Protein extraction and lysis buffer
qRT-PCR:	quantitative reverse transcriptase-polymerase chain reaction
sec:	Second
RNA:	Ribonucleic acid
rpm:	Revolution per minute
rRNA:	Ribosomal RNA
TCM:	Tissue culture medium
TGFB:	Transforming growth factor beta
tRNA:	Transfer RNA
UV:	Ultra-violet light
v/v:	Volume per volume
w/v:	Weight per volume
X-gal:	5-Bromo-4-chloro-3-indolyl-beta-D-galactoside

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1 Introduction

The bi-directional communication between the oocyte and its companion cumulus cells (CCs) is crucial for development and functions of both cell types (Eppig 2001, Gilchrist et al. 2004, Matzuk et al. 2002). This dialogue is vital for the oocyte to acquire meiotic and developmental competence and for proliferation and differentiation of CCs (Brower and Schultz 1982, Calder et al. 2001, Calder et al. 2005, De La Fuente and Eppig 2001, Eppig 1991, Eppig 2001, Eppig et al. 2002, Matzuk et al. 2002).

The oocyte regulates proliferation (Gilchrist et al. 2001, Gilchrist et al. 2003, Gilchrist et al. 2004, Gilchrist et al. 2006, Li et al. 2000), apoptosis (Hussein et al. 2005), luteinization (Eppig et al. 1997, Li et al. 2000), metabolism (Eppig et al. 2005) and expansion (Buccione et al. 1990, Vanderhyden et al. 1990) of CCs through oocyte secreted factors (OSFs) such as growth and differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP15) and possibly others. Competent oocytes also influence the expression of cumulus specific biochemical markers that might be crucial for cumulus expansion and achievement of successful development and maturation (Buccione et al. 1990, Lucidi et al. 2003, Vanderhyden et al. 1990).

CCs play an important role in the utilization of energy substrates by oocyte (Sutton-McDowall et al. 2004), prevent the oocyte from oxidative stress induced apoptosis (Fatehi et al. 2005, Tatemoto et al. 2000) via stimulating glutathione synthesis (de Matos et al. 1997, de Matos et al. 2002) during in vitro maturation (IVM). The ability of the oocyte to form male pronuclei after fertilization strongly depends on the presence of CCs during maturation (Fukui 1990, Moor et al. 1990, Vanderhyden and Armstrong 1989) and fertilization (Suzuki et al. 2000, Tajik et al. 1993, Zhang et al. 1995). Fertilization and development to a healthy blastocysts is limited by the oocyte quality (Eppig et al. 1993) and CCs play a critical role in determining oocyte developmental potential both before and after ovulation (Tanghe et al. 2002).

The interaction between CC derived factors such as kit ligand and oocyte secreted, GDF9 is essential for oocyte growth (Joyce et al. 2000, Otsuka and Shimasaki 2002). Communication between the oocyte and CCs is accomplished mainly through the gap

junction type of intercellular communication (Herlands and Schultz 1984) and the presence of this junction supports oocyte competence in vitro (Hashimoto et al. 1998). For instance, removal of CCs before IVM or blockage of the gap junction inhibits oocyte maturation (Vozzi et al. 2001). Similarly, inhibition of this functional coupling using gap junction inhibitors significantly reduces developmental competence (Atef et al. 2005). As oocytes that fail to expand their CCs can't ovulate and/or are infertile (Hizaki et al. 1999), developmentally competent oocytes are selected based on the number and compactness of the surrounding CCs layers (Gregory 1998, McKenzie et al. 2004).

There is notable species level difference regarding the source and identity of cumulus expansion enabling factor (CEEF). Although OSFs (GDF9 and BMP15) are believed to be the causes of CEEF in rat (Vanderhyden 1993), their presence is not mandatory for bovine (Ralph et al. 1995) and porcine (Prochazka et al. 1991) CCs expansion in vitro as oocytectomised complexes (CCs-OO) expand as equally as the intact ones. Some CCs derived genes, hyaluronan synthase 2 (HAS2), inhibin beta A (INHßA), epidermal growth factor receptor (EGFR), geremlin (GREM1), beta cellulin (BTC), cell cycle division 44 (CD44), tumor necrosis alpha induced protein 6 (TNFAIP6) and prostaglandin synthase 2 (PTGS2) have been associated with better developmental competence and suggested as predictors of embryo quality in women (McKenzie et al. 2004) and cow (Assidi et al. 2008). On the other hand, higher expressions of cysteine proteinases, cathepsin B, S, K and Z have been associated with poor developmental competence in bovine oocytes (Bettegowda et al. 2008).

Although studies were conducted to identify molecular biomarkers for developmentally competent bovine oocytes, large scale expression data on oocyte or CC specific transcripts is still lacking. Removal of oocyte-CCs communication axis before IVM reduces CCs expansion and thereby affects oocyte developmental competence but the effect of removing this communication axis on their corresponding gene expression is poorly understood. Furthermore, functional changes associated with the transition of CCs from germinal vesicle (GV) to metaphase II (MII) stage are not identified. Identification of transcripts that are exclusively and commonly expressed between the oocyte and CCs and those that are affected when the oocyte and its companion CCs

mature in the presence or absence of the other and investigation of functional changes associated with differentially expressed CCs transcripts during cumulus oocyte complex (COC) maturation at a larger scale would enhance our understanding of the molecular mechanisms underlying oocyte-CCs dialogue and oocyte maturation. Therefore, this study was conducted to identify transcripts that are exclusively expressed either in oocyte or CCs, enumerate those which are significantly affected when the two cell types mature with or with out the other and identify significantly changed CCs related biological processes during the transition of COCs from GV to MII stage.

2. Literature review

2.1 Molecular mechanisms of ovulation

Successful ovulation is a complex process whereby ovarian follicles reactivate meiosis, create a rupture pore in the apical follicular wall and initiate tissue restructuring and differentiation to form the corpus luteum. These processes are fundamental to successful establishment of pregnancy and importantly influence the developmental potential of the resulting embryos. A cascade of events drive ovulation, initiated upon receipt by the follicle of a single trigger, the surge of gonadotrophins: luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary. In response, detailed changes in gene expression and follicular structure occur with overlapping control and interdependent consequences in the theca, granulosa, cumulus and oocyte compartments of the ovarian follicle. Systemic and local inputs co-ordinate with signals from the oocyte; thus ovulation is under multipartite control facilitating synchronization of oocyte maturation and permitting the selection of oocytes with full developmental competence for succession to the reproductive pool.

2.1.1 Mural granulosa cells as targets of ovulatory signals

Preovulatory follicles contain two distinct sub lineages of granulosa cells (GCs) that arise during folliculogenesis as the cell population segregate upon formation of a fluid-filled cavity called antrum. Cells lining the wall of a follicle and reside very close to the basement membrane are the mural granulosa cells (MGCs) and those found directly adjacent to the oocyte are the CCs. These two cell populations exhibit highly divergent responses during ovulation. The direct response to the preovulatory LH surge is predominant in MGCs than in CCs due to higher number of LH receptors (LHRs) in MGCs. Binding of human chorionic gonadotrophin (hCG) within intact follicles is consistently reported to be at least 9 fold higher in MGCs than in CCs in rat (Bortolussi et al. 1979, Lawrence et al. 1980), pig (Channing et al. 1981), hamster (Oxberry and Greenwald 1982) and mouse (Wang and Greenwald 1993a). The earliest studies that utilized the binding of radio-labelled hCG to detect sites of action within the ovary have found that CCs bound little or no hCG in contrast to MGCs within the same follicles

(Channing et al. 1981, Oxberry and Greenwald 1982, Wang and Greenwald 1993b). Isolated COCs were similarly reported to have a 10 fold less hCG-binding capacity than isolated MGCs (Channing et al. 1981, Lawrence et al. 1980). Subsequent in-situ hybridization studies in rat preovulatory follicle have found higher LHR mRNA expression in MGCs than in CCs and oocytes (Peng et al. 1991). In vivo observations of follicular LHR mRNA expression have also confirmed low or undetectable levels in freshly isolated murine, bovine and equine CCs but higher levels in MGCs (Goudet et al. 1999, Robert et al. 2003).

Numerous studies indicate that pre-ovulatory CCs respond poorly, if at all, to direct LH exposure. Highly purified LH alone could not cause cumulus expansion in isolated mouse COCs as compared to highly purified FSH (Eppig 1979). Furthermore, although cumulus expansion in vivo is induced by pure hCG treatment, this effect required the presence of other follicular compartments indicating the indirect response in cumulus (Eppig 1980). COCs mucification is an FSH-specific and that FSH is 10 fold more potent than LH in stimulating progesterone (P) secretion by CCs in rats (Hillensjo et al. 1981). Subsequent experiments in mouse showed that FSH and LH can sequentially stimulate cumulus matrix synthesis, with FSH required prior to LH and that FSH or FSH plus oestradiol induce LH-binding activity on CCs, peaking after 8 hr when cumulus expansion is well advanced (Chen et al. 1994). Indeed most studies have demonstrated that the action of LH on COCs or IVM of oocytes requires prior treatment or co-culture in the presence of FSH (Foong et al. 2006, Shimada et al. 2003). Current evidences indicate that the ovulatory LH signal is received and responded to in the mural granulosa and theca cell layers and these may transmit a secondary signal to CCs inducing several changes including the up regulation of LHR expression.

2.1.2 LH-induced intracellular signals

Preovulatory LH surge triggers the activity of multiple intracellular signalling pathways in GCs that culminate in altered transcriptional complexes mediating the expression of ovulatory genes. The LHR, known as a classical Gas G-protein-coupled receptor (GPCR) activates adenylate cyclase (AC). AC in turn converts ATP in to cAMP resulting in a large quantity of intracellular cAMP that activates the cAMP dependent

protein kinase A (PKA), a reaction which has been intensively studied (Marsh 1976, Richards 1994). Downstream of PKA, the cAMP regulatory element-binding protein (CREBP) is phosphorylated at serine 133 (Russell et al. 2003a, Salvador et al. 2002) and then recruits the CBP/p300 transcriptional co activator (Arias et al. 1994). Phosphodiesterases are required to maintain tonic cAMP levels in responsive cells and PDE4D, in particular, regulates cAMP levels in GCs (Tsafriri et al. 1996). The LH surge is thought to stimulate additional signalling pathways, as LH treatment increased inositol triphosphate production in rat GCs (Davis et al. 1986). It is agreed that the action of LH on GCs stimulates the extracellular signal regulated mitogen-activated protein kinase (MAPK) pathway (Choi et al. 2005, Maizels et al. 2001, Salvador et al. 2002). The activation of MAPK is very rapid (Sela-Abramovich et al. 2005) and depends on PKA (Russell et al. 2003a, Salvador et al. 2002). It is not clear whether MAPKs are activated via direct phosphorylation by PKA or through some intermediate step such as inactivation of extracellular signal regulated kinases (ERK) phosphatases (Cottom et al. 2003). Reports to date have primarily focused on their role in progesterone receptor (PR) synthesis, however, the downstream function of ERKs in GCs, particularly in relation to ovulation is poorly understood (Dewi et al. 2002, Tajima et al. 2003). The transcription factor RHOX5 has been identified as a transcriptional target of LH via ERKs in ovulating follicles (Maclean et al. 2005). The effecters of ERK mediated transcriptional regulation of GC gene expression during ovulation remain to be determined. Follicular cyclic guanosine monophosphate (cGMP) levels increase during the course of ovulation and at least one cGMP effecter, the cGMP dependent kinase cGKII, is also up regulated at the time of ovulation by cAMP and ERK1/2 via PR and epidermal growth factor (EGF) mediated pathways (Sriraman et al. 2006).

2.1.3 Preovulatory regulation of gene transcription

Signalling pathways induced by LH rapidly modify the transcriptional machinery of MGCs to reprogram cellular function towards ovulation concurrent with luteinisation. Extensive reprogramming of gene expression after the LH surge is achieved through modulation of several transcriptional regulators, which in turn mediate the transcription of effecter gene. A cohort of preovulatory genes with key roles in ovulation exhibits a

common pattern of transcriptional induction involving binding of Sp1/Sp3 transcription factors to G-C box promoter elements. Sp1 and Sp3 are constitutively present in GCs but exhibit sequence-specific, LH-regulated binding to gene promoters (Russell et al. 2003a). The signal transduction mechanism that modifies Sp1 binding activity in GCs is uncertain, but in most tissues involves post translational phosphorylation or glycosylation (Li et al. 2004). The activity of Sp1/Sp3 complexes induces expression of additional transcription factors that further contribute to a cascade of ovulatory gene expression. Induction of PR in mouse and rat MGCs occurs in response to the LH surge through a cAMP/PKA dependent mechanism (Sriraman et al. 2003, Sriraman and Richards 2004). The cAMP-mediated induction of PR requires Sp1/Sp3 binding to multiple GC box elements in the PR promoter ((Sriraman et al. 2003) and consequent recruitment of additional transcription factors. Two isoforms of PR are produced from distinct promoters on the same gene (Natraj and Richards 1993). Human ovaries express PRA and B isoforms, with PRA most consistently dominant, however, regulation of each isoforms in ovulating follicles is not yet determined (Stouffer 2003).

Early growth response 1 (EGR1) is a transcription factor induced through cAMP/PKA and ERK signalling and its induction requires the binding of Sp1/Sp3 and phosphorylated CREB to the proximal promoter. This may lead to recruitment of a combinatorial transcription initiator complex involving CBP and other cofactors (Russell et al. 2003a). These LH-induced transcription factors in turn activate the transcription of several ovulatory effecter genes. For instance, the protease cathepsin L is induced through Sp1/Sp3, PR, EGR1 and CREB-mediated trans-activation (Sriraman and Richards 2004), and another ovulatory protease, ADAMTS1, is induced through Sp1/Sp3 and PR (Doyle et al. 2004).

The cascade of diverse preovulatory gene expression in MGCs involves the induction and recruitment of a cohort of transcription factors that subsequently induce effecter gene products, including ADAMTS1 and cathepsin L proteases, EGF-like ligands (EGF-L) and others. These ovulatory genes exhibit a marked pattern of mRNA and protein expression: a rapid and transient increase after the LH surge followed by down regulation.

Recurring modifications of the transcriptional machinery interacting with these gene promoters mediate this characteristic expression. The common denominator is Sp1/Sp3 transcription factor binding, suggesting that these are universal mediators of preovulatory gene expression.

The phosphodiesterase PDE4D, an important regulator of LH-mediated cAMP accumulation in GCs (Tsafriri et al. 1996), is required for normal ovulation. PDE4D null mice exhibit dramatically reduced ovulation rates and litter size due to altered responsiveness of GCs to the LH surge and reduced expression of ovulatory genes, including PR, cathepsin L and COX-2 (Jin et al. 1999, Park et al. 2003). Many follicles fail to reach preovulatory stage due to premature luteinisation. However, in-situ hybridization study has shown that morphologically normal pre-ovulatory follicles, although reduced in number, still express ovulatory genes such as the EGF-L (Park et al. 2004). Thus, in addition to rendering the MGCs to respond to ovulatory LH, the lack of PDE4D primarily permits accumulation of cAMP such that follicles exhibit premature luteinisation and oocytes are entrapped in prematurely luteinized follicles.

EGF-L (amphiregulin, epiregulin and betacellulin) are very rapidly induced specifically in MGCs within 1-3 hr after the LH surge and act as major secondary signals that transmit the ovulatory signal to the cumulus complex (Park et al. 2004). Each of the EGF-L factors are synthesized as integral membrane proteins and cleavage of the precursor forms is required for their interaction with cumulus-expressed receptors since protease inhibitors can block transmission of the LH signal to CCs, whereas co-treatment with epiregulin reverses this block (Ashkenazi et al. 2005).

Several processing enzymes play key regulatory roles in intra-follicular co-ordination of the ovulatory processes. These include the extracellular protease ADAMTS1, which is induced under the control of LH and P in MGCs of rat (Espey et al. 2000) and mouse (Robker et al. 2000) and in the ovaries of bovine (Madan et al. 2003), equine (Boerboom et al. 2003) and human ovaries (Freimann et al. 2005) and may be associated with symptoms of polycystic ovarian syndrome in human (Freimann et al. 2005). The dormant pro-form of ADAMTS1 is synthesized by the MGCs, but the secreted, mature form is selectively localized to the extra cellular matrix (ECM) and cell

surfaces of the expanded cumulus complex (Russell et al. 2003b). A dramatic reduction in ovulation rates and litter size has been reported in two lines of mice with null mutation of ADAMTS1 (Mittaz et al. 2004, Shindo et al. 2000) suggesting its importance in mediating the ovulatory effects of LH and PR across species. In summary, many of MGC gene products that are required for successful and optimal ovulation encode proteins that sense and respond to systemic inputs, including the LH surge. A cohort of essential ovulatory mediators are transcription factors that are up and down regulated by the LH surge and help to translate the ovulatory trigger into global reprogramming of gene transcription and ultimately cell function.

2.1.4 The role of cumulus granulosa cells in conducting ovulatory signals

The preovulatory oocyte is surrounded by several cell layers, known as the CCs, which are tightly connected to each other and the oocyte through intercellular membrane processes and gap junctions that facilitate exchange of glucose metabolites, small signalling molecules and ions. Oocytes depend on the CCs for glucose metabolism and pyruvate supply for energy production (Gardner et al. 1996, Preis et al. 2005). The CCs exhibit hormone responsiveness and gene expression profiles distinct from those of the MGCs layers. In preovulatory follicles, cumulus and mural cells present abundant cell surface FSH receptors (FSHR), but the cumulus mass and MGCs very close to the antrum show the greatest proliferative response to FSH during follicle growth (Robker and Richards 1998). CCs are most directly exposed to mitogenic factors secreted by the oocyte (Erickson and Shimasaki 2000). OSFs belonging to the TFGβ super family establish a morphogenic gradient that promotes CC characteristics including enhanced cell proliferation and repression of LHR and transcription and biosynthesis of PR (Eppig et al. 1998, Gilchrist et al. 2004). The rate of proliferation of these cells has been suggested to correlate with implantation potential in human assisted reproduction (Gregory 1998). Following the ovulatory LH surge, CCs respond with a unique pattern of gene induction leading to production and stabilization of a local ECM that envelope the COCs.

2.2 Factors influencing the development competence of an oocyte

Developmental competence is the final measurement of the quality of the oocyte. The oocyte is said to be developmentally competent if it is able to resume meiosis, cleave following fertilization, develop to the blastocysts stage, induce pregnancy and result in a healthy offspring (Sirard et al. 2006). Several criteria including the morphology, cytoplamic structures and molecular attributes of the oocyte have been proposed to evaluate the developmental competence of the oocyte (Wang and Sun 2007). For instance, oocytes are evaluated based on the transparency and darkness of their cytoplasm, the number of CCs layers, extrusion of polar body and spindle formation (Wang and Sun 2007). However, this morphology based type of oocyte selection is inconsistent and sometimes controversial (Wang and Sun 2007), but could be used as a pre-screening parameters for application of other methods. Additionally, the expression levels of some CC (Assidi et al. 2008, Bettegowda et al. 2008) and oocyte ((Patel et al. 2007) transcripts have been considered as molecular markers for selection of quality oocyte. Competent oocytes are also selected based on early cleavage rates of the resulting embryos as early cleaving embryos are more likely to reach blastocysts stage than those cleaving late (Dinnyes et al. 1999, Lonergan et al. 1999). The developmental potential of the oocyte is determined by several factors and all these factors must be considered simultaneously to accurately predict it.

2.2.1 The quality of oocyte

Oocyte quality affects early embryonic survival, establishment and maintenance of pregnancy, foetal development and even adult health. Developmental competence is acquired during folliculogenesis as the oocyte grows and during the period of oocyte maturation. Assisted reproductive technologies involving ovarian hyper stimulation or collection of immature oocytes for IVM affect this process and result in oocytes with reduced developmental competence. Although nuclear or meiotic maturation may be completed successfully, other processes occurring within the ooplasm are required for complete developmental competence following fertilization.

2.2.1.1 Cytoplasmic maturation

In general terms, oocyte cytoplasmic maturation involves the accumulation of mRNA, proteins, substrates and nutrients that are required to achieve oocyte developmental competence that fosters embryonic developmental competence (Krisher 2004, Sirard et al. 2006). The oocyte that has not completed cytoplasmic maturation is of poor quality and can't successfully complete normal developmental processes (Otoi et al. 1997). In human, poor oocyte quality is the cause of infertility in a significant number of couples unable to conceive. The woman's age is the most important factor affecting the chance of live birth (Edwards et al. 1984) and risk of miscarriage (Romeu et al. 1987) when her own oocytes are used. Oocytes from older women (Edwards et al. 1984) and cows (Malhi et al. 2007) have been reported to be less competent. As the oocyte grows and matures, it acquires the ability to resume and complete meiosis, successfully undergo the fertilization process and initiate and sustain embryonic development (First et al. 1988). In the course of acquiring these competencies, cytoplasmic changes including cellular processes such as mRNA transcription (First et al. 1988, Kastrop et al. 1991), protein translation (Sirard and Blondin 1996), post-translational modification of proteins (Levesque and Sirard 1994) and ultra structural changes (Kruip and Dieleman 1982) may occur. Proteins that play important roles during the growth of oocyte within the follicle include maturation promoting factor (MPF) and mos. These proteins are involved in meiotic progression and cell cycle control (Wickramasinghe and Albertini 1993). Other transcripts and their respective protein products may be involved in cellular processes critical for successful development before and after activation of the zygotic genome (Barron et al. 1989, Watson et al. 1999).

2.2.1.2 Morphology of the ooplasm and the number and compactness of the companion CCs

Selection of developmentally competent bovine oocytes based on visual assessment of morphological features was first attempted by (Leibfried and First 1979). Since then, many reports have proposed classification schemes based on the compactness and the number of the companion CCs layers (Hazeleger et al. 1995, Laurincik et al. 1992) and on the appearance of the oocyte itself (de Loos et al. 1989). Oocytes with better

developmental competence have been reported to have smooth or evenly granulated cytoplasms surrounded by less than three compact layers of CCs (Smith et al. 1996). A positive correlation between the morphology of COC and blastocysts yield has been reported in in vitro study (Hazeleger et al. 1995). The number and compactness of the companion CCs have been proposed as the better methods to select developmentally competent immature oocytes as oocytes surrounded by more compact and several layers of CCs develop to the blastocysts stage at a significantly higher rates than those from less compact and fewer layers of CCs, respectively (Madison et al. 1992). Paradoxically, morphological characteristics have been reported as incompetent to predict the developmental competence of the oocyte (Vassena et al. 2003).

2.2.1.3 Origin of the oocyte

It has been indicated that besides the process of fertilization, growth and maturation, the origin of the oocyte is the most critical factor influencing the outcome of embryo production (Merton et al. 2003). Under physiological condition following fertilization, approximately 80% of the ovulated oocytes reach the embryo stage (Merton et al. 2003). Since the ovulation window does not coincide with super ovulation (Callesen et al. 1986, Dieleman and Bevers 1987), some oocytes collected from super stimulated cows may not complete development at the time of collection (Takagi et al. 2001) and hence embryo production rates are relatively low when super ovulation-derived oocytes are matured in vitro (Hendriksen et al. 2004, Rizos et al. 2002). Although their developmental competences are significantly different, there are no noticeable differences between oocytes matured in vivo and in vitro regarding nuclear maturation, fertilization and early cleavage rates provided that they are obtained from the same size follicles (Rizos et al. 2002, Sirard and Blondin 1996). Oocytes matured in vivo attain relatively higher blastocysts rates than those matured in vitro (Humblot et al. 2005, Rizos et al. 2002). Oocytes matured in vitro can't not acquire complete developmental competence due to incomplete oocyte capacitating and synthesis of RNAs, proteins and other molecules (Hyttel et al. 1997). For example, under in vivo condition, bovine oocytes resume meiosis from dominant follicles (DF) that has a diameter of about 15 mm (Pavlok et al. 1992) but oocytes for IVM are usually retrieved from follicles with a diameter of 2 to 6 mm (Sirard et al. 1992).

2.2.1.4 Oocyte and follicular size

Developmental competence is also affected by the sizes of the follicles under the same culture condition (Marchal et al. 2002). During the growth phase of follicular development, arrested oocytes accumulate large amount of mRNAs and proteins that function to support and regulate preimplantation embryonic development until the onset of major embryonic genome activation (De Sousa et al. 1998). In vivo studies in cattle have shown that oocytes from small follicles (< 8 mm) don't resume meiosis due to few number of LH receptors on the GCs (Bevers et al. 1997, Zu et al. 1995). Additionally, oocytes from small follicles appear to have not yet completed cytoplasmic maturation due to deficient mRNA or protein accumulation (Lonergan et al. 1994, Pavlok et al. 1993).

