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Molecular genetic analysis of bovine oocytes with different