In cattle, the oocyte and follicle continue to grow in parallel until the follicle reaches a diameter of 3 mm. The oocyte can attain a diameter of about 120-130 µm, while the follicle can grow up to 15-20 mm in diameter before ovulation (Fair 2003). It has been suggested that bovine oocytes acquire full meiotic competence at a diameter of 115 µm and full developmental competence to blastocysts when they reach a diameter of 120 µm (Otoi et al. 1997). The size of the follicle from which the oocytes are obtained reflects the developmental stage of the follicle and the maturation stages of the oocyte within that follicle (Blondin and Sirard 1995, Lequarre et al. 2005). It has been highlighted that oocyte population acquire developmental competence progressively during folliculogenesis (Eppig and O'Brien 1996). For instance, oocytes from preantral follicles are unable to resume meiosis after prophase I arrest, whereas those from very small antral follicles are competent to progress to metaphase I (MI) and those from larger antral follicles to metaphase II and beyond (Fair et al. 1995, Otoi et al. 1997). No differences have been observed in the developmental competences of oocytes derived from follicles with a diameter of 2 to 4 mm or 4 to 8 mm (Pavlok et al. 1992). However, oocytes from 1 to 2 mm follicles have shown a significantly lower competence to undergo IVM, fertilization and completely lack the capability to cleave beyond the 8-cells stage (Pavlok et al. 1992). Additionally, blastocysts yield of oocytes originating from follicles with a diameter of more than 6 mm has been reported to be twice that of oocytes from 2 to 6 mm (Lonergan et al. 1994).

2.2.2 Stage of follicular wave

It is generally agreed that the stage of follicular wave affects the developmental competence of bovine oocytes after fertilization due to the differences in the size of the follicles from which the oocytes are obtained (de Wit et al. 2000, Hendriksen et al. 2004). During the oestrus cycle, follicle population change under the influence of follicular waves and the interactions between the follicles throughout each follicular wave affect oocyte quality. The pioneer studies by Hagemann et al. (1998, 1999) have shown that oocytes from follicles with a diameter of 3 mm collected on days 2 and 10 of the oestrus cycle, presumably at emergence of a follicular wave, produced significantly more and higher quality embryos than those collected on days 7 and 15. In OPU where follicular wave was induced by ultrasound guided aspiration of all large follicles and oocytes retrieved on either day 2, 5 or 8 of the follicular wave, the proportion of suitable COCs was significantly higher on days 2 and 5 than on day 8 (Hendriksen et al. 2004). Blastocysts rates were also the highest for day 2 and the lowest for day 8 indicating impaired developmental competence during the late stage of dominance phase. This notion is supported by the findings of Hendriksen et al. (2000), where the proportion of oocytes with three or more layers of non-expanded cumulus cells was higher for day 5 than day 8, with no significant difference between days 2 and 5.

Bovine oocytes aspirated from the DFs of the last follicular wave before LH surge exhibit changes in their nuclear and cytoplasmic morphology (Mihm and Bleach 2003) showing that not only final oocyte maturation but also the period before LH surge may be important for the establishment of developmental competence (Hyttel et al. 1997). From these results it can be concluded that developmental competence is relatively low on day 1, increases between days 2 to 5 and decreases on days 7 and 8.

2.2.3 The level of atresia and influence of dominant follicle

Constantly changing microenvironment of the oocyte throughout follicular growth, i.e. whether the follicle is healthy or atretic may be related to its acquisition of developmental competence. The oocyte requires a specific follicular steroid environment to attain complete maturation and normal fertilization (Moor et al. 1998).

Approximately, 85% of the follicles contained in an ovary at a given oestrous cycle are atretic (Kruip and Dieleman 1982). Detection of GCs apoptosis has been widely used to identify atretic follicles but whether this could be used as a marker of oocyte quality is poorly understood. The developmental competence of oocytes from non-atretic, intermediate and slightly atretic follicles has been suggested to be the same (Blondin and Sirard 1995). Except for oocytes retrieved from heavily atretic follicles, the percentage of embryos produced from oocytes with signs of increasing atresia appeared to be higher (de Wit et al. 2000) and early apoptosis is associated with improved developmental potential in in vitro produced bovine oocytes (Li et al. 2009). It has been proposed that, the presence of a DF has a negative effect on the subsequent development of bovine embryos produced in vitro (Bracket and Zuelk 1993, Machatkova et al. 2004). This is due to the direct inhibitory effect of the DF on the development of subordinate follicles causing them to undergo atresia mainly through inhibin and oestradiol β-17 secretion (Law et al. 1992, Wood et al. 1993).

2.2.4 Age of the donor animal

Age of the donor animal is a major factor influencing oocyte developmental competence and the efficiency of in vitro embryo production (Armstrong 2001). Although their antral follicles contain fully grown oocytes, the overall pregnancy successes of embryos derived from prepubertal heifers both in vivo and in vitro are low (Armstrong 2001, Gandolfi et al. 1998). Developmental deficiencies of oocytes derived from such animals are associated with defective molecular mechanisms and biological processes that play pivotal roles in the process of oocyte maturation. For instance, oocytes collected from prepubertal animals exhibit reduced activities of MPF and MAPK, aberrant protein and energy metabolism (Gandolfi et al. 1998, Khatir et al. 1998, Paczkowski and Krisher), less Ca^{2+} influx at fertilization and an overall reduced embryo survival post-fertilization (Armstrong 2001). Reduced developmental capacity of oocytes from prepubertal heifers may also be attributed to their abnormal cytoplasmic maturation (Khatir et al. 1997). Oocytes obtained from prepubertal heifers are capable of undergoing nuclear maturation and fertilization as equally as those obtained from cows but blastocysts yields from such oocytes are significantly lower than those from cows (Damiani et al. 1996, Khatir et al. 1996, Khatir et al. 1998).

Higher blastocysts yield was also obtained from animals between 1 and 3 years of age than older animals (Mermillod et al. 1992). Oocytes from prepubertal lambs (Ledda et al. 1997), gilts (Archipong et al. 1987), goats (Martino et al. 1995) and mice (Eppig and Schroeder 1989) have also shown similar trends. Higher proportion of presumptive zygotes derived from abattoir derived cow oocytes reached blastocysts stage than those from heifers after in vivo culture (Rizos et al. 2005).

2.2.5 In vitro culture conditions

2.2.5.1 Glucose and oxygen concentrations

Oocytes matured in vitro often have altered energy metabolism and reduced developmental competence reflecting deficiency in the maturation medium, the intrinsic ability of the oocyte itself, or both. However, unless the oocyte is able to correctly control its metabolism, it will exhibit reduced viability. Regulation of nutrient metabolism is controlled at several levels, including substrate availability in the environment, transport systems in the plasma membrane and enzyme activity and regulation (Gardner et al. 2000). These mechanisms may enable the oocyte to create an environment conducive for nuclear and cytoplasmic maturation. It has been well demonstrated that embryos lose the ability to regulate their metabolism correctly when cultured for even a short period in vitro (Gardner 1998, Lane 2001, Thompson 1997) and this may be the case for oocytes as well. It has been suggested that oxidative metabolism is the first stage of energy production during maturation of mammalian oocytes (Biggers et al. 1967, Brinster 1971, Rieger and Loskutoff 1994). However, increased glycolytic activity is associated with increased developmental competence in bovine oocytes (Krisher and Bavister 1999, Sutton-McDowall et al. 2010). Thus, the control of oocyte maturation and developmental capacity may be more complex than the provision of adequate energy for cellular processes.

Glucose metabolism through Krebs cycle and oxidative phosphorylation results in much greater ATP production than glycolysis. It may be important for the production of ribose sugar and NADPH required for cellular processes, such as reduction of intracellular glutathione, an important antioxidant, and purine synthesis. Energy

production and reactive oxygen species (ROS) are closely linked. Oxidative damage due to increased ROS results in impaired mitochondrial function, which further contributes to oxygen radical formation, reduced ATP concentration and decreased intracellular glutathione, all of which have been associated with decreased developmental competence (Grupen et al. 1995, Tarin 1996, Van Blerkom et al. 1995). Glucose metabolism is essential in controlling meiosis in the mouse oocyte. But this controlling mechanism is not dependent on energy availability, but potentially on the generation of building blocks for purine metabolism by the pentose phosphate pathway (PPP) (Downs et al. 1998). IVM induced by FSH stimulates hexokinase activity and increases glucose metabolism, resulting in high concentration of phosphoribosyl-pyrophosphate that is required for purine metabolism and germinal vesicle breakdown (Downs et al. 1998). Alternatively, glucose metabolism is tied to environmental oxygen tension and ROS concentrations within the oocyte. Excessive oxidative stress appears to contribute to reduced development of oocytes and embryos in vitro (Guerin et al. 2001, Orsi and Leese 2001, Tatemoto et al. 2001). Under conditions of high oxygen concentration during IVM of bovine oocytes, low glucose is necessary to maintain developmental competence. Low glucose concentrations results in decreased ROS and increased glutathione concentrations. High glucose may inhibit enzymes responsible for glutathione synthesis, thus impairing the ability of the oocytes to reduce ROS. Thus, low glucose is necessary to protect the oocyte against oxidative stress under high oxygen conditions (Hashimoto et al. 2000b). Low oxygen tension during IVM of bovine oocytes is beneficial for developmental competence, potentially due to decreased ROS. Although ATP production and progression to metaphase II is inhibited in a low oxygen environment, addition of glucose increases ATP concentrations and the proportion of oocytes reaching MII. ATP that may be necessary for completion of nuclear maturation, under low oxygen conditions results from glucose metabolism via glycolysis. Thus, low oxygen and high glucose result in increased developmental competence in bovine oocytes by decreasing ROS and increasing ATP production via glycolysis (Hashimoto et al. 2000b). Although exceedingly high levels of ROS in the oocyte can cause oxidative damage to the cell, H_2O_2 can also function as a second messenger for gene expression, including some enzymes involved in glucose metabolism (Hashimoto et al. 2000a).

Increased ROS at the beginning of IVM improves subsequent developmental competence (Blondin et al. 1997). The follicular environment is hypoxic for the majority of the oocytes and the follicle signal to increase blood flow in preparation for ovulation and luteinisation at the time of LH surge, when maturation is reinitiated. Studies on early embryonic development in diabetic mice have also demonstrated the importance of glucose metabolism in developmental potential. Oocytes and embryos from diabetic mice display delayed meiotic maturation and preimplantation development, an effect reversible with insulin administration (Diamond et al. 1989). This effect was duplicated when in vitro culture conditions contained elevated glucose levels (Diamond et al. 1991). Embryos cultured in high glucose concentrations display elevated, dose-related intracellular glucose concentrations, high concentrations of metabolites and developmental delays, suggesting an involvement of metabolic abnormality in delayed development (Moley et al. 1996). It is interesting that high glucose culture conditions induce embryo fragmentation and modulate expression of an apoptotic gene, Bax, in the mouse blastocysts (Moley et al. 1998a). Specifically, culture environments with high glucose content alter gene expression, triggering a decrease in facilitative glucose transporter 1 and intracellular glucose, which acts as a cell death signal inducing apoptosis (Chi et al. 2000, Moley et al. 1998b). Hyperglycemia results in the accumulation of ROS and mitochondrial damage within embryonic cells, resulting in ATP depletion and triggering apoptosis (Moley 2001, Wiznitzer et al. 1999). These studies demonstrate that high glucose levels during mouse preimplantation embryo development cause metabolic anomalies, resulting in diminished ATP stores, increased oxygen radicals and altered gene expression, leading to apoptosis and potential malformations in the resulting foetus. Exposure of oocytes to elevated glucose concentrations during maturation may cause similar deleterious effects in the subsequent embryo. It is believed that glucose metabolism, particularly via the PPP, is involved in the normal mechanisms regulating meiotic resumption and maintenance of developmental competence. Although there are several possibilities, the mechanisms remain unknown. The end products of glucose metabolism may be important to meiotic resumption, as has been suggested in mice (Downs et al. 1998) and in cattle (Hashimoto et al. 2000a). Glutathione and ROS may also play a role in meiotic resumption (Hashimoto et al. 2000b).

One common fact unifying all of these hypotheses is the redox potential of the cell. Glucose metabolisms via glycolysis results in limited ATP production, but the end products, pyruvate and lactate, can be metabolized in the Krebs cycle to produce high quantity of ATP under aerobic conditions. Pyruvate can also be reduced to form lactate, thus altering the NAD: NADH ratio and the redox potential. The PPP offers even more opportunities for the cell to control its redox potential. The pathway itself requires NADP and produces NADPH. In addition, glutathione recycling can occur, changing glutathione from its reduced to oxidized form as it reduces ROS within the cell and then back to the reduced form using NADPH. All of these molecules contribute to the redox potential of the cell. Nuclear and cytoplasmic maturation may be controlled not by energy requirements (ATP), building blocks (ribose-5-phosphate), or oxidative stress but by the redox potential within the oocyte. Measurements of glutathione, ATP or ROS are simply indicative of this state. A similar hypothesis has recently been proposed for developmental capacity of mammalian preimplantation embryos (Harvey et al. 2002).

Further evidence of metabolic influence on oocyte developmental competence arises from in vitro studies in mouse (Eppig and O'Brien 1996)). During the culture of COCs, FSH and insulin stimulate abnormal LH receptor expression in the surrounding CCs and this reduces developmental potential demonstrating the deleterious effects of inappropriate culture conditions (Eppig and O'Brien 1998). Oocytes from preantral follicles grown in vitro have resulted in successful preimplantation development and birth of a single offspring (Eppig and O'Brien 1996, Eppig and O'Brien 1998). Although normal at birth, these mice developed severe health problems with age, suggesting that suboptimal in vitro growth and development of oocytes may contribute to delayed onset of health issues (Eppig and O'Brien 1996, Eppig and O'Brien 1998).

2.2.5.2 Ionic concentration

Transient increases in intracellular Ca^{2+} concentration may spontaneously activate and regulate the meiotic and cytoplasmic maturation of the oocyte. Whereas many studies have focused on Ca^{2+} oscillations during fertilization of matured oocytes, few studies have examined intracellular Ca^{2+} levels within the immature oocytes prior to metaphase II arrest. In immature mouse oocytes, Ca^{2+} transients occur every 1 to 3 min following

the release of the oocyte from the ovarian follicle and persist for approximately 1 to 3 hr (Carroll et al. 1994). Yet, it remains controversial whether Ca^{2+} transients in immature mouse oocytes actually influence germinal vesicle breakdown (GVBD). In bovine oocytes, chelation of intracellular Ca^{2+} results in the blockage of spontaneous GVBD (Homa 1995). Porcine oocytes cultured in a Ca^{2+} free medium in the presence of a Ca^{2+} chelator, experience a Ca^{2+} transient before the oocyte undergoes GVBD suggesting an increase in intracellular Ca^{2+} triggers GVBD in pig oocytes (Kaufman and Homa 1993). The source of the Ca^{2+} required during oocyte maturation is also of significant interest. Certain mammalian oocytes, including porcine, matured in a Ca^{2+} free environment, apparently rely only on intracellular sources of Ca^{2+}, like endoplasmic reticula and mitochondria, to initiate GVBD (Kaufman and Homa 1993). Recently inositol 1, 4, 5-trisphosphate (IP3) and ryanodine release channel receptors were found to be located on the surface of the endoplasmic reticulum within porcine oocytes. Interestingly, these receptors are active from the germinal vesicle stage throughout maturation to metaphase II and possibly even to interphase (Machaty et al. 1997), enabling increased amounts of Ca^{2+} to be released from the intracellular stores.

GVBD in pigs may depend on internal stores of Ca^{2+}, continuation of meiosis after metaphase I and polar body formation may require extracellular Ca^{2+} (Homa 1995, Kaufman and Homa 1993), provided by the reproductive tract or the culture medium. Elevated intracellular Ca^{2+} levels, the result of cellular stress or high extracellular Ca^{2+} concentrations, may perturb normal maturation. Under physiological conditions, Ca^{2+} transients serve as second messengers in mammalian cells and stimulate mitochondrial oxidative metabolism and ATP production (Rizzuto et al. 1994). Under pathological conditions, a prolonged increase in intracellular Ca^{2+}, may activate destructive enzymatic pathways and inhibit energy production in the cell (Beitner 1993). Excess Ca^{2+} directs the opening of the mitochondrial permeability transition pore, or mega channel (Krieger and Duchen 2002), resulting in the release of cytochrome C and the activation of proteolytic caspases that lead to apoptotic cell death. A disrupted mitochondrial membrane also diminishes the mitochondrial membrane potential. Without a functional mechanism to produce energy, the oocyte undergoes necrotic cell death upon consumption and depletion of energy stores. Cytosolic magnesium (Mg^{2+}) inhibits the opening of this mega channel. As a counter action to cellular Ca^{2+}, Mg^{2+} has

the ability to lower intracellular Ca^{2+} concentrations and therefore regulate and alleviate the adverse effects of elevated Ca^{2+} (Altura et al. 1982). In addition to Ca^{2+}, energy in the form of ATP is critical for nuclear and cytoplasmic maturation. Because mitochondria synthesize ATP and serve as an internal source of Ca^{2+}, it is possible that mitochondria redistribute and aggregate within the oocyte to concentrate ATP (Ducibella et al. 1977) and Ca^{2+} (Li and Fan 1997, Mehlmann et al. 1996) at sites of high demand. During oocyte maturation, breakdown of the nuclear membrane likely requires elevated ATP levels. Determining the distribution and activity of mitochondria in response to a changing ionic environment may be vital to understand the role of mitochondria during the maturation of porcine oocytes.

2.2.6 Plane of nutrition

Plane of nutrition is one of the main reasons for the decline in the fertility of modern dairy cows. During early lactation, high-yielding dairy cows are typically in a state of negative energy balance because the amount of energy required for milk production and maintenance exceeds the amount of energy the cows obtain from feeds. Insufficient energy supply results in poor reproductive performances including delayed onset of oestrous cycles postpartum (Butler 2000, Reist et al. 2000, Staples et al. 1990) and reduced oocyte quality (Snijders et al. 2000, Walters et al. 2002), resulting in low conception and high early embryonic death rates (Lucy 2001). Cows fed with high energy diets produce better quality oocytes than those fed with low energy diets (Butler and Smith 1989, Kendrick et al. 1999). Similarly, higher level of dietary fat significantly increases the rate of blastocysts production from both in vitro matured oocytes and cleaved embryos (Fouladi-Nashta et al. 2007). Higher level of dietary fat improves embryo quality via increased number of total and trophectoderm cells. Short-term changes in plane of nutrition have been shown to have a direct effect on ovarian follicular dynamics in cattle, without any changes in the concentrations of circulating gonadotropins (Armstrong et al. 2003, Webb et al. 2003). It has been hypothesized that endocrine and metabolic signals that regulate follicular growth also influence oocyte development either through changes in hormone/growth factor concentrations in follicular fluid or via oocyte-granulosa cells interactions and are highly influenced by dietary level and composition (Webb et al. 1999, Webb and Campbell 2007). For

example, short-term changes in dietary energy intake influence both oocyte morphology and developmental potential (O'Callaghan et al. 2000, O'Callaghan and Boland 1999). However, a higher level of dietary protein reduces oocyte quality (Armstrong et al. 2001, Sinclair et al. 2000). In high-yielding dairy cow, supplementary dietary carbohydrates can reduce the quality of oocytes and embryo production (Fouladi-Nashta et al. 2007). Oocyte quality is also affected by the interaction between feeding level and body condition score; high level of feeding is beneficial to oocytes from animals of low body condition but detrimental to those from animals of moderate to high body condition score (Adamiak et al. 2005).

2.2.7 Genetic factors

Modern high yielding dairy cows have been selected on the basis of their milk yield as the expense of fertility parameters and this has resulted in reduced fertility (Lopez et al. 2005, Pryce et al. 2000). Selection of dairy cattle for milk yield has linked the endocrine and metabolic controls of nutrient balance and reproductive events so that reproduction in dairy cattle is compromised during periods of nutrient shortage, such as in early lactation. The energy required to synthesize and secrete hormones, ovulate a follicle, and sustain an early developing embryo are probably lower compared to the energy needs for maintenance and lactation. Oocytes from high yielding dairy cows result in fewer blastocysts and lower cleavage rates than oocytes from medium yielding ones (Snijders et al. 2000). Notable differences have also been reported in the number of oocytes recovered and blastocysts rate from cows with varied genetic origins (Tamassia et al. 2003).

2.3 Mammalian cumulus oocyte complex matrix as a regulator of meiotic maturation

In nearly all mammals, the development of ovarian follicles, ovulation and the formation of the corpus luteum are complex processes accompanied by dramatic changes in follicular cells under the specific and strict regulation of pituitary gonadotrophins, steroids and growth factors. The growth and development of the oocyte and its companion somatic cell compartment in the follicle take place in a highly

coordinated and mutually dependent manner (Eppig 2001, Matzuk et al. 2002, Thomas et al. 2003).

Owing to the meiotic initiation, mitosis in the oogonial germ cells is terminated and the number of oocytes that a female will be endowed with is finally set. Oocytes that enter the diplotene stage, a prolonged dictyate stage of prophase I (first meiotic division) become surrounded by a single layer of somatic cells to form non-growing primordial follicles (Figure 1). With the formation of the primordial follicles, oocytes suddenly stop their meiotic progress at the dictyate stage. This large population of resting primordial follicles serves as the source of developing follicles and oocytes until the end of a female's reproductive life. Initial growth from primordial follicles to pre-antral follicles is a gonadotrophin independent process occurring during the foetal life and continuing to the prepubertal stage. Throughout prepuberty, the resting pools of primordial follicles are continuously recruited into the growing follicle pool. After puberty, gonadotrophin-dependent growth occurs in a cohort of antral follicles, selected from the growing follicle pool, which will supply the DFs. While very few follicles develop to the ovulatory stage (< 1% of primordial follicles present at the time of birth), the majority of non-ovulatory follicles undergo atresia during the adult life. In vivo studies have shown that apoptosis is the underlying mechanism of oocyte depletion and follicular atresia during folliculogenesis (Fujino et al. 1996, Perez et al. 1999, Perez et al. 2007).

Ovarian follicles, the basic functional units of an ovary, start their development as primordial structures that consist of an oocyte arrested at the dictyate stage of prophase I. Once a limited number of primordial follicles are activated in response to an unknown signal, enlargement of the oocyte commences and the epithelial cells become cuboidal in shape and begin to divide. As the granulosa cells proliferate, the number of cell layers around the oocyte increases, and the basal lamina expand. Outside the basal lamina, the pre-theca cells are organized concentrically to form the theca layer. As the follicular maturation proceeds, zona pellucida develops on the surface of oocyte. Later in development, a fluid-filled cavity called the antrum develops within the granulosa cell layers. Antrum formation forces the oocyte into a more eccentric site and leads granulosa cells to differentiate into two specialized cell subpopulations: cumulus cells,

which are closely connected with the oocyte and mural granulosa cells, which are organized as a stratified follicular epithelium encapsulated by a basal lamina.

Bidirectional cross-talk occurs between the granulosa cells and theca cells as well as between granulosa cells and oocytes, via both paracrine and gap-junctional signalling (Eppig 2001). These exchange of small regulatory molecules, play critical roles in the normal growth and development of follicles (Albertini et al. 2001, Kidder and Mhawi 2002, Knight and Glister 2003, Rodgers et al. 2003). It also requires appropriately-timed endocrine signals, pituitary gonadotrophins and metabolic hormones, which act on receptors in the each of the cell types and interact with local autocrine/paracrine signalling pathways.

The COCs, a structural unit of the antral follicle, includes several layers of CCs (approximately 1,000-3,000 cells/COC) around the oocyte in mouse (Salustri et al. 1992). Just before ovulation, following the endogenous gonadotrophin surge, a graafian follicle rapidly increases in volume by the accumulation of follicular fluid and intrafollicular pressure. Concomitantly, the COCs form muco-elastic ECM containing a large amount of hyaluronan (HA) and specific HA-binding proteins (PTX3 and TNFAIP6), causing a radical volumetric enlargement called cumulus expansion (Dekel and Kraicer 1978, Eppig 1979, Salustri et al. 1992). The expanded COCs become detached from membrane granulosa and freely float in the follicular fluid. The follicular wall becomes thin and is broken down by proteolytic enzymes (collagenase and plasmin) produced by MGCs. As a result, the COC matrix mass is expelled from the follicle and reaches the oviductal ampulla (Smith et al. 2002).

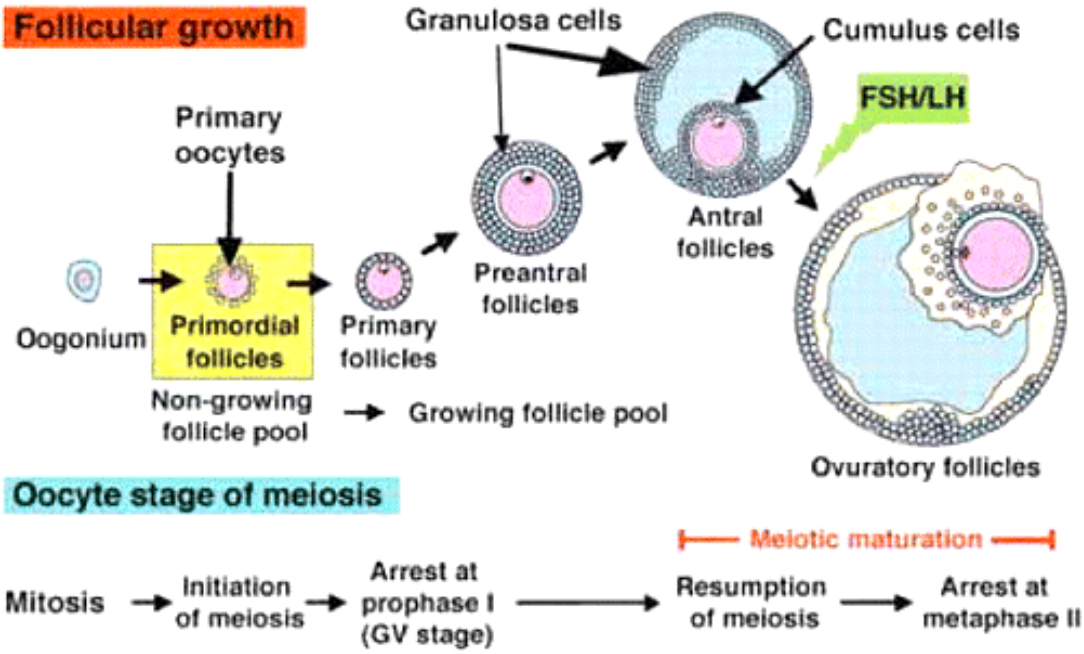


Figure 1: Follicular growth and oocyte meiotic maturation (Kimura et al. 2006). During prenatal life of the female animal, oogonial germ cells divide mitotically and form pools of primordial follicles containing primary oocytes arrested at prophase stage of the first meiotic division. Primordial follicles gradually grow to primary, preantral and antral follicles and form a fluid filled cavity referred to as antrum. At this stage, granulosa cells form two cell lines: mural and cumulus granulosa cells and COC detach from follicular wall and floats on the antral fluid and finally ovulate. The arrested germinal vesicle oocytes resume meiosis at antral follicular stage, undergo maturation processes and arrested again at MII meiotic stage.

2.4 Follicular dynamics in cattle

Trans-rectal utrasonogaphic imaging of follicular populations at different size categories have convincingly documented that follicular growth in cattle occurs in a wave-like pattern and the majority of oestrous cycles are characterized by two or three waves (Adams et al. 2008, Ginther et al. 1989, Knopf et al. 1989, Sirois and Fortune 1988).

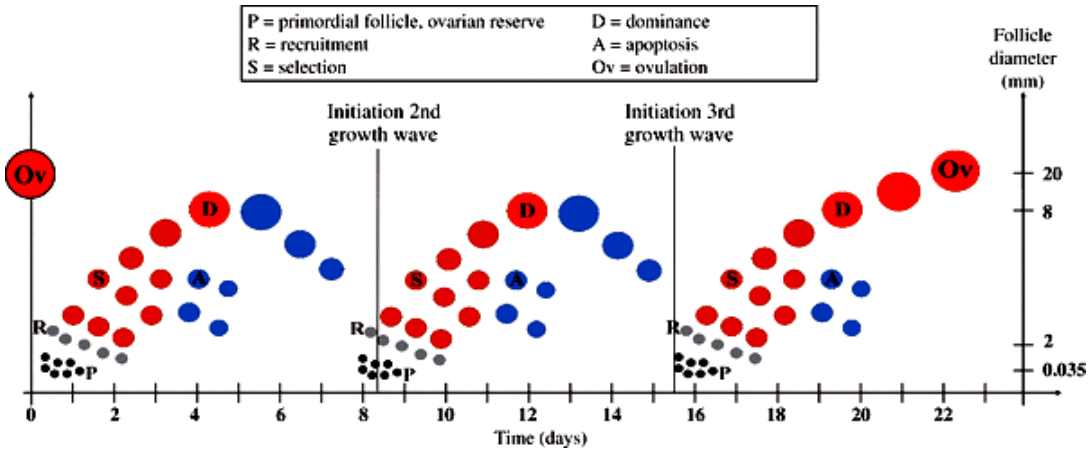


Figure 2: Dynamics of ovarian follicular wave in cows exhibiting three wave patterns (Aerts and Bols). In each wave, a cohort of antral follicles (black) escaping apoptosis is recruited; the recruited follicles (gray) are subjected to selection process, whereby in monovular species such as cattle and horses a single follicle develops in to DF (red) while the remaining follicles regress (Ginther et al. 2000). The DFs of the first and second waves grow up to 8 mm diameter and become atretic while the DF of the last wave becomes ovulatory.

The emergence of each wave is initiated by an increase in the concentration of circulating FSH and a surge in the concentration of circulating LH precedes ovulation. The LH surge is preceded and succeeded by a period of high-LH pulse frequency as a result of low circulating P concentrations (Adams et al. 2008). The pattern of follicular wave in cattle is controversial. While more than 80% of 2 wave patterns has been reported by Ahmad et al. (1997) and Rajamahendran and Taylor (1990), the occurrence of more than 80% of the 3-wave patterns has been reported by Celik et al. (2005) and Noseir (2003) and still others have reported a more even distribution of 2 and 3 wave patterns (Price and Carriere 2004).

The emergence of a follicular wave in cattle is characterized by a sudden growth of ultrasonographically detectable, 8-14 small antral follicles with in 2-3 days (Adams et al. 2008). This periodic emergence of ovarian follicular waves in ruminants is regulated by a series of tightly timed systemic feedback mechanisms between the ovary and the pituitary gland (Adams 1999). Each follicular wave is preceded by a surge of FSH (Adams et al. 1992) that stimulates follicular growth. In cows, with three follicular

waves, the first, second and third waves are detected on days (0 to 2), (9 to 10) and (15 to 16) of the estrous cycle, respectively. Cattle having two-wave pattern showed the first and second follicular waves on days (0 to 2) and (8 to 12) of the cycle (Savio et al. 1990, Sirois and Fortune 1988). In cattle with four wave patterns, the waves begin on 2, 8, 14 and 17 days of the oestrus cycle, respectively (Sirois and Fortune 1988).

2.4.1 Large preovulatory follicles maintain their dominance by producing inhibitory factors

Follicular dominance appears to be controlled by a number of mechanisms acting together. These include alterations in peripheral FSH concentrations by oestradiol and inhibin secreted by the dominant (ovulatory) follicle, as well as the possible production of local ovarian factors, which can inhibit the development of subordinate follicles directly (Campbell et al. 1995). Dominance and inhibition of the growth of subordinate follicles may be attributed to the production of follicular growth inhibitory factors (FGIFs) by the DFs. It has been demonstrated that, factors other than inhibin, are also capable of inhibiting follicular development in sheep and cattle (Campbell et al. 1991, Law et al. 1992). The cow is an excellent model system for investigating these putative FGIFs. Unlike sheep and pigs, cows are monovulators and the action of FGIFs probably ensures that > 95% of cows ovulate only once per oestrous cycle. FGIFs are expected to act via systemic mechanisms since DFs can inhibit the growth of follicles in the contra lateral ovary. However, the mechanism by which FGIFs inhibit the growth of subordinate follicles is unknown. Since they inhibit FSH-stimulated granulosa cell proliferation and aromatase activity, it is possible that FGIFs interfere with the activity of locally produced growth factors that control the response of granulosa cells to FSH. Another possibility is that FGIFs inhibit vascularisation of subordinate follicles. It has been noted that DFs have a more extensively vascularised theca layer compared with other antral follicles and this is associated with an increased uptake of gonadotrophins from the circulation (Redmer and Reynolds 1996, Zeleznik et al. 1981). Therefore, any factor that affects the blood supply of a developing follicle should have a pronounced effect on its development. Whatever the mechanism(s) through which these putative FGIFs acts, the DF must be resistant to their actions (perhaps through the loss of specific receptors) to allow its continued preovulatory development.

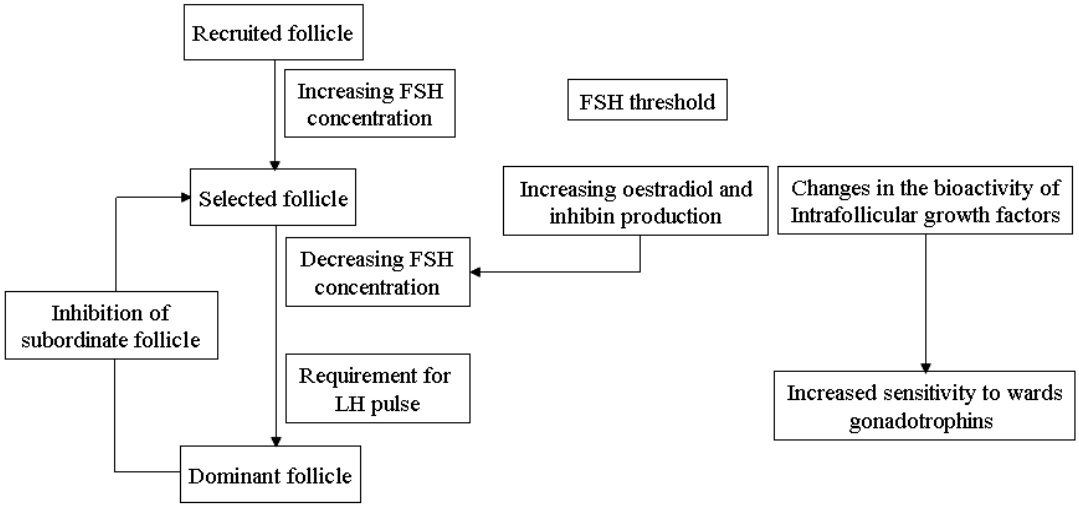


Figure 3: A model for the development of follicular dominance in cattle (Armstrong and Webb 1997, Ginther et al. 2001). Follicle selection occurs when FSH concentrations are increasing and surpass the FSH threshold which regulates the number of follicles selected for further growth (Baird 1987). Differentiation of GCs during the selection process results in increased concentrations of circulating oestradiol and possibly of bioactive inhibin which inhibit the release of FSH from the pituitary gland. The selected follicles then change their dependence from FSH to LH (Campbell et al. 1995). Thus, mechanisms controlling the initiation of dominance occur in an environment of decreasing FSH support and rely on intraovarian mechanisms that regulate both the bioactivity/bioavailability of ovarian growth factors and the vascularisation of the DF. These changes result in an increase in both the sensitivity and exposure of the selected follicle towards gonadotrophins. The inhibition of subordinate follicle growth relies on the production of specific FGIFs that selectively inhibit the growth of subordinate follicles.

3 Materials and methods

3.1 Materials

3.1.1 Oocyte recovery and sample preparation

Bovine ovaries were collected from local abattoirs and transported to the laboratory within 2-3 hrs in a thermo flask containing 0.9% physiological saline solution (NaCl) at 39ºC. Before aspiration of COCs, the ovaries were washed twice in 70% ethanol. COCs were aspirated from antral follicles having 2-8 mm diameter using 5 ml syringe attached to 18 gauge needle. The aspirated follicular fluid was collected in 50 ml sterilized tube and allowed to precipitate for 15 min. COCs with evenly granulated cytoplasm surrounded by multiple layers of CCs were picked using glass-pipette and washed three times in drops of modified parker medium (MPM) supplemented with 12% oestrus cow serum (OCS). In order to increase the homogeneity of the experimental samples, COCs were further screened for developmental competence using brilliant cresyl blue (BCB) staining (Pujol et al. 2004, Rodriguez-Gonzalez et al. 2003, Torner et al. 2008). BCB positive (BCB^+) COCs were assigned randomly in to the following four experiments using three pools of biological replicates (Figure 4). In experiment 1A, CCs were physically removed from oocytes at GV stage by repeated in and out pipetting and the resulting denuded oocytes (DOs) and the companion CCs were frozen. In experiment 1B, intact COCs were cultured; CCs were physically removed and the resulting denuded MII oocytes and CCs were frozen. In experiment 2, CCs were physically removed at GV stage and the resulting (OO-CCs) and other pools of intact oocytes (OO+CCs) were cultured; CCs were physically removed and both oocytes were frozen. In experiment 3, the ooplasm was microsurgically removed at GV stage as described previously (Ralph et al. 1995) and the resulting oocytectomized complexes (CCs-OO) and other intact complexes (CCs+OO) were cultured. The ooplasm were removed from CCs+OO and the resulting MII CCs frozen. In experiment 4, CCs were physically removed from their enclosed oocytes both at GV and MII stages and frozen for subsequent total RNA isolation. Cells were cultured for 22 hrs when applicable and each experiment was repeated three times using pools of biological replicates (n=150).

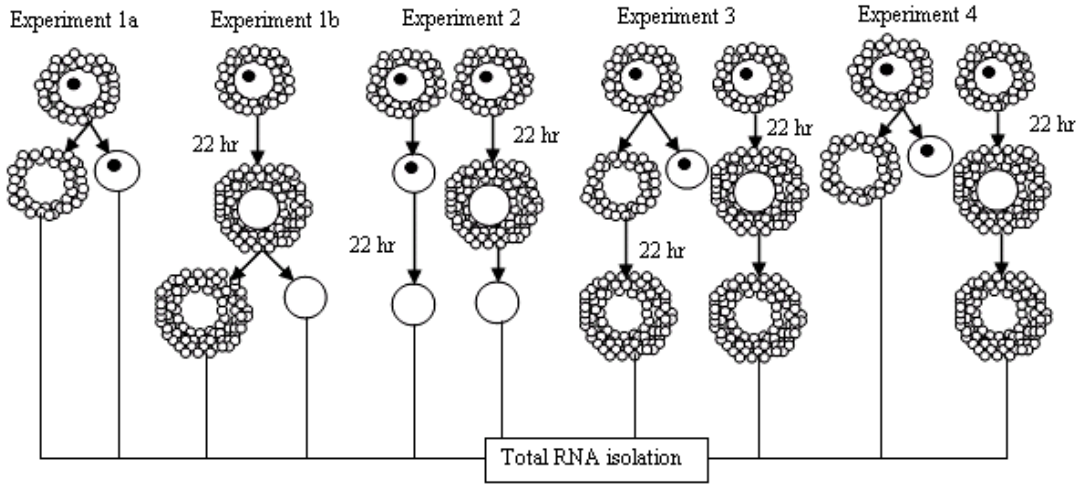
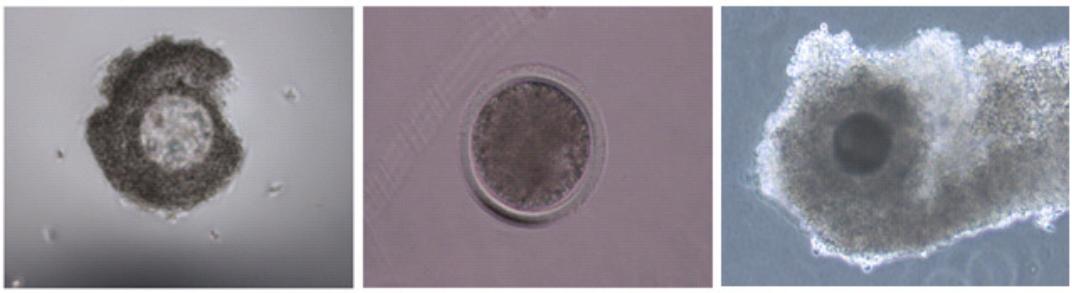


Figure 4: Diagrammatic illustration of the experimental design showing groups that were used for total RNA isolation, cDNA synthesis, array hybridization and semi-quantitative and quantitative RT-PCR validation of the array data. For all experimental groups, the samples were derived from GV stage COCs and cells were cultured for 22 hrs when applicable.

Complete removal of either cell from one or the other was confirmed by microscopic examination of the corresponding samples (Figures 5A and B) as described in (Memili et al. 2007) and by confirming the absence of either cell specific transcripts using semi-quantitative RT-PCR. COCs, DOs and CCs-OO were cultured in groups of 50 in 400 µl MPM medium supplemented with 12% oestrus cow serum and 10 µg mL-1 FSH for 22 hrs at 39ºC in an incubator with humidified atmosphere containing 5% CO_2. Oocyte and CCs samples from each group were stored at -80ºC until subsequent RNA isolation.



(A) (B) (C)

Figure 5: Photographs showing CCs from which the enclosed ooplasm was completely removed using oocytectomy (A), the oocyte from which the companion CCs were completely removed mechanically at GV stage using pipettes (B) and an intact COC (C). Photographs were taken using Leica DM-IRB microscope with a magnification of 20X.

3.1.2 Equipments and kits

Equipments /Kits	Manufacturer
CEQ TM 8000 genetic analysis system	Beckman Coulter, Krefeld, Germany
7000 sequence detection system	Applied Biosystems, Foster city, USA
Laminar flow chamber	Heraeus, Geramny
Thermocycler	BIO-RAD, Munich, Germany
MAXQ6000 shacking incubator	Thermo scientific, Germany
Nanodrop 8000	Thermo scientific, Germany
Agilent 2100 bioanalyser	Agilent technologies, Germany
RNA Nano Labchip	Agilent technologies, Germany
Chip priming station	Agilent technologies, Germany
Windows XP	Fujitsu-Siemens, Germany
IKA vortex mixer	Agilent technologies, Germany
16-Pin bayonet electrode cartridge	Agilent technologies, Germany
GeneChip™3000 laser confocal slide scanner	Affymetrix, Inc. Santa Clara, USA
Gene Chip Operating Software	Affymetrix, Inc. Santa Clara, USA
Affymetrix Bovine Genome 430 v. 2.0 GeneChip® arrays	Affymetrix, Inc. Santa Clara, USA
Hybridization oven	Affymetrix, Inc. Santa Clara, USA

450 Fluidics station	Affymetrix, Inc. Santa Clara, USA
Leica DM-IRB microscope	Leica, Solms, Germany
Carbon dioxide incubator (BB16)	Heraeus, Hanau, Germany
Centrifuges (small, medium, large)	Heraeus, Hanau, Germany
Carbon dioxide incubator (MCO-17AI)	Sanyo, Japan
Cryo-tube	Nunc, Roskilde, Germany
Electrophoresis chamber	BioRad, Munich, Germany
Epifluorescence microscope	Leica, Bensheim, Germany
Four-well dish	Nunc, Roskilde, Germany
Incubator (BB16)	Heraeus, Hanau, Germany
Femtojet II injection capillary	Eppendorf, Hamburg, Germany
Micro wave	Micro maxx®, Germany
PCR thermal cycler (PTC 100)	MJ Research, USA
Power supply PAC 3000:	BioRad, Munich, Germany
Power supply Mini-Protan®	BioRad, Italy
Schüttel water bath incubator	Gerhardrt, Bonn, Germany
Ultraspec 2100 pro spectrophotometer	Amersham Biosciences Buckinghamshire, UK
Variable temperature oven	IMP, Vienna, Austria
Video graphic printer-UP-890CE	BioRad, Munich, Germany
UV light transmitter	Benda, Wiesloch, Germany
Mini centrifuge	Labnet, UK
GenElute ^{TM} HP plasmid isolation kit	Sigma-Aldrich, St. Louis, MO, USA
Picopure RNA isolation kit	Arcturus, Bioscience Mt. View CA
MEGA Script in vitro transcription kit	Applied biosystems, Ambions
ApoTome microscope	(ApoTome MicroImaging Inc., Carl-Zeiss, Germany).

3.1.3 Chemicals

Chemicals	Supplier
Dye terminator cycle sequencing (DTCS)	Krefeld, Germany
Sample loading solution (SLS)	Beckman Coulter, CA, USA
Superscript II reverse transcriptase	Invitrogen, Karlsruhe, Germany
5X First-Stand buffer	
DTT	
BME (essential amino acids)	Gibco BRL, Life technologies,
MEM (non essential amino acids)	Karlsruhe, Germany
RNase free-DNase	Promega, Mannheim, Germany
Ribo-nuclease inhibitor (RNasin)	
T4 DNA ligase	
2X rapid ligation buffer	
pGEM®-T vector	Roth, Karlsruhe, Germany
5-bromo-4-chloro-3-indolyl-β-	
D-galactopyra-noside (X-gal)	
Acetic acid	Roth, Karlsruhe, Germany
Agar-Agar	
Ampicillin	
Ammonium peroxydisulfate (APS)	
Boric acid	Roth, Karlsruhe, Germany
Calcium chloride	
Chloroform	
dNTPs	
Ethylenediaminetetra acetic acid (EDTA)	
Ethanol	Roth, Karlsruhe, Germany
Ethidium bromide	
Formaldehyde	
Glycerine	
Isopropyl β-D-thiogalactoside (IPTG)	
Korsolin® FF Peptone	Roth, Karlsruhe, Germany

Proteinase K	
Yeast extract	
T-octylphenoxypolyethoxyethanol (Triton X-100)	Roth, Karlsruhe, Germany
Anti-human rabbit monoclonal MSX1 primary antibody	Lifespan Biosciences, USA
Sheep anti-rabbit FITC conjugated gG-secondary antibody	Lifespan Biosciences, USA
10X PCR buffer	GeneCraft, Germany
Anti-human rabbit polyclonal IRF6 primary antibody	Santa Cruz Biotechnologies Inc., Germany
Agarose	Sigma, Steinheim, Germany
PBS	
Heparin	
Hepes	Sigma, Steinheim, Germany
Hypotaurin	
Igepal	
Isopropanol	
L-Glutamine	
Magnesium chloride	
TCM-Air	Sigma, Steinheim, Germany
Mineral oil	
Penicillin	
Polyvinyl pyrolidone (PVP)	
Propidium iodide	
Protease inhibitor cocktail	Sigma, Steinheim, Germany
Sodium hydrogen carbonate	Strata gene, Amsterdam, Netherlands
Sodium hydrogen sulphate	
Sodium lactate solution (60%)	
Sodium pyruvate	
Streptomycin sulphate	
SYBR® Green JumpStartTM ReadyMixTM Taq DNA polymerase	BIO-RAD, Munich, Germany Strata gene, Amsterdam, Netherlands

3.1.4 Reagents and media

All reagents and media used in these investigations were prepared with deionized millipore water (ddH_2O) and pH was adjusted with sodium hydroxide (NaOH) or hydrochloric acid (HCl).

LB-agar	Sodium chloride	8.0 g
	Peptone	8.0 g
	Yeast extract	4.0 g
	Agar-Agar	12.0 g
	Sodium hydroxide (40 mg/ml)	480.0 µl
	ddH_2O added to	800.0 ml
LB-broth	Sodium chloride	8.0 g
	Peptone	8.0 g
	Yeast extract	4.0 g
	Sodium hydroxide (40 mg/ml)	480.0 µl
	ddH_2O added to	800.0 ml
BSA (3%)	Bovine serum albumin	0.15 g
	added to PBS+PVA	5 ml
CR1-aa culture medium (50 ml)	Hemi-calcium lactate	0.0273 g
	Streptomycin sulphate	0.0039 g
	Penicillin G	0.0019 g
	Sodium chloride	0.3156 g
	Potassium chloride	0.0112 g
	Sodium hydrogen carbonate	0.1050 g
	Sodium pyruvate	0.0022 g
	L-Glutamine	0.0073 g
	Phenol red solution (5% in D-PBS)	100 µl
DEPC-treated water (1000 ml)	DEPC	1 ml
	added to water	1000 ml
Lysis buffer (100 µl)	Igepal (0.8%)	0.8 µl
	RNasin	5 µl

	DTT	5 µl
	added to water	100 µl
Modified Parker Medium (110 ml)	Sodium hydrogen carbonate	0.080 g
	HEPES	0.140 g
	Sodium pyruvate	0.025 g
	L-Glutamin	0.010 g
	Gentamicin	500 µl
	Medium 199	99 ml
	Hemi calcium lactate	0.06 g
	added to water	110 ml
TAE (50x) buffer, pH 8.0	Tris	242.0 mg
	Acetic acid	57.1 ml
	EDTA (0.5 M)	100.0 ml
	ddH_2O added to	1000.0 ml
TE (1x) buffer	Tris (1 M)	10.0 ml
	EDTA (0.5 M)	2.0 ml
	ddH_2O added to	1000.0 ml
X-gal	X-gal	50.0 mg
	N, N'-dimethylformamide	1.0 ml
(16%) Para formaldehyde (10 ml)	Para formaldehyde	1.6 g
	added to water	10 ml
PBS + PVA (50 ml)	Polyvinyl alcohol (PVA)	300 mg
	added to PBS	50 ml
Permeabilizing solution (10 ml)	Triton X-100	5 µl
	Glycine + PBS added	10 ml
Physiological saline solution (1000 ml)	Sodium chloride	9 g
	added to water	1000 ml
Agarose loading buffer	Bromophenol blue	0.0625 g
	Xylencyanol	0.0625 g
	Glycerol	7.5 ml
	ddH_2O added to	25 ml

dNTP solution	dATP (100 mM)	10.0 µl
	dCTP (100 mM)	10.0 µl
	dGTP (100 mM)	10.0 µl
	dTTP (100 mM)	10.0 µl
	ddH_2O added to	400.0 µl
IPTG solution	IPTG	1.2 g
	ddH_2O added to	10.0 µl
M Sodium Acetate, pH 5.2	Sodium Acetate	123.1 g
	ddH_2O added to	500 ml
1M EDTA, pH 8.0	EDTA	37.3 g
	ddH_2O added to	1000 ml
Phenol Chloroform	Phenol : Chloroform	1 : 1 (v/v)

3.1.5 Software and statistical packages

Software and packages	Source
Primer Express® Software v. 2.0	Applied Biosystems, Foster City, USA
R-software environment	http://www.r-project.org
Bio conductor	http://www.bioconductor.org
Weight to molar quantity converter	http://www.molbiol.ru/eng/scripts/01_07.html
NCBI blast system	http://www.ncbi.nlm.nih.gov
ABI Prism® 7000 sequence detection system	Applied Biosystems, Foster city, USA
Ingenuity pathway analysis s	http://www.ingenuity.com/

3.2 Methods

3.2.1 Primer design

Names of the primers, their accession numbers and sequences, product sizes and their annealing temperatures are shown in Table 1.

Table 1: Pairs of primers that were used for semi-quantitative and qRT-PCR validation of the microarray data

Gene name	Accession number	Forward (5' - 3')	Reverse (3' - 5')	Ann.Tem (ºC)	Product length (bp)
GDF9	NM_174681	AGCGCCCTCACTGCTTCTATAT	ACACCCTCAGCAGCTTCTTCTC	57	152
MSX1	NM_174798	AAGGTATCCACAGTCCCCAGC	TCTGCCTCTCCTGCAAAGTTC	56	180
IRF6	NM_001076934	GGACTCCAAACGCTTCCAGA	TCCTTGGTGCCATCATACATCA	54	212
SPARC	NM_174464	CGATGATGGTGCTGAGGAAA	TGGTGGCAAAGAAGTGGCA	53	220
HPSE	NM_1744082	ATGGGCATAGAAGTGGTGATGA	TGTTTGGTGTTTGTGCAATGAA	TD 54-50	202
DGAT2	NM_205793	TGAACCGGGACACCATAGACTA	CACCTCATTCTCCCCAAAGGA	55	205
SOX2	NM_001105463	GCGGCAACCAGAAGAACAG	GCTTCTCCGTCTCGGACAAA	55	170
HAS2	NM_174079	CCAAATGAAATGCCAAAGGAA	CAACGTCAACCAAGCTTCACA	TD 54-50	237
PTX3	NM_001076259	TTTATTCCCCATGCGTTCCA	CTCCACCCACCACAAGCATT	53	205
IGF2BP3	XM_588560	GACGCGAAAGTGAGGATGGT	TGCACTTGACAAATTCTGGAGC	54	215
ADAMTS1	NM_001101080	TCGTCATACAGCTCCCCTCC	ATTGACACACCATTTCCCCTCT	TD 57-54	220
PDK4	XM_583960	ATTTTTGCGACAAGAGTTGCCT	GGATTCCTTGTGCCATTGTAGG	TD 56-52	250

CCRK	XM_879150	GGCCCCACTCATGGCTACTT	TCCTGAGGGTGATGCTGGTAA	57	170
FOSB	XM_880646	TTCCTGAATCTCTCCCGCC	TGCTCACAGCCTCACACTCG	56	205
POU5F1	NM_174580	AGAAGGGCAAACGATCAAGC	GGTGACAGACACCGAGGGAA	TD 57-53	205
VEGFA	NM_174216	GGTTTCGGGAACCAGACGT	GGCAATCCAATTCCAAGAGGA	55	233
18S	NR_003286	GTGCCCTTCCGTCAATTCCT	ACGAAAGTCGGAGGTTCGAA	55	184
GPT	XM_585516	CCGATGAGGTGTACCAAGACAA	CCATATTCACCACCTCCACGT	55	185
DAPL1	NM_001025346	AAATTTCCAGCAGTAGCGCAC	AGCTCTCAGACATTCGAGGCA	55	158
IRF7	NM_001105040	CTCCCCGCACTACACCATCTA	GCCTGTTCCACCTCCATCA	TD 58-55	245
GADD45A	NM_001034247	GACCGAAAGGATGGATAAGGTG	TGGATCAGGGTGAAGTGGATCT	56	200
DPP4	NM_174039	CAACTGGGCTACTTACCTTGCA	TTACGTACCCTCCGTATGACCA	56	223
IFI6	NM_001075588	ACGGTGACAAAGCCTTGAGC	AGGTCACCATGCCCCAGAA	55	158
ADAMTS4	NM_181667	CCATTGTGGAGGATGATGGG	AGGAAGTCAGTGATGAAGCGG	55	210
CASP1	XM_592026	CTCACTCAGAGCATCGGACCT	GTTTACCCATACCATCCCTTGC	57	229
IFIT5	NM_001075698	CACAGTGTATCGGCTGGATGA	GGTTGGGCTGATATCTGGTCC	56	200
DDX39	NM_001034752	GCAGTTCAAGGACTTCCAGC	GCTCTGCCACATTCACTTCA	TD 55-52	228
GTF2A2	NM_001037619	AGAAAGAGGTTCTGCCCGGA	CTTATCGGCTGCATTGAAAGC	55	150
TSSC1	XM_001789233	AGCAGCGACAGCAGAGTCATC	TAGCTCAGGGAGGCGAACAG	57	229
FSHR	NM_174061	GCTGGATCTTTGCTTTTGCAGT	ATGCGCTTGGCTATCTTGGT	54	243
GPT	NM_001083740	CCGATGAGGTGTACCAAGACAA	CCATATTCACCACCTCCACGT	56	185
PLAUR	NM_174423	GGATTCCACAACAACCACACCT	TCGCTTCCAGACATTGATTCAT	TD 56-52	204
CA2	NM_178572	CAAAGCAGTGCTGAAAGATGGA	AAAACACCCACAACAGCCAGTC	55	212

Touch down (TD)= The forward and the reverse primers of a given gene have different annealing temperatures.

3.2.2 PCR and purification of PCR products

PCRs were performed in 20 µl total reaction volume containing 2 µl of 10X PCR buffer (Sigma), 0.5 µl of each primer (10 pmole), 0.5 µl of dNTP (50 µM), 0.1 µl of Taq DNA polymerase (Sigma), 14.4 distilled water (dwater) water and 2 µl of template cDNA. 2 µl of genomic DNA (50 ng/µl) and 2 µl dwater were used as a positive and negative controls respectively. The reactions were carried out in a PT-100 Thermocycler (MJ Research) and thermal cycling program denaturizing at 95°C for 5 min, followed by 35 cycles at 95°C for 30 sec, annealing at their corresponding temperatures (Table 1) for 30 sec and extended at 72°C for 1 min, final extension step at 72°C for 10 min. 5 µl of the PCR products were taken and loaded with 2 µl of loading buffer on 2% agarose gel to check for amplification of the desired product. Electrophoresis of the amplified PCR products were done for 20 min at 120 voltages and visualized under UV light trans-illuminator.

The remaining 15 µl of the PCR products were purified using QIAGEN PCR purification kit (QIAGEN GmbH, Germany) according to the manufacturer's instruction. Briefly, 5 volumes of Buffer PB was added to 1 volume of the PCR sample and mixed. QIAquick spin column was placed on 2 ml collection tube provided and the samples were centrifuged for 30-60 sec at 12000 rpm. The resulting flow-through was discarded and the column was placed back into the same tube. The column was washed by adding 0.75 ml Buffer PE and centrifuged for 30-60 sec. The flow-through was discarded and the column was placed back into the same tube. The column was centrifuged for additional 1 min and the residual ethanol was removed completely by discarding the flow-through. Next, the column was placed in a clean 1.5 ml micro centrifuge tube and the resulting DNA was eluted by adding 30 µl elution buffer EB (10 mM Tris·Cl, p^H 8.5) or water (p^H 7.0-8.5) to the centre of the QIAquick membrane and centrifuged for 1 min. The quality of purified DNA was analyzed on 2% agarose gel by adding 1 volume of the loading dye to 5 volumes of purified DNA after pipetting the mixture up and down and the DNA was stored at 4°C.

3.2.3 Ligation, transformation and colony screening

The purified DNA fragments were ligated in to the pGEM®-T vector (Promega). The ligation reaction was performed in 5 µl total reaction volume containing 2.5 µl ligation buffer, 0.5 µl of T-vector, 0.5 µl T4 DNA ligase (3 U/µl) and 1.5 µl of template DNA and was incubated at 4°C overnight. For cloning of PCR fragments, 3 µl of the ligation reaction was co-incubated with 60 µl DH5 alpha E. coli competent cells (Stratagen) for 20 min on ice. The mixture was heat shocked in water bath at exactly 42°C for 90 sec and immediately transferred on to ice for 2 min. 750 µl LB-broth was added to the competent cells and the cells were incubated at 37°C for 90 min on a shaking incubator at 110 rpm. Then bacterial cells containing the insert DNA fragments were distributed on two ampecillin containing LB-agar plates with 20 µl X-gal and IPTG solutions and incubated at 37°C overnight. The following morning, colonies containing the DNA insert were identified using blue-white colony screening method.

In order to screen for the colonies containing the target DNA insert, three white colonies were picked from each plate and inoculated in 2 ml test tube containing ampicillin added, 650 µl LB-broth in a PCR tube containing 27 µl dwater and 3 µl 10X PCR buffer (Sigma) one after the other. Similarly, one blue colony was picked and inoculated in a PCR tube containing 27 µl dwater and 3 µl 10X PCR buffer. M13 PCR was run using M13 primers at 60°C annealing temperature and colonies containing the target insert were identified by comparing the length of amplified DNA fragments from white and blue colonies. Colonies containing the target insert were transferred into 15 ml tube containing 5 ml LB-broth and incubated overnight on a shaking incubator at 37°C for plasmid DNA isolation.

3.2.4 Plasmid DNA isolation and confirmation of sequence identity

After an over night incubation in LB-broth, the colonies were centrifuged at 18,000 rpm for 1 min to collect the pellet at the bottom of the 15 ml tube. 200 µl of suspension buffer was added to the pellets and the pellets were transferred into a new 2 ml test tube. 200 µl of

lysis solution was added and the solution was mixed by gently inverting the tubes. After adding 350 µl of neutralization/binding solutions, 500 µl column preparation solutions was added to the column tube. Following this, the solution was centrifuged shortly and the flow-through was discarded and the cleaned column was kept for the next step. The cleaned lysate was transferred into the cleaned and prepared column and was centrifuged for 1 min and the flow through was discarded. 750 µl wash solution was added and centrifuged for 1 min and the column was transferred into a new 2 ml tube. 50 µl of double distilled water was added exactly in the middle of the column and the sample was incubated for 5 min and centrifuge for 1 min to collect the plasmid DNA at the bottom of the tube. Sequencing reaction was made using 5 µl of plasmid DNA and specific primers. After the sequencing reaction is over, 3 M NaOAc, 100 mM EDTA and glycogen were added and the solution was well mixed by vortexing and transferred to 1.5 µl test tube. Next, 60 µl of 100% ethanol was added and the mixture was then centrifuged at 18,000 rpm at 4°C for 15 min. All liquid was removed and the pellets were washed 2 times in 200 µl 70% ethanol. Finally, the remaining alcohol was removed, the pellets were air dried and eluted in 40 µl of sample loading solution (SLS) (Beckman Coulter). The eluted samples were transferred to a CEQ sample plate, covered with mineral oil and sequenced using CEQ™ 8000 Genetic Analysis System (Beckman Coulter, GmBH, Germany) and the identity of a particular DNA fragment was confirmed by blasting its sequence with the bovine gene data bank (www.ncbi.nlm.nih.gov) and sequences with more than 95% identity were considered to be significant.

3.2.5 RNA isolation and array processing

Total RNA was isolated using PicoPure RNA Isolation Kit according to the manufacturer's instruction (Arcturus Biosciences, Mt. View, CA). RNA quality and yield of each sample were determined using Bioanalyzer 2100 and RNA 6000 Pico LabChip assay (Agilent Technologies Inc, Palo Alto, CA) in combination with Quant-iT™ RiboGreen Reagent according to supplied protocols (Invitrogen, Carlsbad, CA). Initial total RNA concentration across all samples were adjusted to the lowly concentrated sample (12 ng) and this amount

of total RNAs were used for the two round cDNA synthesis and subsequent in vitro-transcription according to the two-cycle eukaryotic target labelling assay (Affymetrix expression analysis technical manual: Eukaryotic sample and array processing (http://www.affymetrix.com/support/technical/manual). MEGA script in vitro transcription kit containing T7-Oligo (dT) primer and other components (MEGAscript® high yield transcription kit, Applied Biosystems, Ambions) and random primers (Invitrogen, Karlsruhe, Germany) were used for the first and second cycles of cDNA synthesis. 15 µg of fragmented and biotin-labelled complementary RNA (cRNAs) were hybridized with Affymetrix bovine Genome 430 v. 2.0 GeneChip® arrays for 16 hrs at 45°C. Post-hybridization staining and washing were performed according to manufacturer's protocols using the Fluidics Station 450 instrument.

3.2.6 Image capturing, quantification and data analysis

Array slides were scanned with a GeneChip™3000 laser confocal slide scanner (Affymetrix) and the images were quantified using Gene Chip Operating Software (GCOS, Affymetrix) version 1.2. Probe level data were imported into the R software environment (http://www.r-project.org). Data normalization and background correction were performed using guanine-cytosine Robust Multichip Average (gcRMA) function (Irizarry et al. 2003). The presence or absence of probe sets were detected using microarray suit 5 (MAS 5, Affymetrix) present/absent call (Assou et al. 2006, Mah et al. 2004). To minimize false positive signals, probe sets called absent were avoided and these called present in at least two of the three replicates were used for further analysis. Differential gene expression was analyzed using linear models for microarray (LIMMA) (Smyth 2004).

3.2.7 Gene ontology classification and identification of functional changes

MAS 5 detected and differentially expressed genes in experiments 1A, 1B and 4 were classified according to their GO terms using GO consortium (Ashburner et al. 2000) and lists of genes over expressed in OO+CCs and CCs+OO relative to those OO-CCs and CCs-

OO respectively, were uploaded into Ingenuity Pathways Analysis (IPA), (Ingenuity Systems, www.ingenuity.com) to identify relationships between the genes of interest and to uncover common processes and pathways in the positive phenotypes.

3.2.8 Semi-quantitative and qRT-PCR validation of microarray data

Independent oocyte and CCs samples representing five pools of biological replicates (n=150) were used for total RNA isolation and cDNA synthesis to validate the microarray data using qRT-PCR. In order to reduce the variability in the concentration of the initial RNA populations across samples and to take equivalent RNA quantity to that used for array hybridization, 12 ng of the total RNAs of each sample were used for cDNA synthesis using oligo (dt)25 and random primer as described elsewhere (Whelan et al. 2003). The ABI prism® 7000 apparatus (Applied Biosystems) was used to perform the quantitative analysis using SYBR® Green JumpstartTM Tag Ready MixTM (Sigma) incorporation for dsDNA specific fluorescent detection dye. Standard curves were fitted for both target genes and internal control (18S rRNA) using serial dilutions of plasmid DNAs containing 10^1-10^9 molecules and run in separate wells. The appropriateness of 18S as internal control for this study was confirmed by the results of semi-quantitative and qRT-PCR showing its stability across all samples (Figure 24). PCR was assembled using 20 µl total reaction volume containing dwater, forward and reverse primers, SYBR green universal master mix (Sigma) and 2 µl template cDNAs using five replicates for each sample. 2 µl of dwater was used as a negative control for the RT-PCR. During each reaction, samples from the same cDNA were run in duplicate to control the reproducibility of the results. A universal thermal cycling parameter (initial denaturizing step at 95ºC for 3 minutes, 45 cycles of denaturizing at 95ºC for 30 seconds and 58ºC for 30 seconds) were used to quantify the mRNA expression level. After the end of the last cycle, dissociation curve were fitted by starting the fluorescence acquisition at 60ºC and taking measurements every 7 sec interval until the temperature reaches 95ºC. Final quantitative analysis was done using relative standard curve method and the expression values of the target transcripts were normalized to that of 18S. The mean normalized data were reported as the amount of a given target gene

transcript in the two samples compared and significantly different means were identified using t-test ($P < 0.05$).

3.2.9 Immunofluorescence staining

In order to localize the proteins of some differentially expressed transcripts, ovarian sections were washed three times in PBS and fixed in 4% (w/v) Paraformaldehyde overnight at 4°C. The fixed specimens were permeabilized during 2.5 hr incubation in 0.5% (v/v) Triton-X100 (Sigma) in PBS. To inhibit non-specific binding of the antibodies, samples were subsequently blocked in 3% (w/v) bovine serum albumin (BSA) in PBS for 1 hr. The sections were then incubated for 1 hr at 39°C and mounted onto glass slides with gelvatol. The primary antibody for IRF6 (rabbit anti-human polyclonal antibody, Santa Cruz Biotechnologies Inc., Germany) was used at 1:100 in PBS and for MSX1 (rabbit anti-human polyclonal antibody, Lifespan Biosciences, USA) at 1:50 in blocking solution. The samples were incubated for 15 and 1 hr with primary and secondary antibodies (FITC conjugated goat anti-rabbit secondary antibody (Lifespan Biosciences, USA), respectively using 1:100 ratio in both cases. Negative controls were processed in the same manner by omitting the use of primary antibody. In order to visualize the nucleus, the sections were finally incubated in 0.1 mg/ml 4'-6-Diamidino-2-phenylindole (DAPI, Sigma) or propidium iodide (Sigma). After the final wash in PBS, the sections were mounted on glass slides and visualized on ApoTome microscope (ApoTome MicroImaging Inc., Carl-Zeiss, Germany).

4 Results

4.1 Specific transcription programs is exhibited by bovine oocytes and CCs

The Affymetrix Bovine Genome 430 v. 2.0 GeneChip® Array contains 24016 probe sets that represent 11073 full-length genes, 5004 ESTs and 7939 non-ESTs. In order to get an insight into specific transcription program in bovine oocytes and CCs, we analyzed transcriptome profile of GV and MII oocytes and their companion CCs using MAS 5 present or absent call as described else where (Assou et al. 2006, Su et al. 2007). The analysis showed that of 13162 detected probe sets, 1516 and 2727 are exclusively expressed in GV oocytes and their companion CCs, respectively, while 8919 are expressed in both (Supplemental Tables 1, 2, 3). Similarly, of 13602 detected probe sets, 1423 and 3100 are expressed exclusively in MII oocytes and their companion CCs, respectively and 9079 are expressed in both (Supplemental Tables 5, 6, 7). Differential gene expression analysis of these detected probe sets showed that a total of 8612 transcripts are differentially expressed between GV oocytes and CCs of which 4304 and 4308 are over expressed in oocytes and CCs respectively, (Supplemental Table 4). Similarly, a total of 8863 transcripts were differentially expressed between MII oocytes and CCs of which 4271 and 4592 were over expressed in MII oocytes and CCs, respectively (Supplemental Table 8). Supplemental tables from all microarray data are available online at lwf.uni-bonn.de/institute/itw/tierzucht und tierhaltung. The heat map and hierarchical clustering of the top differentially expressed genes between oocytes and CCs at the two developmental stages are shown in figures 6 and 7.

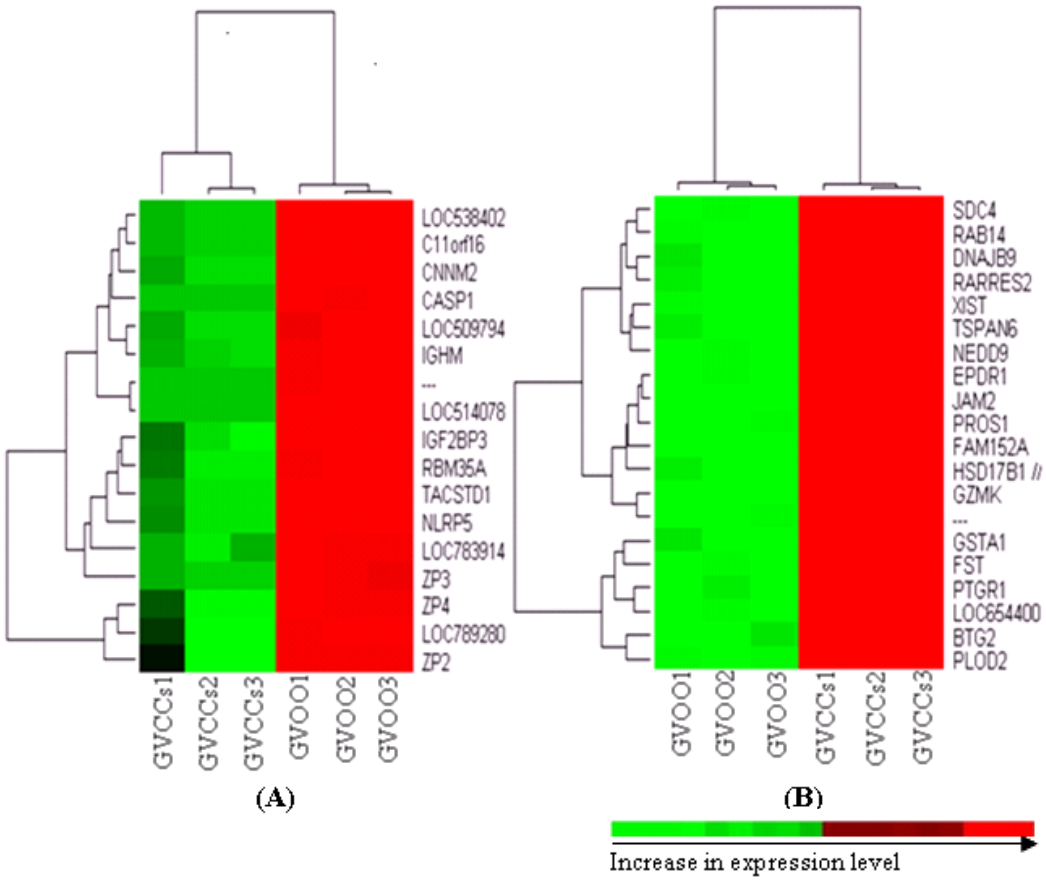


Figure 6: Hierarchical clustering and heat map of the top genes over expressed in GV oocytes (A) and CCs (B) with a fold change of more than 1024. Abbreviations, GVOO and GVCCs stand for germinal vesicle oocyte and cumulus cells, respectively. Numbers, 1, 2, and 3 indicate the three technical replicates used for microarray hybridization.

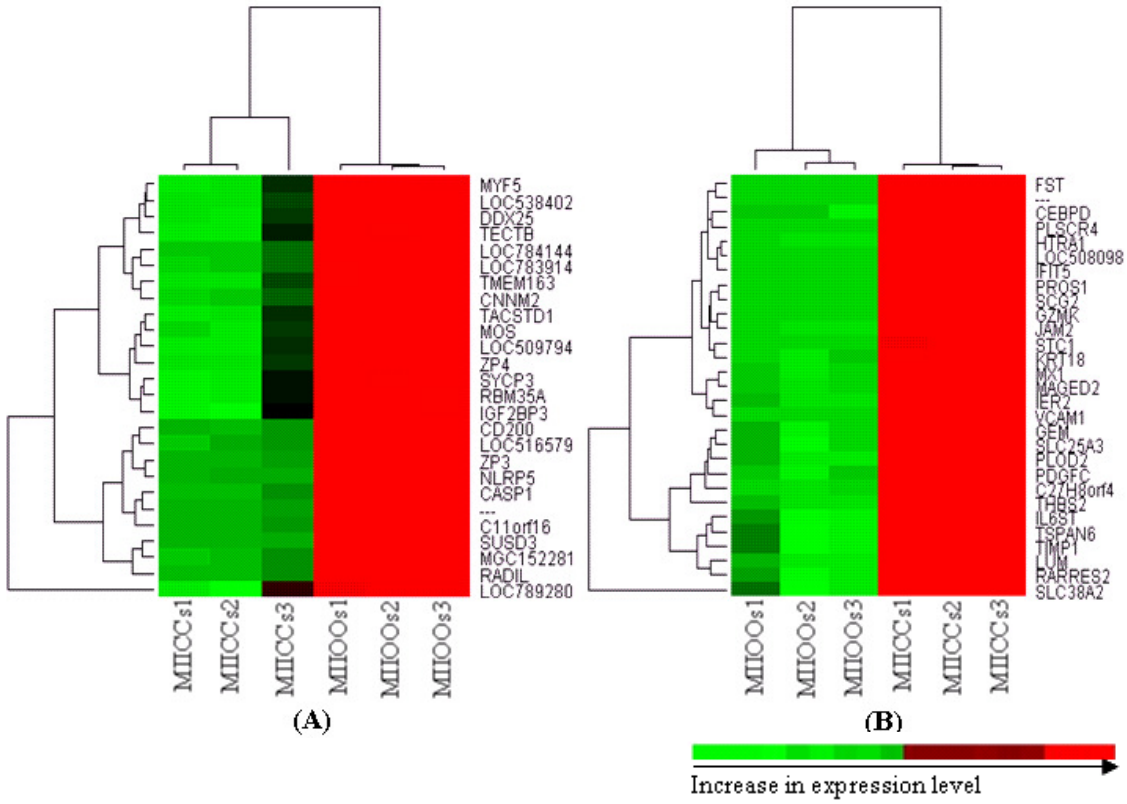


Figure 7: Hierarchical clustering and heat map of the top genes over expressed in MII oocytes (A) and CCs (B) with a fold change of more than 512. Abbreviations, MIIOO and MIICCs stand for metaphase II oocyte and cumulus cells respectively. Numbers (1, 2, and 3) indicate the three technical replicates used for microarray hybridization.

The GO analysis revealed that genes differentially expressed between oocytes and CCs at the two stages are involved in various molecular functions (Figures 8 and 9). Transcripts over expressed in GV oocytes relative to their companion CCs include those involved in transforming growth factor beta receptor activity (ACVR2B, ACVR1, BMPR1A, ACVR2A), nucleic acid binding (GTF2A2, GTF2E2, GTF2F1, GTF2F2, GTF2H2, GTF2H5), DNA dependent ATPase activity (MCM2, 3, 4, 5, 6, SMARCAL1), protein kinase activity (MAP2K6, MAP2K7, MAP3K3, MAP4K2, MAPK10, MAPK6, MAPKAPK2, MAPKAPK3, MAPKAPK5, MAP2K1). Similarly, transcripts over expressed in GV CCs relative to oocytes include those involved in structural molecular activity (RPL23, 24, 30, 34, 37, RPLP2, RPL23), isomerase activity (FKBP10, FKBP11,

FKBP1A, FKBP2, FKBP3, PTGDS, PTGES, PTGES3), oxido-reductase activity (ACADVL, ACADM, ACAD10, ACADL), translation regulator activity (TCEA2, TCEA3, EIF4B, EEF2, EEF1A1, EIF3D, EIF2S3, EEF1E1, ETF1), catalytic activity (ATP1A1, ATP1B1, ATP1B3, ATP2A2, ATP2B1, ATP5A1, ATP5B, ATP5D), insulin like growth factor binding (IGF1R, IGFBP4, IGFBP5).

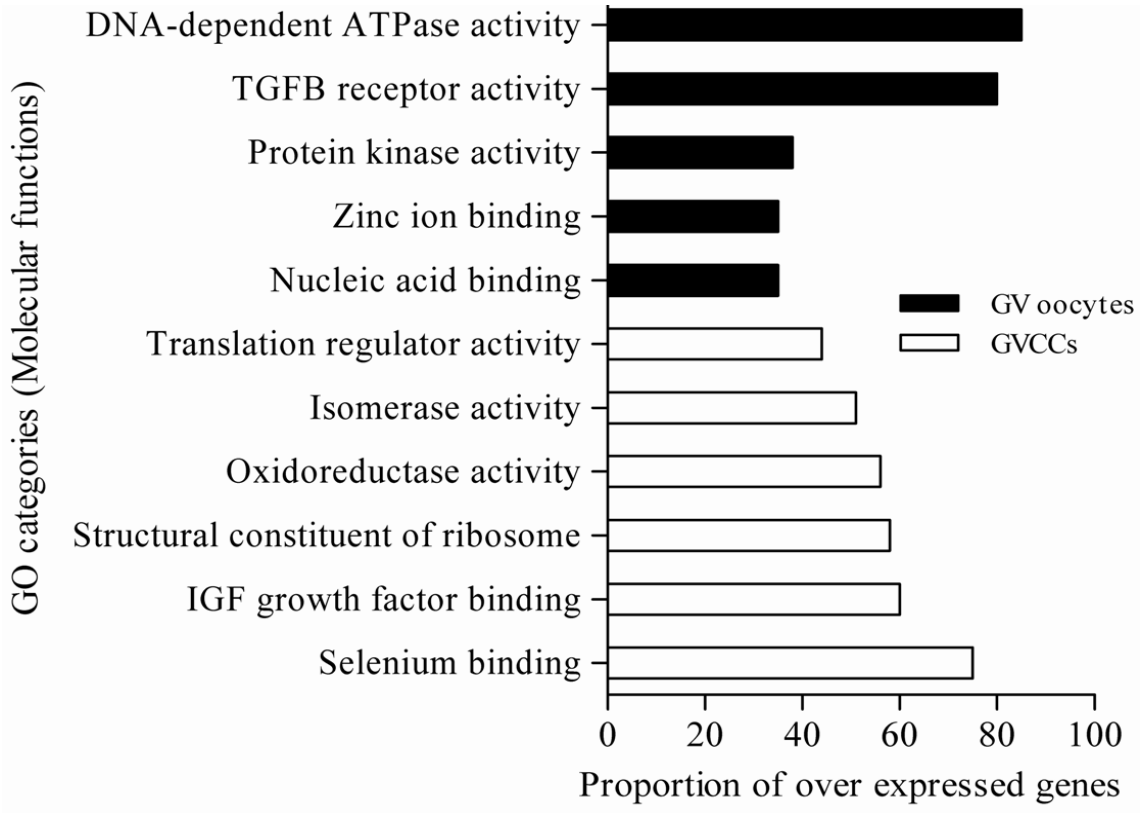


Figure 8: The top significantly changed GO terms (molecular functions) with the proportion of transcripts over expressed in GV oocytes and CCs relative to each other ($P < 0.001$). The proportion of transcripts in a GO term was calculated as the number of genes over expressed in one sample divided by the total number of genes that are involved in a given GO term multiplied by 100.

Transcripts over expressed in MII oocytes relative to their companion CCs are involved in homocysteine S-methyltransferase activity (MTR, BHMT), 6-phosphofructo-2-kinase activity (PFKFB1, 2), DNA dependent ATPase activity (MCM2, 3, 4) and those over expressed in MII CCs relative to their enclosed oocytes are involved in nucleic acid binding

(CREB1, 3, CREB3L3, CREBL2, EIF2AK1, 2, EIF2B1, B2, B5), nucleotide binding (MAP2K1, MAP2K7, MAP3K4, MAP4K2, 3, MAPK13, MAPK3, 6), aminoacyl-tRNA ligase activity (HARS, FARS2, LARS2, MARS, MARS2), nuclease activity (RNASE1, RNASE6, RNASEH1, RNASEH2A, RNASET2), transcription factor binding (RAB1A, RAB2A ,NCOA1, 2, 3) and others.

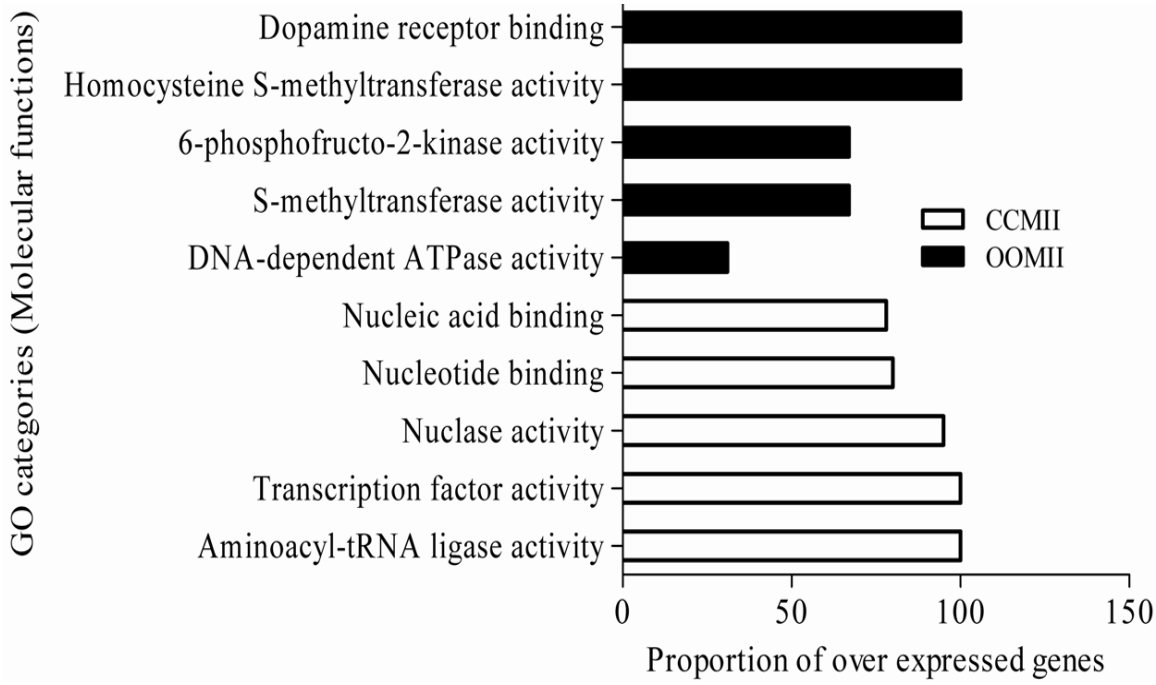


Figure 9: The top significantly changed GO terms (molecular functions) with the proportion of transcripts over expressed in MII oocytes and CCs relative to each other ($P < 0.001$).

Additionally, transcripts differentially expressed between GV oocytes and CCs are involved in various biological processes (Figure 10). While those over expressed in GV oocytes are involved in biopolymer metabolic process (BMPR1A, BMPR2, FBXO15, 25, 32), RNA metabolic process (EXOSC3, 4, 8, 9), response to DNA damage stimulus (LIG3, POLB, SMC3, UBE2N), DNA metabolic processes (POLD1, POLD3, POLG2, PTTG1), DNA replication (RFC2, 3, 4, 5), transcription regulation (GTF2A2, GTF2E2, GTF2F1, GTF2F2, GTF2H2), those over expressed in GV CCs are involved in macromolecule biosynthetic process (RPL19, 21, 23, 24, 26, 27), translation (EEF1B2, EEF1G, EEF2, EIF1, EIF2B2, EIF2B5, EIF2S2, EIF2S3, EIF3D, EIF3G), metabolic process (ALDH18A1,

ALDH3B1, ALDH7A1, ALDH9A1), cell cycle arrest (IL8, INHBA, GADD45A, CDKN1A, DDIT3), response to oxidative stress (GCLC, GCLM, GPX1, GPX3, GPX4) and others.

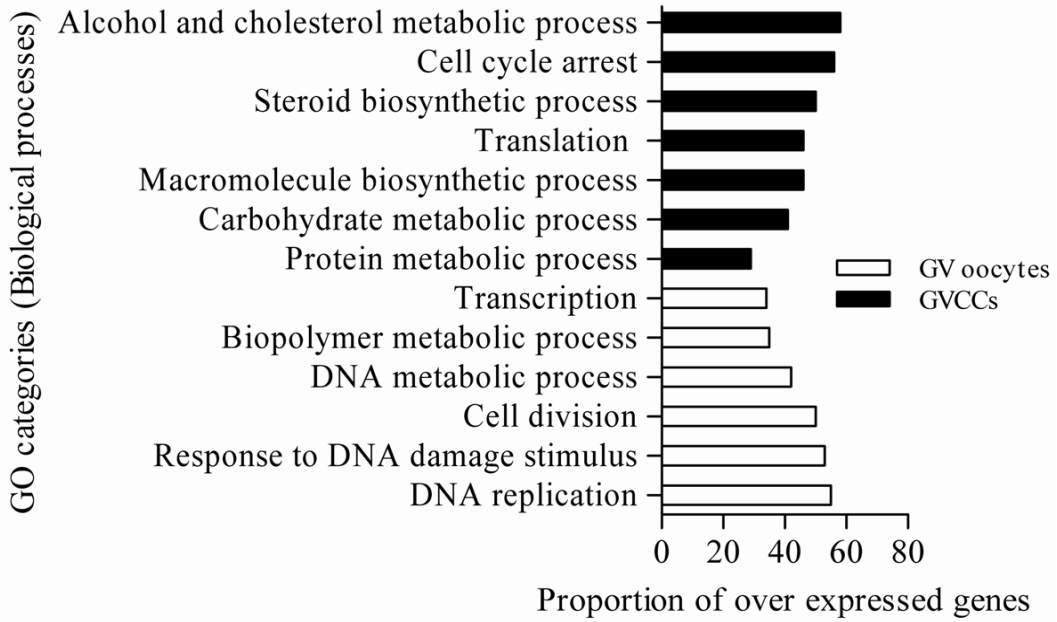


Figure 10: The top significantly changed GO terms (biological processes) with the proportion of transcripts involved among those over expressed in GV oocytes and CCs ($P < 0.01$).

4.2 Removal of ooplasm or CCs before in vitro maturation alters the gene expression of either cell types at MII

In order to investigate the transcriptome profile changes when the oocyte matures with or with out its companion CCs and when CCs mature with or with out their enclosed ooplasm, we analyzed their corresponding transcriptome profiles using LIMMA as described previously (Smyth 2004). The analysis showed that a total of 265 genes are differentially expressed between OO+CCs and OO-CCs of which 217 and 48 are over expressed in OO+CCs and OO-CCs, respectively (supplemental Table 9). Similarly, 566 genes are differentially expressed between CCs that were matured with (CCs+OO) or with out (CCs-OO) their enclosed ooplasm of which 320 and 246 are over expressed in CCs+OO and CCs-OO, respectively (supplemental Table 10). The heat map and hierarchical clustering of

the top differentially expressed genes between OO+CCs and OO-CCs and OO+CCs and OO-CCs respectively, are presented in figures 11 and 12.

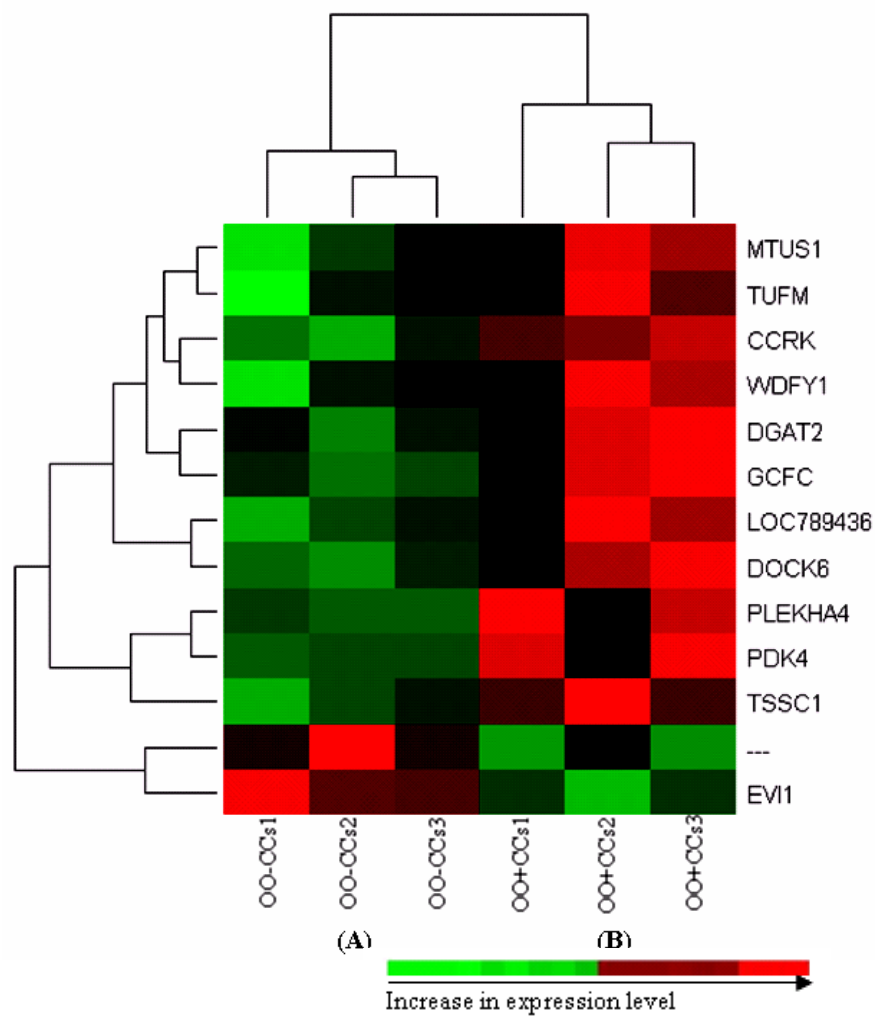


Figure 11: Hierarchical clustering and heat map of the top differentially expressed genes between oocytes matured with (red bar) or with out (green bar) their companion CCs with a fold change of more than 4. Abbreviations, OO+CCs and OO-CCs stand for oocytes matured with or with out their companion CCs, respectively and numbers 1, 2 and 3 indicate the three technical replicates that were used for microarray hybridization in each group.

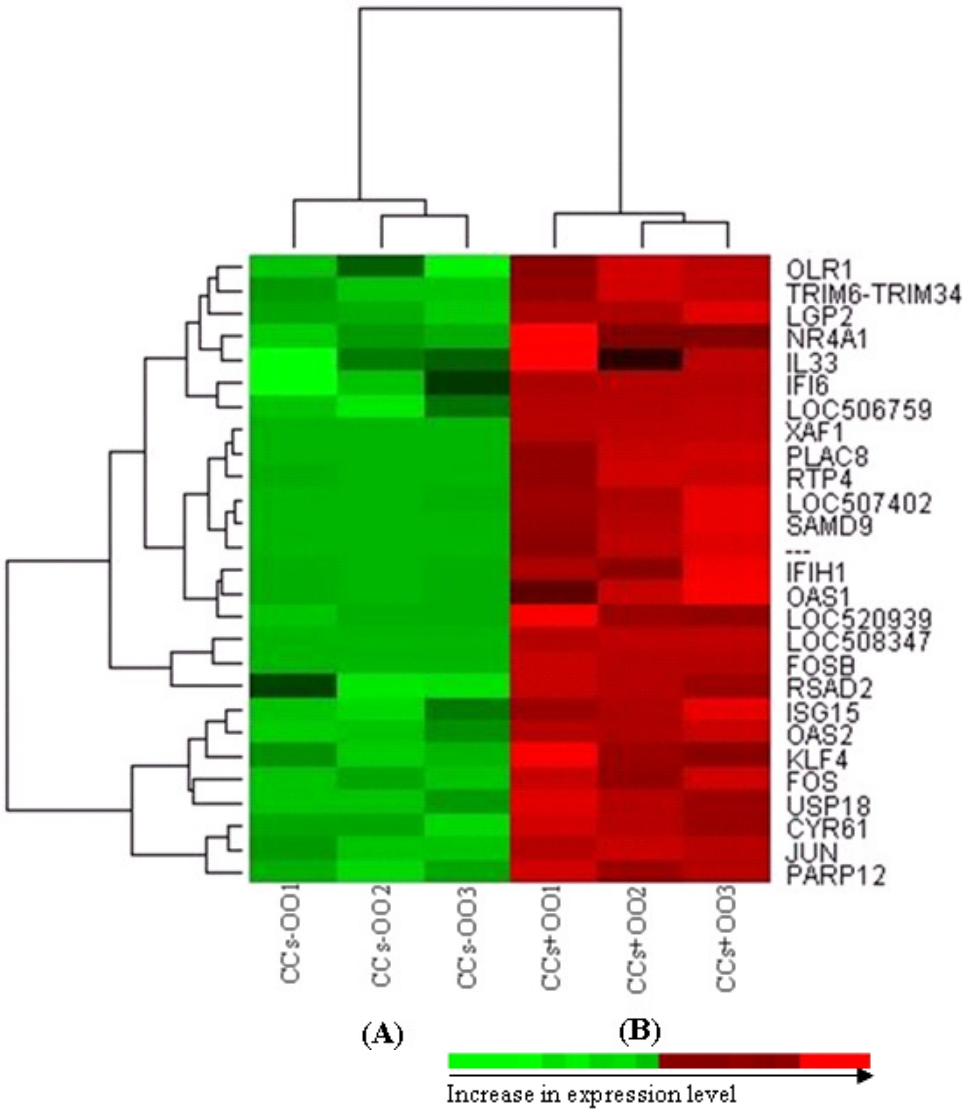


Figure 12: Hierarchical clustering and heat map of the top differentially expressed genes between CCs matured with (red bar) or with out (green bar) their enclosed ooplasms with a fold change of more than 16. Abbreviations, CCs+OO and CCs-OO stand for cumulus cells matured with or with out ooplasm respectively and numbers 1, 2 and 3 indicate the three technical replicates that were used for microarray hybridization in each group.

We found that 36 and 375 of the genes over expressed in OO+CCs and CCs+OO respectively could be assigned to a specific functional group based on the information in the IPA Knowledge Base. Only 4 of the mapped genes over expressed in OO+CCs group,

representing about 1.5% of the total, are classified under the functional group "Carbohydrate metabolism," which contains genes involved in energy conversion and modulation. Other functional groups, including molecular transport, nucleic acid metabolism, small molecule biochemistry and RNA post transcriptional modification are also observed. Similarly, 90 of the genes over expressed in CCs+OO, representing 34% of the total are classified under cellular growth and proliferation. A graphical representation of this functional classification of the genes over expressed in OO+CCs and CCs+OO relative to OO-CCs and CCs-OO respectively are shown in figures 13 and 14, in which 16 and 12 functional groups with higher P values are noted. Some of these groups shared several common genes.

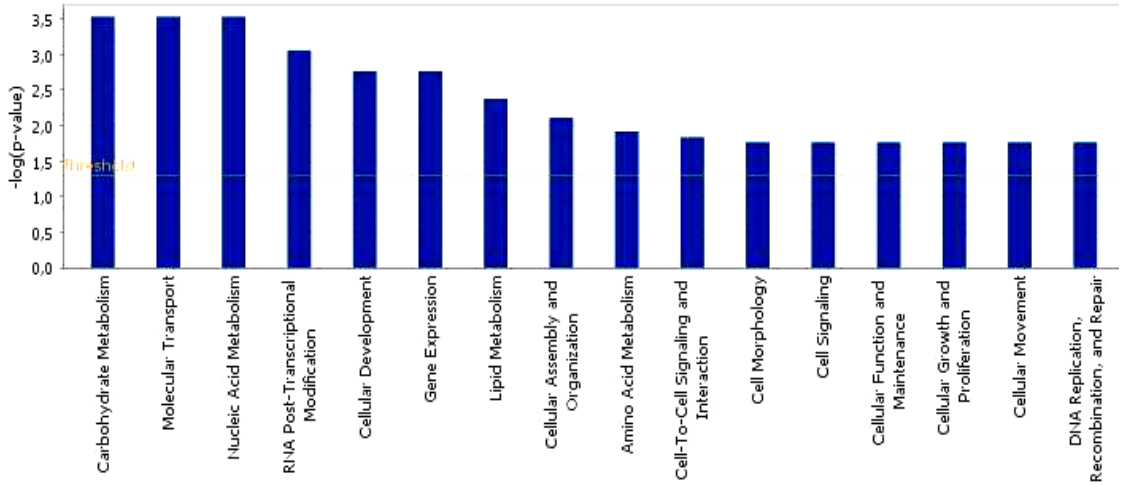


Figure 13: Functional grouping of the genes that are over expressed in the OO+CCs relative to OO-CCs samples showing the most significant functional groups ($P < 0.05$). The bars represent the P-value in logarithmic scale for each functional group.

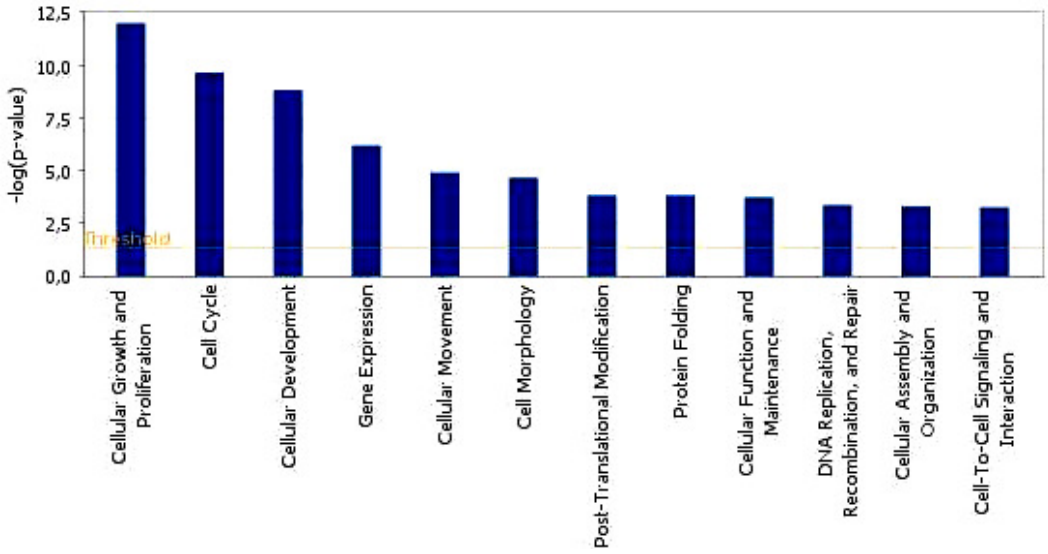


Figure 14: Functional grouping of the genes that are over expressed in the CCs+OO relative to CCs-OO samples showing the most significant functional groups ($P < 0.05$). The bars represent the P-value in logarithmic scale for each functional group.

In addition, 28 and 23 of the genes over expressed in OO+CCs and CCs+OO, relative to OO+CCs and CCs-OO, respectively, are assigned to 5 and 8 different canonical pathways (Figures 15A and B).

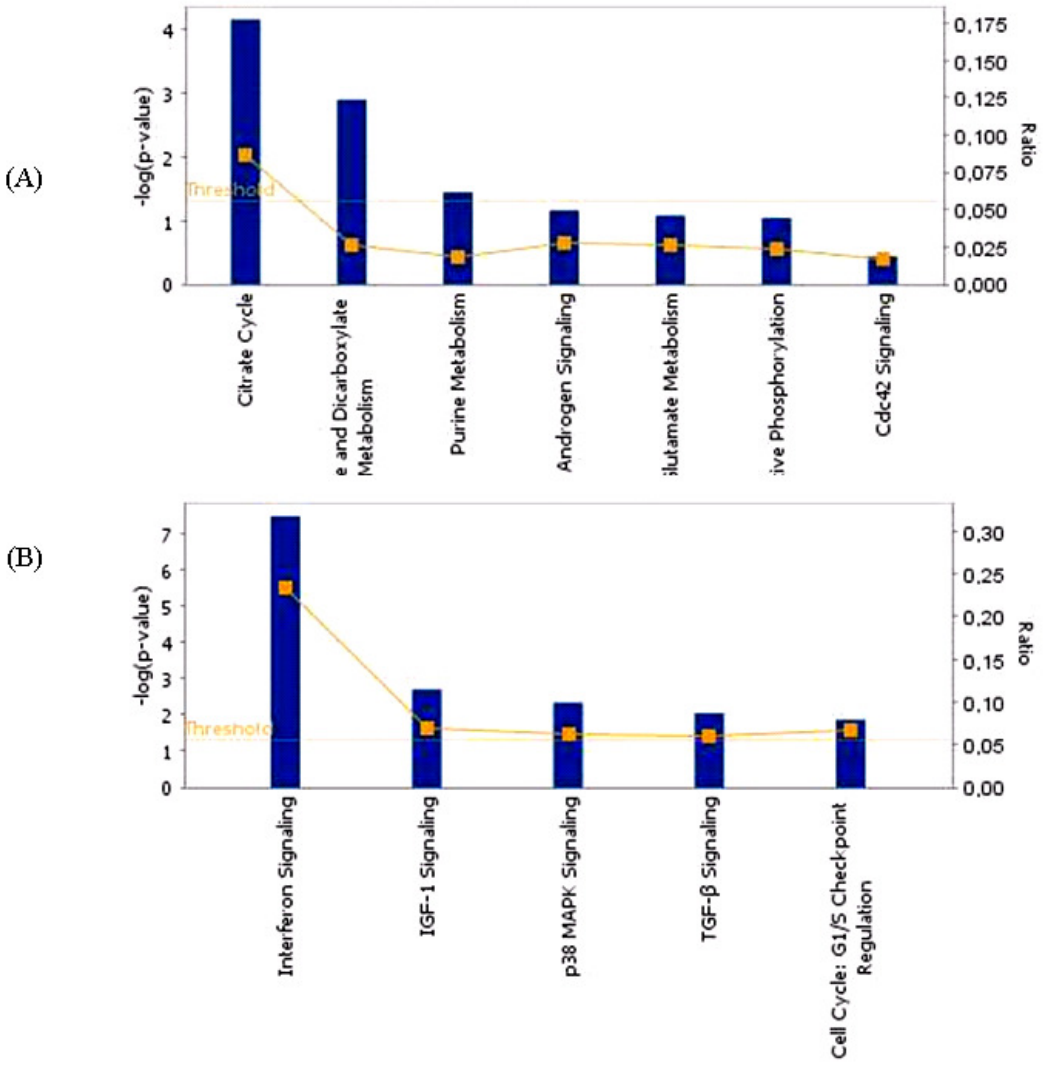


Figure 15: The most prominent canonical pathways involving genes that are over expressed in OO+CCs relative to OO-CCs (A) and those over expressed in CCs+OO relative to CCs-OO (B) ($P < 0.05$). The bars represent the P-value for each pathway. The orange irregular line is a graph of the ratio of genes from the data set to the total number of genes involved in the pathway for the different pathways.

Finally, these genes from the two groups are mapped on 5 top networks each network containing genes from the input data that shared known direct or indirect relationships. Examples of networks created from these data are shown in figures 16 and 17, where the relationships between molecules that are over expressed in OO+CCs and CCs+OO relative

to OO-CCs and CCs-OO respectively, are represented by the arrows that connect them. Figure 16 shows a complex network that plays an important role in gene expression, small molecule biochemistry and carbohydrate metabolism while figure 17 shows a network that plays a role in cellular development.

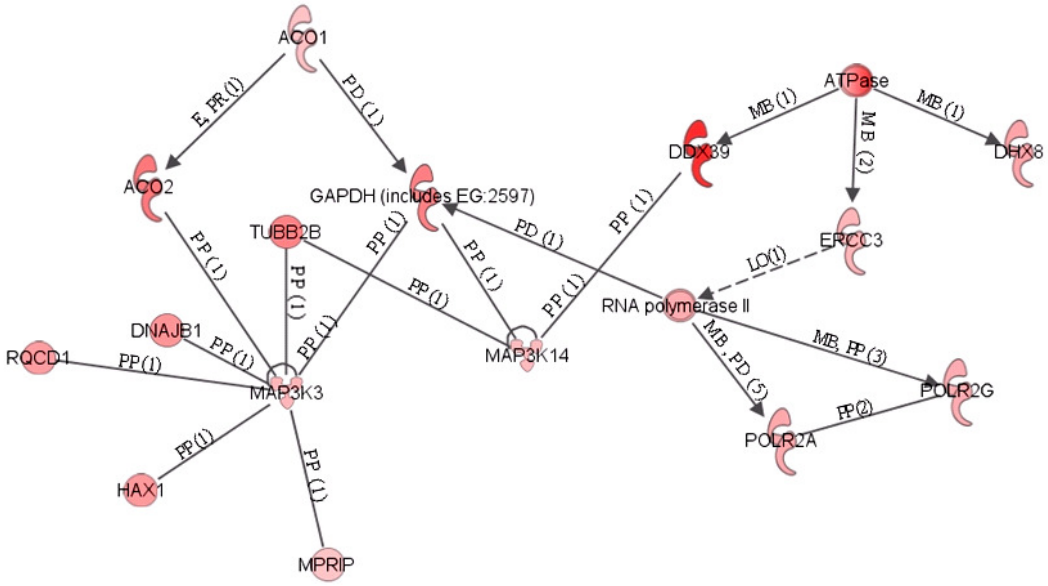


Figure 16: An example of a gene network, showing the relationships between molecules identified by the microarray and over expressed in oocytes matured with their companion CCs. The type of the association between two molecules is shown as a letter on the line that connects them. The number in parenthesis next to the letter represents the number of bibliographic references currently available in the Ingenuity Pathways Knowledge Base that support each one of the relationships. Direct or indirect relationships between molecules are indicated by solid or dashed connecting lines, respectively. P = phosphorylation, A = gene activation, E = increase in expression, PP = protein-protein interaction, PD = protein-DNA binding, MB = membership in complex, LO = localization, L = proteolysis, RB = regulation of binding

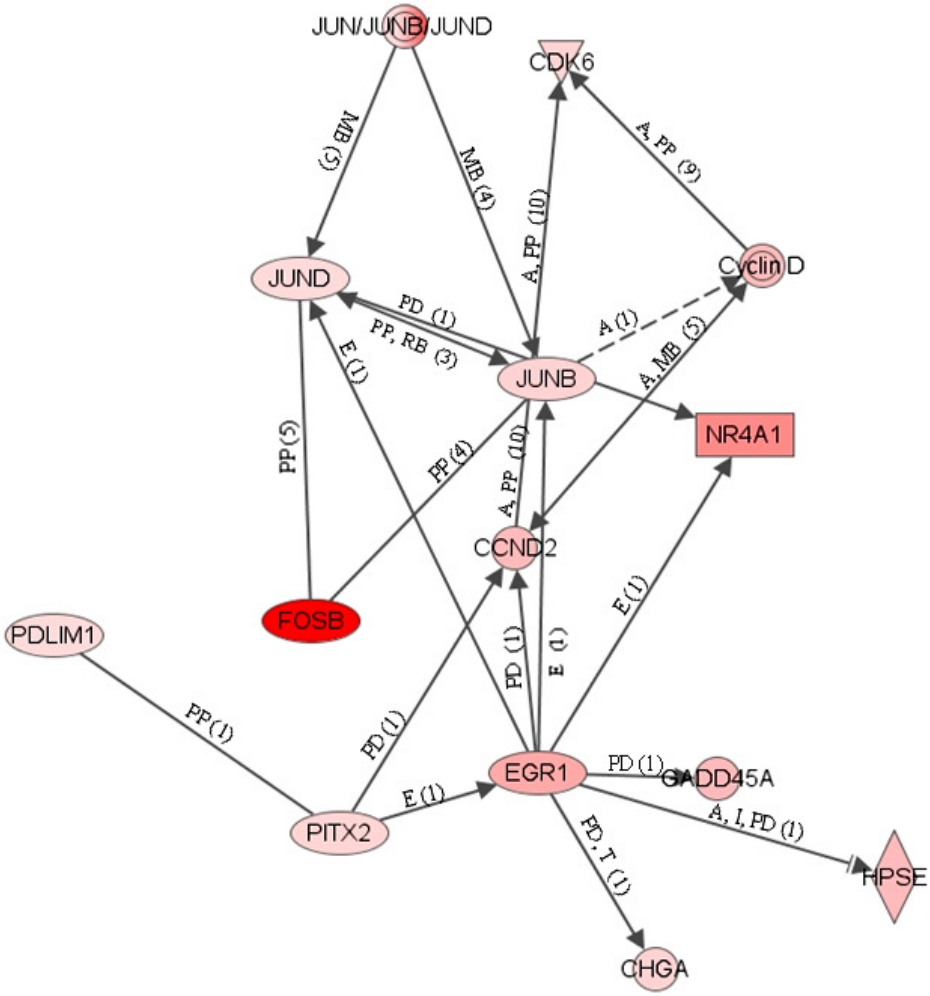


Figure 17: An example of a gene network, showing the relationships between molecules identified by the microarray and over expressed in CCs matured with the enclosed ooplasm relative to those matured with out.

4.3 Global transcriptome profile change and the associated functional shifts in the CCs during COCs in vitro maturation

In this experiment, we analyzed CCs transcriptome profile changes during COCs IVM. The results of MAS 5 present and absent call showed that of 12827 detected probe sets 4689 and 834 are expressed in GV and MII CCs respectively while 7304 are expressed commonly at both stages (Supplemental Tables 11, 12, 13). Differential expression analysis of these detected probe sets showed that a total of 4677 genes are differentially expressed

between the two samples of which 2397 and 2280 are over expressed in GV and MII stages respectively (Supplemental Table 14). The heat map and hierarchical clustering of the top differentially expressed genes between CCs from the two stages are indicated in figure 18.

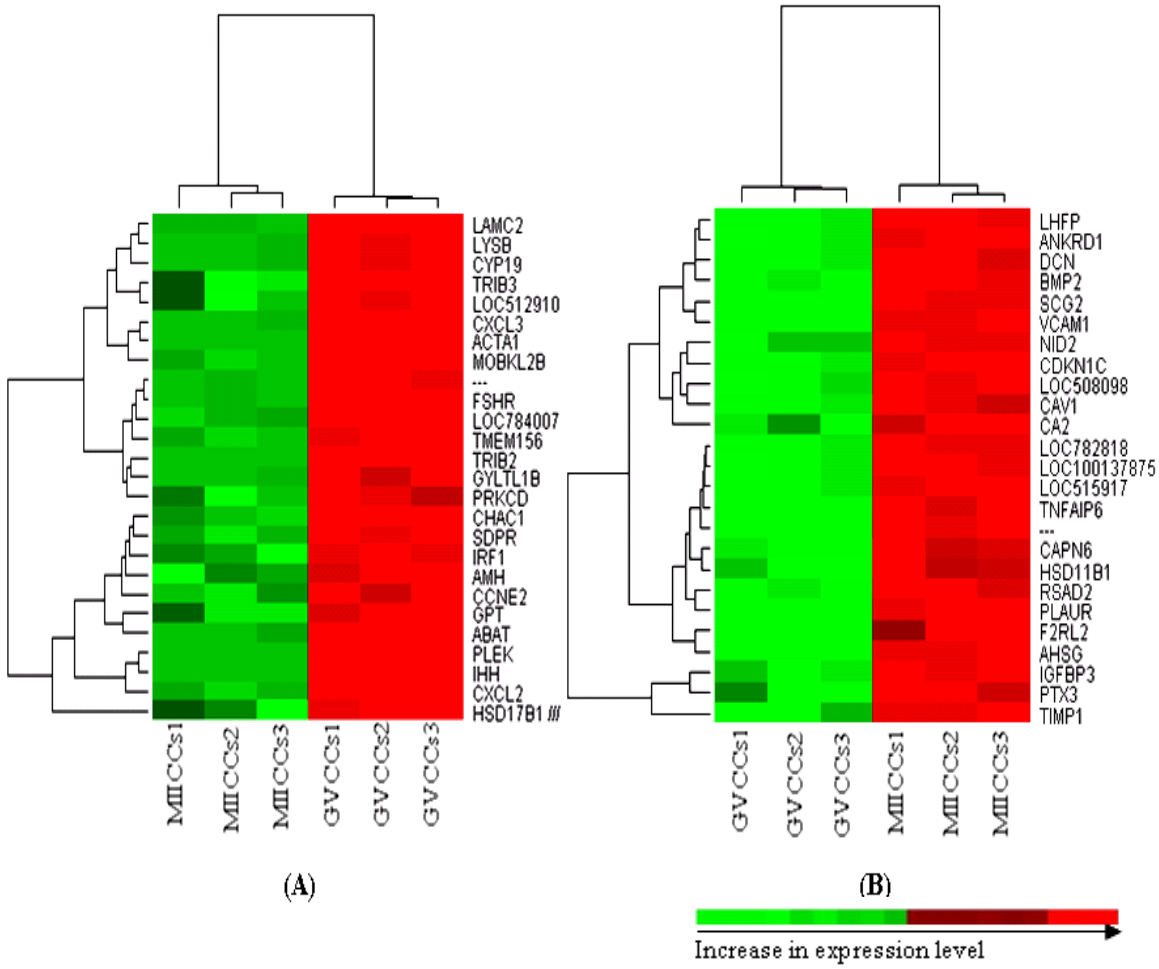


Figure 18: Hierarchical clustering and heat map of some of the top differentially expressed genes between GV CCs (A) and MII CCs (B) with a fold change of more than 256. Abbreviations, GVCCs and MIICCs stand for germinal vesicle and metaphase II cumulus cells respectively, and numbers 1, 2 and 3 indicate the three technical replicates that were used for microarray hybridization in each group.

Analysis of the GO terms revealed that genes over expressed in GV CCs are involved in various cellular and molecular functions including those involved in DNA replication, recombination and repair (MCM2, 3, 4, 5, POLA1, POLB), cell cycle (CCNA2, CCNB1,

CCNB2, CCND2, CCNE2), cellular assembly and organization (ACTA1, ACTA2, ACTN1) (Figure 19) and biological processes such as signal transduction (CDC2, UACA, SFN, MSH2), cholesterol biosynthetic process (IDI1, MVD, FDFT1, APOA1), DNA replication (ORC1L, ORC3L, ORC4L, RFC4, RFC5), steroid biosynthetic process (FDXR, LSS, IDIL, DHCR7, CYP51) and others (Figure 20).

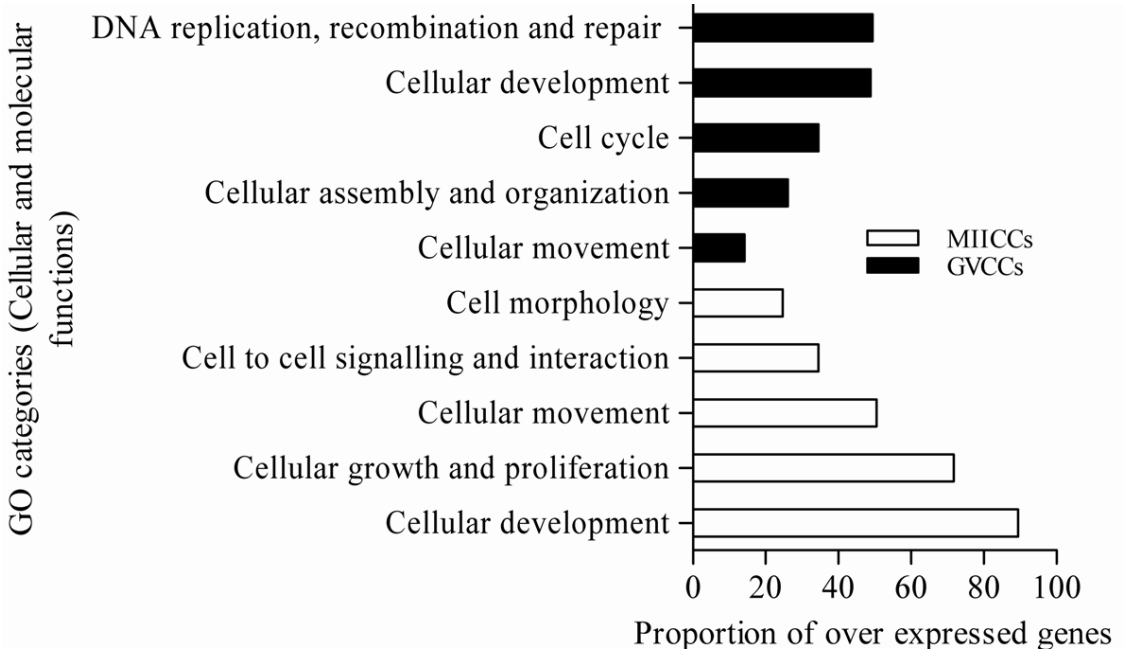


Figure 19: The top significantly changed GO terms (molecular functions) with the proportion of transcripts involved among those differentially expressed between in GV and MII CCs ($P < 0.001$). The proportion of transcripts in a GO term was calculated as the number of genes over expressed in one sample divided by the total number of genes that are involved in a given GO term multiplied by 100.

Similarly, genes over expressed in MII CCs are involved in various cellular and molecular functions including cellular development (IFI6, MMP9, SCG2, CASP4), cellular growth and proliferation (BMP4, IGFBP3, 5, 6, TGFB2), cellular movement (CXCR4, NR2F1, ALCAM) (Figure 19) and biological functions including actin filament polymerization (ANG, ARPC5L, TMSB10), cellular component organization and biogenesis (MAP2, MAP6D1, CAPG, GSN), protein metabolic process (ANG, CAPG, TMSB10) and others (Figure 20).

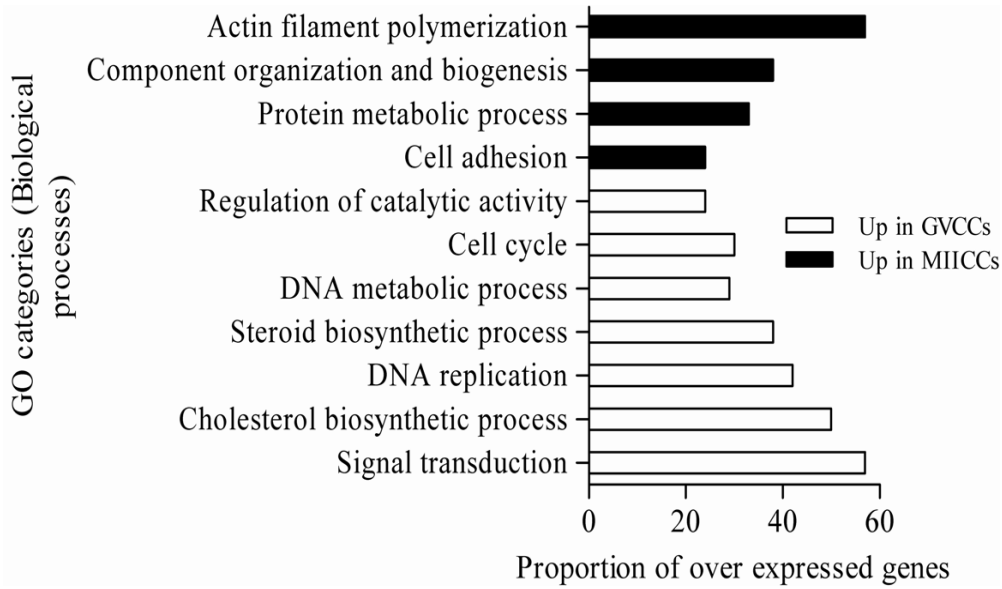


Figure 20: The top significantly changed GO terms (biological processes) with the proportion of transcripts involved among those over expressed in GV and MII cumulus cells relative to each other ($P < 0.001$).

4.4 Validation of the microarray data using quantitative and semi quantitative RT-PCR

In order to validate the micro array data, a total of 23 differentially expressed transcripts were selected and quantified using qRT-PCR as described in materials and methods section. With the exception of one transcript, the results of qRT-PCR analysis validated the array data as the expression levels of a given transcript in the two samples compared are significantly different ($P < 0.05$) (Figures 21, 22 and 23).

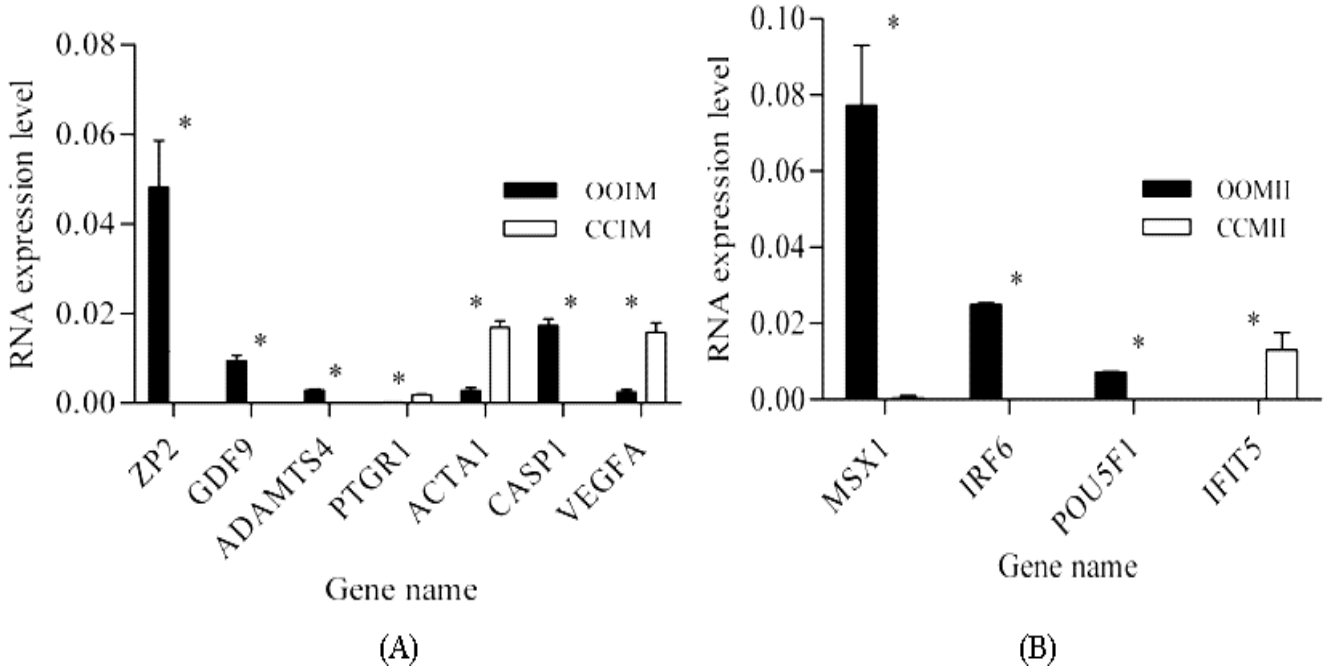


Figure 21: qRT-PCR validation of the microarray data showing some selected transcripts that are differentially expressed between GV stage oocytes and CCs (A) and between MII oocytes and CCs (B). Two bars representing the same gene and marked with star (*) between them are significantly different ($P < 0.05$). OOIM = immature (GV) stage oocytes and CCIM = immature (GV) stage CCs, OOMII = matured metaphase II oocytes and CCMII = matured metaphase II CCs.

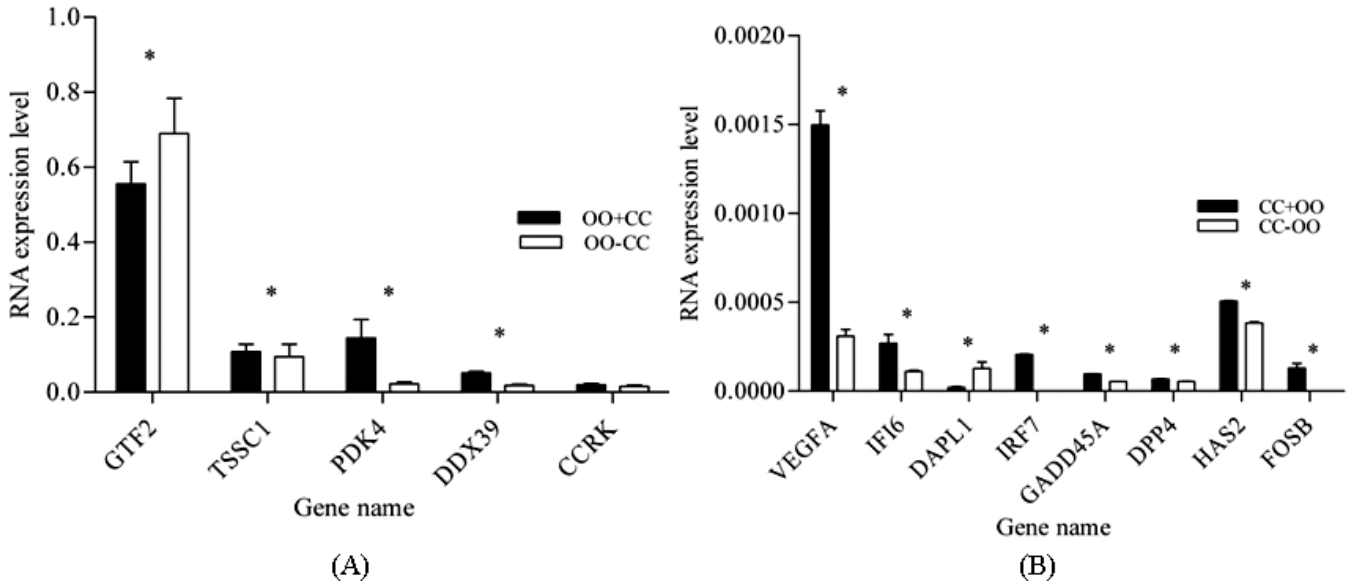


Figure 22: qRT-PCR validation of the microarray data showing some selected transcripts that are over expressed in oocytes matured with their companion CCs compared with those matured alone (A) and those over expressed in CCs matured with their ooplasm compared with those matured alone (B). Two bars representing the same gene and marked with star (*) between them are significantly different ($P < 0.05$). OO+CC = oocytes matured with CCs and OO-CC = oocytes matured with out CCs, CCs+OO = cumulus cells matured with their ooplasm, CCs-OO = cumulus cells matured with out their ooplasm.

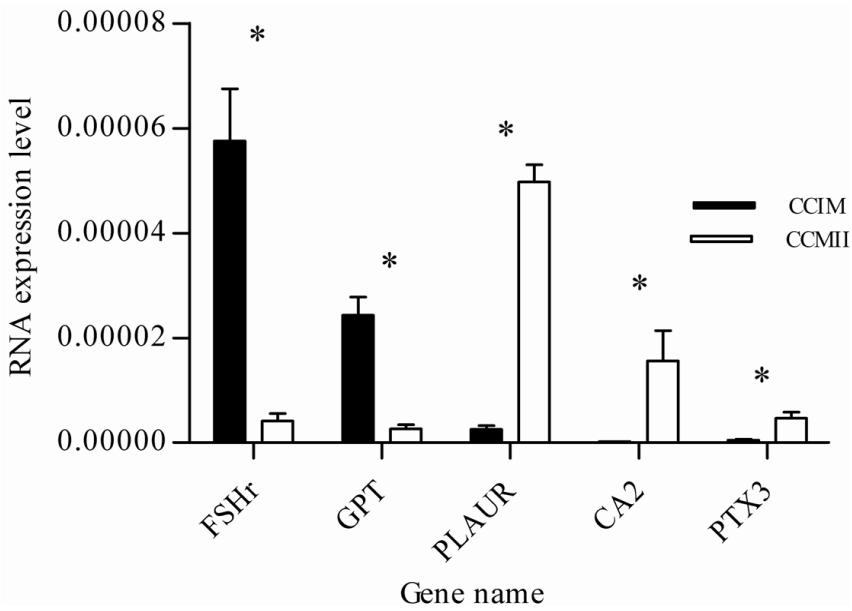


Figure 23: qRT-PCR validation of the microarray data showing some selected transcripts that are differentially expressed between GV and MII CCs. Two bars representing the same gene and marked with star (*) between them are significantly different ($P < 0.05$). CCIM = immature or GV stage CCs and CCMII = matured or metaphase II CCs.

4.5 Protein localisation using immunofluorescece staining

In addition to real time qRT-PCR and semi-quantitative PCR, the microarray data was also validated at protein level for few selected genes (IRF6 and MSX1). The results of immunofluorescence staining clearly indicated that IRF6 is exclusively expressed in oocyte while MSX1 is expressed in both oocyte and CCs but more abundantly in the former (Figure 25).

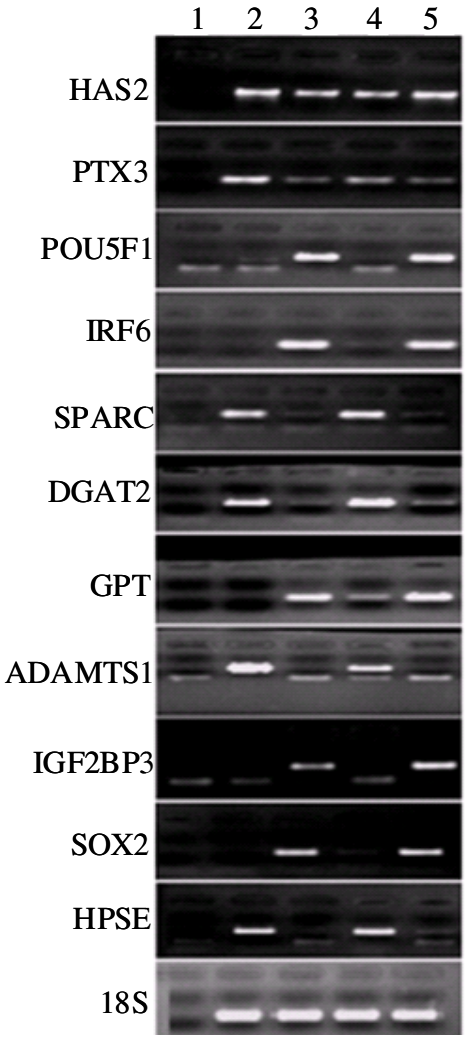


Figure 24: Semi-quantitative RT-PCR validation of the microarray data for transcripts that are commonly expressed between oocytes and CCs and those exclusively expressed either in oocytes or CCs. Number 1 shows a negative control (no template), 2, 3, 4 and 5 show the abundance levels of each transcript in MIICCs, MII oocytes, GVCCs and GV oocytes, respectively. 18S was used as a loading control for total RNA.

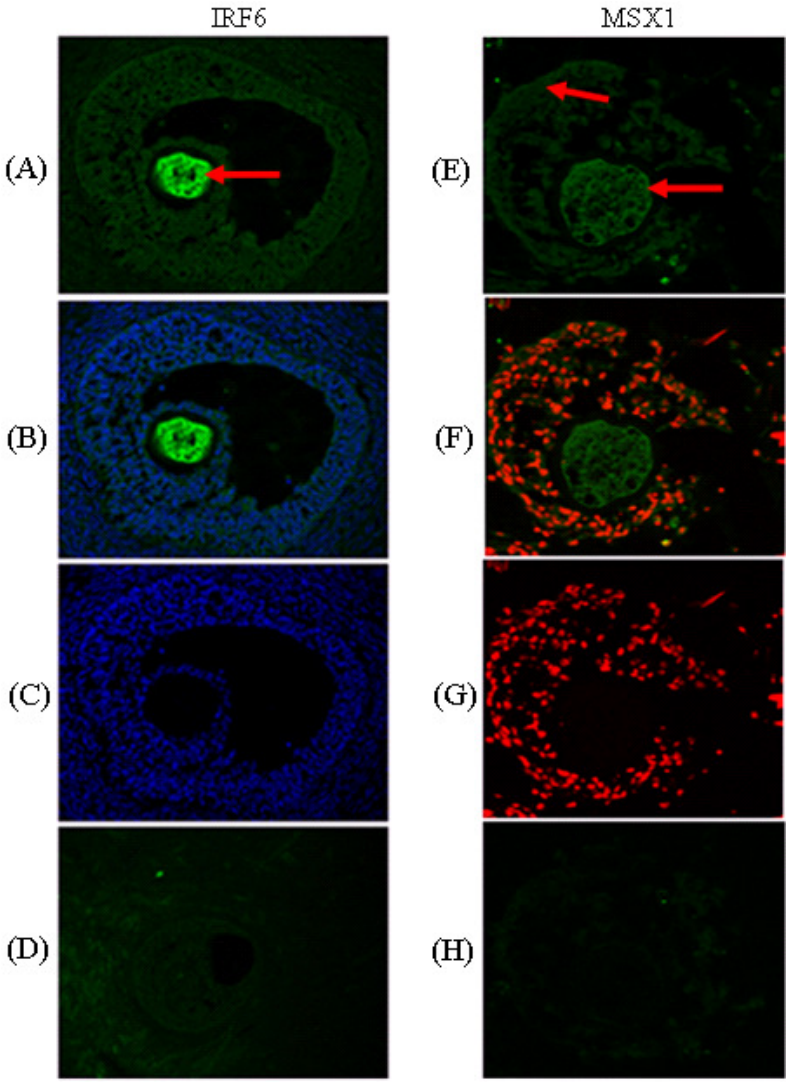


Figure 25: Immunoflourescence images taken from sections of ovarian follicles showing the protein of IRF6 expressed only in oocytes and that of MSX1 expressed in both oocyte and CCs. Lanes A and E. = ovarian sections incubated with IRF6 and MSX1 primary antibodies showing the location of IRF6 and MSX1 proteins, respectively. B and F = Protein and nuclear staining showing IRF6 and MSX1 proteins and DNAs together. C = Sections incubated only with DAPI showing the nucleus of the cells D. Negative control staining in which the sections were incubated only with secondary antibodies. The red arrows indicate the location of each protein.

5 Discussion

The bidirectional communication between an oocyte and its companion CCs is crucial for development and functions of both cell types. The transcriptome profiles of bovine oocytes and their companion CCs and transcriptome profile changes when either cell matures with or with out the other was analysed using Affymetrix GeneChip Bovine Genome array containing more than 24000 probe sets. It is for the first time that transcripts that are exclusively expressed in bovine oocytes or CCs at GV and MII stages are identified. Additionally, tThe effect of removing the bidirectional communication axis on the gene expression profile of either cell during in IVM and the transcriptome profile of CCs at GV and MII and functional changes associated with differentially expressed genes between the two stages was investigated at a large scale.

5.1 Specific expression program is exhibited between bovine oocyte and CCs

In addition to the previously identified ones, this study identified several oocyte or CCs specific transcripts that might play important biological roles in the bidirectional communication of the two cell types during IVM and for the acquisition of developmental competence at latter stages. Hierarchical clustering of these genes demonstrated that oocyte expression profile is markedly different from that of its companion CCs with the latter having more number of transcripts than the former.

Significantly higher (more than 1024 fold change) and exclusive expression of GDF9, BMP15, MOS, zona pellucida proteins (ZP2, 3, 4), NLRP5, RBM35A, TACSTD1, GAS7 and others was found in oocytes compared with their companion CCs. Since the roles of GDF9 and BMP15 in oocyte growth and maturation have been widely discussed else where (Gilchrist et al. 2004, Hussein et al. 2006, McNatty et al. 2004), they are not discussed here. The c-mos proto-oncogene product MOS is believed to be an active component of the cytostatic factor that stabilizes and sustains the activity of maturation-promoting factor. Notable interspecies differences exist among different vertebrates regarding its physiological effect on oocyte maturation. However, its higher expression in oocytes both at GV and MII stages in the current study supports previous reports claiming that MOS is required both for the activation of MPF during meiosis I

and II and for the meiotic arrest of oocytes at the metaphase of meiosis II (Hunt 1992, Yew et al. 1992).

NLRP5 also known as maternal antigen that an embryo requires (MATER) is an oocyte-specific maternal effect gene required for early embryonic development beyond the 2-cells stage in mouse (Tong and Nelson 1999, Tong et al. 2000). Its transcript and protein have been detected only in oocyte from primary follicles onwards and accumulated in the cytoplasm during follicular growth (Pennetier et al. 2006) indicating its important role in acquiring development competence. RBM35A has been reported to act as a tumour suppressor in colon cancer cells and has been proposed to be involved in posttranscriptional regulation of a number of genes by exerting a differential effect on protein translation via 5' UTRs of mRNAs (Leontieva and Ionov 2009). However, data associating its expression with the development of mammalian oocytes is not available.

GAS7 has been suggested to be involved in cell migration and cell protection in mouse embryonic cells (Moorthy et al. 2005) and enhances microtubule polymerization by stabilizing sheet intermediates and is a useful tool for analyzing microtubule transformation (Uchida et al. 2009). Higher expression of this transcript in bovine oocyte is suggesting its involvement in the formation of microtubules and microfilaments, the essential components of cytoskeleton.

Transcripts detected exclusively and highly in oocytes compared with CCs also include various members of the zinc finger proteins family (ZNF10, 165, 187, 304, 397, 529), many of the members of trans-membrane proteins (TMEM30B, TMEM163, TMEM32, TMEM120B and TMEM52) and Rho GTPase activating proteins (ARHGAP10, 17, 18, 22, 24, 26, 27, 28), various members of the mitogene activated protein kinases (MAP4K2, MAPK10, MAPK8IP2), and phosphatases (PPP1R1B, PPP2R2B, PPP3R1, PPP1R3D). Although two zinc finger containing proteins have been reported to play vital role in the maturation of nematode oocytes, data showing their roles in the development of mammalian oocyte is not available.

Trans-membrane proteins are involved in oocyte-granulosa cell regulatory loop and Rho proteins play a role in GTP-bound active state and can interact with a number of

effecters to transduce signals leading to diverse biological responses including actin cytoskeletal rearrangements, regulation of gene transcriptions, cell cycle regulation, control of apoptosis and membrane trafficking (Bishop and Hall 2000, Hall 1998). Phosphorylation and dephosphorylation of proteins is also crucial and control nearly every cellular activities, including metabolism, gene transcription and translation, cell-cycle progression, cytoskeletal rearrangement, protein-protein interactions, protein stability, cell movement, and apoptosis. These processes in turn depend on the highly regulated and opposing actions of protein kinases (PKs) and phosphatases (PPs) where the balance between the two plays an important role in the control of oocyte meiotic resumption (Liang et al. 2007). Thus, higher expression of these genes may evidence that they are involved in meiotic maturation.

Like wise, some of the transcripts that are highly expressed in CCs relative to oocyte include IFIT5, BMP2, FSHR, GSTA1, FST, PTGR1, hormonal receptors and hormones such as INHA, INHBA, PGR and PGRMC2.

BMP2 is expressed in bovine antral follicles and plays a role in the development and functioning of ovarian follicles (Fatehi et al. 2005). Human oocyte and CC genes expression study has revealed that the receptor of BMP2, also a receptor for GDF9, is expressed only in CCs (Assou et al. 2006). However, the current array data showed that BMP2 is expressed only in CCs whiles its receptor mRNA is expressed in both suggesting higher activity of BMPR2 in CCs than in oocyte. GSTA1 is highly expressed in steroidogenically active cells of bovine ovarian follicle and suggested to intervene in folliculogenesis and oocyte maturation (Rabahi et al. 1999) and steroid receptor cells are found only in CCs evidencing the involvement of CCs derived GSTA1 in oocyte maturation. On the other hand, higher expression of FST and INHBA has been reported in cumulus oophorus that were obtained from in vivo produced COCs compared to these derived from in vitro produced ones (Tesfaye et al. 2009).

In order to further validate the array data, the expression of some selected genes was analysed using semi-quantitative RT-PCR. A 2% agarose gel pictures showing transcripts that are specific to either cell types are shown in figure 24. Previous studies have suggested that higher expression of HAS2, PTX3, TNFAIP6, PTGS2, CD44,

INHBA and BTC in CCs can be used as molecular bio-markers to select quality embryo in women (McKenzie et al. 2004) and cow (Assidi et al. 2008). Higher expression of PTX3, PTGS2, ADAMTS1, INHA and INHBA was also reported in human CCs (Assou et al. 2006). However, data that clearly demonstrate whether these transcripts are oocyte or CC specific or expressed in both is not available. Here, we show that none of HAS2, PTX3, INHA, INHBA and CD44 is CC specific as they are expressed in both samples (Supplemental Tables 3 and 6, Figure 25).

In addition to these findings, we report for the first time that POU class 5 homeobox 1 (POU5F1), interferon regulatory factor 6 (IRF6), sex determining region Y box2 (SOX2) and insulin like growth factor 2 binding protein 3 (IGF2BP3) are expressed only in oocytes and secreted protein, acidic, cysteine-rich (SPARC), glutamate pyruvate transaminase (GPT), ADAM metallopeptidase with thrombospondin type 1 (ADAMTS1) and heparanase (HPSE) are expressed only in CCs.

Significantly higher expression of POU5F1 has been reported in developmentally competent bovine oocytes (Donnison and Pfeffer 2004) and its loss of function resultes in preimplantation lethality in mouse embryos as it is a central regulator of pluripotency (Nichols et al. 1998). Another transcript we found only in the oocyte is SOX2. It is a developmental pluripotency marker, which has been hypothesized as a regulator of POU5F1 controlled genes (Botquin et al. 1998) suggesting a possible synergetic effect of the two genes during oocyte maturation. IRF6 has been suggested as a key mediator of cellular proliferation and differentiation in mammary epithelial cells by facilitating entry into the G0 phase of the cell cycle (Bailey et al. 2008) and is essential for normal skin, limb and craniofacial morphogenesis in mice (Ingraham et al. 2006). Recently, it has been implicated to play a role in the development and differentiation of several epithelial tissues in Danio and Xenopus embryos (Sabel et al. 2009). As it regulates cell proliferation and differentiation in different cell types, its higher expression in oocyte sample in the present study is evidencing that it's a maternal transcript that play a role in acquiring developmental competence.

Insulin like growth factor 2 binding protein3 (IGF2BP3) is also expressed only in oocytes relative to oocytes. Intra follicular insulin like growth factor binding proteins

(IGFBPs) play a key role in the regulation of follicular development (Monget et al. 1996). However, the role of IGF2BP3 in the developmental biology the oocyte is poorly understood.

Higher expression of ADAMTS1 was found in MII CCs compared to GV stage (Figure 24). Interestingly, increased expression of ADAMTS1 protein has been reported in mouse GCs in response to preovulatory LH surge (Russell et al. 2003b) where it is targeting Versican (VCAN), one of the proteins that cross link HA rich CCs matrix and contribute to oocyte maturation, ovulation and/or fertilization (Dunning et al. 2007).

SPARC is a multifunctional calcium-binding glycoprotein that modulates cell-extracellular matrix interactions and influences cell–cell adhesion, migration and invasion in vitro and in vivo (Martinek et al. 2008). Its present CC specific expression supports the results of previous study where it was expressed only in the somatic cells of germarium and follicles during oogenesis (Martinek et al. 2008).

HPSE, which according to the NCBI knowledge base is an upstream regulator of the glycosaminoglycan degradation pathway, is also expressed only in the CCs. It has been suggested as a novel member of the LH-induced extracellular matrix-degrading enzyme family and may contribute to follicular rupture during ovulation (Klipper et al. 2009). Its CCs specific expression in this study is consistent with the notion that transcripts that are involved in the rapture of follicular fimbria during the process of ovulation are expressed by GCs (Richards et al. 2002).

Although several biologically important transcripts are expressed either as oocyte or CCs specific, a number of genes that play important roles in oocyte-granulosa cells regulatory loop and oocyte maturation are expressed in both cell types (Supplemental Table 3). These include members of the MPF (CDK1 and Cyclin B), several cell cycle related genes (cyclin A2, B2, C, D3, E1, G1, H, I, K, L2, T, Y, CCRK and CCPG1), activating transcription factors (ATF1, 2, 3, 4 and 7), Aurora kinases (AURKA, AURKB), vascular endothelial factors (VEGFA and B) and others (MSX1, IPO8, MARK1, TSPAN6, VCAN).

As a member of the tetraspanin family, the protein of TSPAN6 mediates signal transduction events in regulating cell development, activation, growth and motility. Inline with the importance of these processes in oocyte maturation, TSPAN6 can be suggested as a candidate transcript that involves in oocyte maturation. ATF3 has been implicated to contribute to the pro-apoptotic effects of curcumin (Yan et al. 2005), a compound that regulates tumor development in experimental systems (Aggarwal et al. 2003). Here, we propose that ATF3 could be one of the mediators of the anti-apoptotic effects in in vitro environments.

VEGFA is expressed in both the oocyte and CCs but with a higher level in the latter. In human, optimal follicular growth and oocyte maturation require an adequate ovarian vascularisation for oxygen and nutrient supply (Luttun and Carmeliet 2003). VEGFA is a potent inducer of vasculogenesiss (Robinson and Stringer 2001). It is essential for endothelial cell differentiation and angiogenesis during development of embryonic vasculature and stimulates the primary to secondary follicle transition in bovine follicles in vitro (Yang and Fortune 2007). Its higher expression in CCs may indicate that it has some role in the latter stages of bovine follicular development and oocyte maturation as the oocyte depends on the surrounding CCs for oxygen and nutrient supply (Sutton-McDowall et al. 2004).

Another interesting transcript is MSX1, which is expressed in both but at higher level in oocytes than in CCs. It has been noted that MSX1 maintains cyclin D1 expression and prevents its exit from the cell cycle and thereby regulates cellular proliferation and differentiation during embryogenesis (Hu et al. 2001). Significantly higher expression of this gene has been reported in developmentally competent bovine oocytes (Donnison and Pfeffer 2004) and its selective degradation reduces the development of polar body formation in bovine oocytes (Tesfaye et al. 2010).

5.2 Removal of CCs before in vitro maturation alters the gene expression profile of MII oocyte

Oocyte maturation is a long process during which oocytes acquire their intrinsic ability to support the subsequent stages of development in a stepwise manner, ultimately

reaching activation of the embryonic genome. This process involves complex, distinct and linked events of nuclear and cytoplasmic maturation. Nuclear maturation mainly involves chromosomal segregation, whereas cytoplasmic maturation involves organelle reorganization and storage of mRNAs, proteins and transcription factors that act in the overall maturation process, fertilization and early embryogenesis. Molecular maturation consists of transcription, storage and processing of maternal mRNA, which is stored in a stable, inactive form until translational recruitment. It has been reported that oocytes can attain nuclear maturation but not cytoplasmic maturation if cultured in the absence of their companion CCs (Atef et al. 2005).

The gap junctional communication between the oocyte and its adjacent somatic CCs is vital not only to transfer nutrients and metabolites from CCs to the oocyte but also has a significant physiological implication. In vitro studies have shown that FSH dependent cAMP, the activator of mitogen-activated protein kinase (MAPK) signalling, is produced by CCs and diffuses to the oocyte via this junction (Thomas et al. 2004, Webb et al. 2002). cAMP dependent activation of MAPK triggers the expression of one of the components of MPF, CDK1, to initiate meiotic resumption (Tremblay et al. 2005) and simulates mos mRNA cytoplasmic polyadenylation during Xenopus (Howard et al. 1999, Zhang and Sheets 2009) and mouse (Gebauer et al. 1994) oocyte maturation. Consistent with this, several members of the MAPK signalling pathway (MAP3K2, MAP3K3 and MAP4K14) are over expressed in OO+CCs group compared to OO-CCs groups evidencing that the presence of CCs during IVM is vital for oocyte to attain cytoplasmic maturation.

PDK4, EIF3A, TSSC1, TBRG4, PTTG1IP and some cell cycle related genes (CCRK, GAK and CDCA4) are also over expressed in OO+CCs relative to OO-CCs. Although data that directly relate the activity of PDK4 to oocyte maturation is not available, a 3 fold increase in the content of the predominant isoform of PDK genes family has been reported during Xenopus oocyte maturation (Tokmakov et al. 2009). However, whether this increased expression is connected to the cytoplasmic polyadenylation of PDK mRNA is some thing that needs further investigation.

CCRK encodes a protein that contains a kinase domain most closely related to the cyclin-dependent protein kinase which in turn is involved in cell growth and CDCA4 is involved in cell proliferation (Watanabe-Fukunaga et al. 2005).

Dramatically elevated expressions of mitochondrial DNAs have been reported in matured Xenopus oocytes as a preparation for fertilization and development. Oocyte maturation, fertilization and early embryo development have also been associated with changes in active mitochondrial distribution in pig oocytes (Sun et al. 2001). Over expression of several genes encoding for mitochondrial proteins (MRPL10, MRPL3, MRPL4, MRPS6, MRPS9) in OO+CCs group compared with OO-CCs suggests a possible roles of these genes in bovine oocytes maturation.

In general, removal of companion CCs at GV stage appears to affect the gene expression of MII oocytes as a number of genes are over expressed in OO+CCs relative to OO-CCs. As explained earlier, some of these genes have been implicated to be involved in various biological functions that are pertinent to oocyte meiotic resumption and maturation supporting the notion that the presence of CCs during IVM is crucial for oocyte developmental competence. However, the majority of these differentially expressed genes are uncharacterized and/or their biological functions, particularly with regard to oocyte development and maturation, are poorly understood.

Paradoxically, several previously identified and biologically important OSFs (GDF9, BMP6, 15, TGFBs), Zona pellucuda proteins (ZP2, 3, 4), the components of MPF (CDK1 and Cyclin B1) and others are missing from the list of these differentially expressed genes. From these data one can argue that either these over expressed genes have functional redundancy with those missing genes or the expression of the latter is completed prior to CCs removal at GV stage and consequently they are detected as equally as those matured with out their CCs. One plausible explanation in line of the latter argument is the fact that bovine oocytes are transcriptionally active during folliculogenesis and transcriptional activity decreases at later stages of follicular development (Memili and First 1998). Additionally, our present microarray data between GV and MII oocytes (data not shown) revealed that only GDF9 and CDK1 are slightly over expressed (fold change = 2.46) at MII relative to GV stage. Interestingly,

while BMP15 and TGFB2 are over expressed at GV stage, ZP2, 4 and cyclin B1 are equally expressed between the two stages. From these results, it is difficult to conclude that removal of CCs at GV stage significantly affects the developmental competence of in vitro produced bovine oocytes and this needs further investigation.

5.3 The absence of ooplasm during in vitro maturation alters the gene expression profile of CCs at MII

Notable interspecies differences exist whether OSFs are mandatory for FSH induced CCs expansion in vitro. In rat, the presence of OSFs are crucial for in vitro CCs expansion as CCs-OO failed to expanded their cumulus oophorus (Vanderhyden 1993). In cattle and pig CCs expansion doesn't depend on the presence of these factors as CCs that were matured with out their enclosed ooplasm (CCs-OO) expanded as equally as those matured with their ooplsams (CCs+OO) (Prochazka et al. 1991, Ralph et al. 1995). The result of a modified experimental model in which intact bovine COCs were cultured with denuded oocytes has shown an improved blastocysts rate relative to those cultured alone (Hussein et al. 2006). Because CCs-OOs expand as equally as the intact ones, we hypothesized that transcription profile of CCs that are matured with their enclosed oocytes is the same as those matured alone. However, contradictory to our hypothesis, several transcripts are differentially expressed between the two groups.

The IPA showed that some of the genes under expressed due to removal of ooplasm before IVM are classified in to cellular growth and proliferation (VEGFA, GADD45A, FOS, EGR1, HAS2), cell cycle (CCND2, CDCA8, CDK6) and gene expression (FOSB, TGFB2, ATF3) functional groups (Figure 14). These genes are also mapped in to a complex gene network that includes genes involved in cellular development such as Cyclin D, HPSE, JUNB, D and others (Figure 17). Some of these genes are not well characterized and their direct role in the biology of COCs is poorly understood. However, results of previous studies in different species have shown that some of these genes are involved in cumulus expansion and oocyte maturation. For instance, genes from FOS family have been implicated as regulators of cell proliferation, differentiation and transformation and insulin like growth factor binding proteins stimulate the growth

promoting effects of *IGF1* which in turn is important for oocyte growth and maturation and granulosa cells proliferation (Zhou et al. 1991).

Increased expression of EGr1, one of the genes which play active roles in biochemical pathways associated with meiotic maturation and subsequent oocyte developmental competence and HAS2, an enzyme which is required for biosynthesis of HA during CCs expansion (Eppig 1979, Fulop et al. 1997) was observed in CCs matured with their ooplasm compared with those matured with out.

Increased expression is also observed for members of vascular endothelial growth factors, (VEGFA), protein kinases (AURKA), cell cycle molecules (CCND2, CDK6) and growth factors (TGFB2) in CCs+OO group relative to CCs-OO. Results of previous studies in different species appear to provide possible roles for some of these genes in cumulus expansion and oocyte maturation. For instance, AURKA is one of the member of protein kinase signalling pathways that is involved in oocyte nuclear maturation (Bettegowda et al. 2008, Saskova et al. 2008) and a member of transforming growth factor beta super family, TGFB2 has been suggested to constitute CEEF that signal through SMAD 2/3 to enable the initiation of mouse cumulus expansion (Dragovic et al. 2007). While CCNB2 has been highly associated with developmentally competent bovine oocytes (Mourot et al. 2006), increased expression of CDK6 mRNA has been reported in rat granulosa cells after six hr of hCG hormone treatment (Cannon et al. 2005).

Although a number of genes are differentially expressed between CCs that mature with or with out their enclosed ooplasms, it is not easy to conclude that removal of ooplasm at GV stage completely changes the expression of CCs genes during IVM. For example, except HAS2, the majority of genes expressed in CCs and previously identified as molecular bio markers for developmental competence with a particular reference to CCs expansion including INHßA, EGFR, BTC, CD44, TNFAIP6, PTX3 and PTGS2 (Assidi et al. 2008, McKenzie et al. 2004) are not differentially expressed between the two samples. From these results, we hypothesize that either these differentially expressed genes predict oocyte developmental competence better than the previously indentified ones or the transcription of previously identified genes is completed earlier at GV stage

before the ooplasm is removed and hence they are not differentially expression at MII stage. However, the assertion of both scenarios requires further investigation.

5.4 The dynamics of CCs transcriptome during the transition of COC from GV to MII stages is associated with functional changes

Oocyte developmental competence is progressively acquired during follicular development and the oocyte plays a dominant role in regulating granulosa cell functions and maintaining the microenvironment appropriate for acquiring development competence. Hence, granulosa cell functions are reflective of oocyte competence and molecular markers of granulosa cells are potentially reliable predictors of oocyte quality. In vitro studies have revealed that FSHRs are found only in CCs (Nuttinck et al. 2004, Webb et al. 2002) and cAMP, a mediator of MAPK signalling pathway for oocyte meiotic resumption and maturation is primarily synthesized by CCs (Webb et al. 2002).

In this experiment global transcriptome profile and functional changes in the CCs during IVM of bovine COCs was analyzed. Massive transcript destruction during IVM of bovine (Fair et al. 1995), human (Assou et al. 2006, Su et al. 2007) and mouse (Su et al. 2007) oocytes has been reported. Similarly, considerable transcript and associated functional changes are observed during the transition of CCs from GV to MII stage. While transcripts that are involved in cell cycle, DNA replication, metabolic process, steroid and cholesterol biosynthesis, signal transduction and regulation of catalytic activity are over expressed in GV CCs, those involved in cell adhesion, protein metabolic process, regulation of cellular component organization and biogenesis and actin filament polymerization are over expressed in MII CCs (Figure 20). The most interesting finding of this experiment is not only the change in the number of transcripts but also the groups of transcripts that are involved in a given biological process at the two developmental stages.

Cell cycle and DNA replication are the two successive events which play significant roles in meiotic process resulting in the formation of four haploid cells. Over expression of transcripts involving in cell cycle and DNA replication in GV CCs compared to their

MII counterparts may demonstrate that these transcripts are important for meiotic resumption in vitro.

Metabolism is the process of taking in raw materials, building cell components, creating energy molecules and releasing by-products. The functioning of a cell depends upon its ability to extract and use chemical energy stored in organic molecules via metabolic pathways. On the other hand, MAPK activation in CCs rather than in oocytes exerts essential functions during mammalian oocyte meiotic resumption (Liang et al. 2005, Liang et al. 2007) and steroids such as P have been suggested to induce cAMP dependent MAPK signalling cascade leading to meiotic resumption (Shimada and Terada 2002). Hence, over expression of transcripts that are involved in metabolic and steroid biosynthetic pathways in GV CCs may suggest that these pathways are more active in GV stage than in MII.

As the oocyte lacks gonadotropin receptors, it has been hypothesized that FSH exerts, its effect via a positive meiosis factor, epidermal growth factor (EGF) synthesized by CCs indirectly via signal transduction pathway that involves cAMP dependent MAPK to induce meiotic resumption via GVBD (Downs et al. 1988). From these findings, we also propose that over expression of transcripts that are involved in signal transduction pathway in GV CCs relative to MII CCs is an indication that this pathway is working more actively in GV than in MII CCs.

Focal adhesions are specific types of large macromolecular assemblies through which both mechanical force and regulatory signals are transmitted (Chen et al. 2003). More precisely, they can be considered as sub-cellular macromolecules that mediate the regulatory effects. They serve not only to anchor the cell, but also to carry signals, which inform the cell about the condition of the ECM and thus affect their behaviours (Riveline et al. 2001). In view of their increased expression at MII stage relative to their expression at GV, we propose that these genes are involved in one of the regulatory network that connects the oocyte to its companion cumulus cells during IVM.

Actin filaments localize to specific regions within mammalian oocytes and their modelling including polymerization are important for oocyte maturation, fertilization

and embryo development (Rawe et al. 2006, van Tol et al. 1996, Webb et al. 2002). Interestingly, we found higher expression of transcripts that are involved in actin filament polymerization pathway in MII than in GV CCs.

Prior to in vitro meiotic resumption, FSH is received by GCs via FSHR and this activates the release of cAMP and MAPK signalling pathways to initiate meiotic resumption (Shimada and Terada 2002). Consistent with the perception that in vitro meiotic resumption in bovine oocytes is triggered by FSH (van Tol et al. 1996, Webb et al. 2002); we observed higher expression of FSHR mRNA in GV CCs than in MII stage.

CCs prevent the oocytes from oxidative stress induced apoptosis (Fatehi et al. 2005, Tatemoto et al. 2000). Accordingly, genes involving in the protection of tumor cell development including TNFRSF6B, TNFRSF1A, TNFRSF12A and TNFAIP8L3 are over expressed in MII CCs relative to GV stage. Additionally, molecules that play significant role in CCs expansion (HAS2, TNFAIP6, PTX3, CD44) and several uncharacterized genes (IGFBP3, SCG2, VCAM1, ANKRD1, RSAD2, CAV1, PLAUR) are highly over expressed in MII CCs compared with their GV counterparts.

The binding proteins of HA (TNFAIP6 and PTX3) and its receptor protein (CD44) have been implicated to play significant roles in attaining full CC expansion, a key biological event for successful oocyte maturation, ovulation and fertility (Assidi et al. 2008, Kimura et al. 2002, McKenzie et al. 2004, Ochsner et al. 2003). CD44 is a widely expressed cell adhesion molecule that binds the extracellular matrix component, HA in a tightly regulated manner (Lesley et al. 1993). HAS2 is an important enzyme for the biosynthesis of HA which in turn forms stable matrix during CCs expansion (Simpson et al. 2002). The interaction between HA and CD44 is the key molecular mechanism for the activation of signalling cascades that contribute to cell adhesion, proliferation, migration and differentiation (Bourguignon 2001, Turley et al. 2002). This interaction is also important for MAPK signalling pathway in oocyte and may promote meiotic resumption (Kimura et al. 2007). Oocytes can't attain cytoplasmic maturation when matured in the absence of their companion CCs as they can't store sufficient mRNAs, proteins and transcription factors that are important for maturation process due to

removal of the bidirectional communication axis. The interaction between HA-CD44 is critical for modification of this communication axis during CCs expansion (Yokoo and Sato 2004) and relatively higher expression of these molecules in matured CCs supports the previous impression that HA-CD44 interaction is vital for oocyte maturation (Kimura et al. 2007).

As a general remark, the majority of genes differentially expressed between different oocyte and CCs samples are poorly characterized and their roles in the biology of bovine COC is not known. Moreover, due to the dynamic nature of gene expression in different species, tissues and follicular stages, what have been reported so far for other organisms may not necessarily hold true for bovine oocytes and CCs. Thus, detailed gene by gene study is required to come up with specific roles of these genes in the biology of bovine COCs. However, with the previously existing body of scientific knowledge, this study has generated several valuable gene expression data that would enhance our understanding of the molecular mechanisms underlying oocyte-CCs dialogue in general and oocyte maturation in particular.

5.5 Future prospects

This study has generated a long list of genes that are exclusively expressed either in oocytes or CCs and those under expressed due to disruption of the bidirectional axis of communication between the two cell types. Although few of them have been implicated to be involved in oocyte maturation, the roles of the majority of these newly identified genes in the biology of oocyte are poorly understood. Therefore, RNA interference based functional analysis is necessary to better uncover the pathways and gene networks that are affected due to suppression of one of these transcripts and how this impact oocyte maturation.

6 Summary

The bi-directional communication between an oocyte and its companion CCs is crucial for development and functions of both cell types. This dialogue is vital for the oocyte to acquire meiotic and developmental competence and for proliferation and differentiation of CCs.

Although studies were conducted to identify molecular biomarkers for developmentally competent bovine oocytes, large scale expression data on oocyte or CC specific transcripts is still lacking. Removal of oocyte-CCs communication axis during IVM reduces CCs expansion and thereby affects oocyte developmental competence but the effect of removing this communication axis on their corresponding gene expression is poorly understood. Furthermore, differentially expressed CC genes and functional changes associated with these genes during the transition of COCs from GV to MII stage are not identified.

Different types of oocyte and CC samples were obtained from abattoir ovaries and randomly assigned in to five groups. Total RNA of all samples was isolated, fragmented, biotin labelled and hybridized to Affymetrix Bovine Genome Array and the data were analyzed using linear model for microarray. Significantly changed GO terms and gene networks and canonical pathways were analyzed using GO consortium and IPA, respectively.

Experiments 1A and B were conducted to identify transcripts that are co and exclusively expressed between the oocyte and CCs. Experiments 2 and 3 were conducted to enumerate those which are significantly affected when the oocyte or CCs mature with or with out the other and experiment 4 was conducted to identify differentially expressed CC genes and significantly changed biological processes associated with the transition of COCs from GV to MII stage. In experiment 1A, of 13162 detected probe sets, 1516 and 2727 are exclusively expressed in GV oocytes and CCs respectively and 8919 are expressed in both. Similarly, in experiment 1B, of 13602 detected probe sets, 1423 and 3100 are exclusively expressed in MII oocytes and CCs respectively and 9079 are expressed in both. In experiment 2, 265 transcripts are differentially expressed between

OO+CCs and OO-CCs of which 217 and 48 are over expressed in OO+CCs and OO-CCs respectively. In experiment 3, of 566 differentially expressed transcripts between CCs+OO and CCs-OO, 320 and 246 are over expressed in CCs+OO and CCs-OO respectively. In experiment 4, of 9201 detected probes 4690 and 835 are exclusively expressed in GV and MII CCs respectively while 7303 are expressed in both.

Oocyte specific transcripts include those involved in transcription (IRF6, POU5F1, MYF5, MED18), translation (EIF2AK1, EIF4ENIF1), biopolymer metabolic process (MOS, ACVR1, ZNF529, MAP3K3), DNA replication (MCM6, NASP, ORC6L), protein amino acid phosphorylation (MAP4K2, PRKCH, MOS) and CCs specific ones include those involved in macromolecule biosynthetic process (APOA1, USPL1, APOE, NANS), carbohydrate metabolism (HYAL1, PFKL, PYGL, MPI), protein metabolic processes (IHH, APOA1, PLOD1), steroid biosynthetic process (APOA1, CYP11A1, HSD3B1, HSD3B7). While transcripts over expressed in OO+CCs are involved in carbohydrate metabolism (ACO1, 2), molecular transport (GAPDH, GFPT1) and nucleic acid metabolism (CBS, NOS2), those over expressed in CCs+OO are involved in cellular growth and proliferation (FOS, GADD45A), cell cycle (HAS2, VEGFA), cellular development (AMD1, AURKA, DPP4) and gene expression (FOSB, TGFB2). Signal transduction, cholesterol biosynthetic processes, DNA replication, cellular growth and proliferation, actin filament polymerisation and cell adhesion are among the top significantly changed biological functions associated with transcripts that are differentially expressed between GV and MII CCs.

In conclusion, this study generated large scale gene expression data from different oocyte and CCs samples that would enhance our understanding of the molecular mechanisms underlying oocyte-CCs dialogue in general and oocyte maturation in particular.

7 Zusammenfassung

Die bidirektionale Kommunikation zwischen Oozyten und ihren umgebenden CCs ist für die Entwicklung und Funktion beider Zelltypen entscheidend. Diese Kommunikation ist für die Meiotische- und Entwicklungskompetzenz der Oozyte wichtig, sowie für die Proliferation und Differenzierung von CCs.

Obwohl Studien zur Identifizierung von molekularen Biomarkern für entwicklungskompetente Rinder Oozyten durchgeführt wurden, fehlen Umfangreiche Expressionsdaten über Oozyten oder CC spezifische Transkripte. Die Aufhebung der Oozyten-CCs Kommunikation während der IVM verringert die Entwicklung der CCs und hat dadurch Einfluss auf die Entwicklungskompetenz der Oozyten. Allerdings ist der Effekt, die Aufhebung dieser Kommunikation und die daraus resultierende Genexpression, noch nicht vollständig geklärt. Außerdem, sind keine unterschiedlich exprimierten CC Gene sowie funktionelle Änderungen, die mit diesen Genen assoziieren, während des Übergangs des COCs von GV zum MII identifiziert.

Unterschiedliche Proben von Oozyten und CC wurden aus Schlachthof-Ovarien gesammelt und in fünf zufällige Gruppen eingeteilt. Die Gesamte RNA aller Proben wurde isoliert, zerkleinert mit, Biotin gelabelt und auf Affymetrix Bovine Genom Array hybridisiert. Zur Analyse der Data wurde ein lineares Modell für Microarray verwendet. Für die Analyse von signifikanten Veränderungen des GO, der Gennetzwerke und von anerkannten Pathways wurden GO und IPA verwendet.

Experiment 1A und B dienten der Identifizierung von Transkripten die entweder eine gemeinsame oder ausschließliche Expression in den Oozyten und den CCs zeigten. Mittels Experiment 2 und 3 wurden die aufgezählt, die signifikant an der Maturation von Oocyten und CC mit oder ohne den jeweiligen anderen beteiligt sind. In Experiment 4 wurden unterschiedlich exprimierte CC Gene identifiziert sowie signifikante Veränderungen in biologischen Prozessen, die mit der Entwicklung von den COCs von CV bis zum MII Stadium assoziiert sind. Im Experiment 1A wurden 13162 Probensets entdeckt, davon waren ausschließlich 1516 in GV Oozyten und 2727 in CCs und 8919 in Beiden exprimiert. Ähnliche Expressionsmuster spiegelten sich im

Experiment 1B wieder, in diesem wurden 13602 Probensets entdeckt, von denen ausschließlich 1423 in MII Oozyten und 3100 in CCs exprimiert wurden und in Beiden 9079. In Experiment 2 zeigten 265 Transkripte einen Unterschied in der Expression zwischen OO+CCs und OO-CCs, wobei 217 in OO+CCs und 48 in OO-CCs überexprimiert waren. Das Experiment 3 weist 566 unterschiedlich exprimierte Transkripte zwischen CCs+OO und CCs-OO auf, von denen 320 in CCs+OO und 246 in CCs-OO exprimiert wurden. Im 4. Experiment waren von 9201 entdeckten Proben ausschließlich 4690 in GV und 835 in MII CCs bzw. 7303 in Beiden exprimiert.

Die Oozyten spezifischen Transkripte umfassen jene die an der Transkription (IRF6, POU5F1, MYF5, MED18), der Translation (EIF2AK1, EIF4ENIF1), an dem biopolymeren metabolischen Prozess (MOS, ACVR1, ZNF529, MAP3K3), der DNA Replikation (MCM6, NASP, ORC6L) und an der Protein Aminosäure Phosphorylierung (MAP4K2, PRKCH, MOS) beteiligt sind. Die CCs spezifischen umfassen jene die in marcomolekülen biosynthese Prozessen (APOA1, USPL1, APOE, NANS), am Kohlehydrat Metabolismus (HYAL1, PFKL, PYGL, MPI), an Protein metabolischen Prozessen (IHH, APOA1, PLOD1) und am steroid biosynthetischem Prozess (APOA1, CYP11A1, HSD3B1, HSD3B7) beteiligt sind. Indem die Transkripte von OO+CCs überexprimiert sind, haben sie Einfluss auf den Kohlehydrat Metabolismus (ACO1, 2), molekularen Transport (GAPDH, GFPT1) und Nukleinsäuse Metabolismus (CBS, NOS2). Die Überexpression in CCs+OO hat Einfluss auf den Zell Wachstum und Proliferation (FOS, GADD45A), Zellzyklus (HAS2, VEGFA), Zellentwicklung (AMD1, AURKA, DPP4) und Genexpression (FOSB, TGFB2). Signal Transduktion, Colesterin biosynthetische Prozesse, DNA Replikation, Zellwachstum und Proliferation, Actin filament Polymerisierung und Zelladhäsion zeigten die stäksten signifikanten Veränderungen in biologischen Funktionen, die mit den Transkripten assoziiert sind, welche unterschiedliche Expressionen zwischen GV und MII CCs zeigten.

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Curriculum vitae

1. Personal information

- -

2. Educational background

- -

3. Professional experiences

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4. Publications

1. Tesfaye A., Alemu R. and Lemma F (2000): Preliminary production and reproduction performance evaluation of Borana and rift valley Goats. In: The challenges and opportunity of enhancing goat production in east Africa. A paper presented on an International conference held at Debub University in collaboration with Langston University, Awassa College of Agriculture, Ethiopia

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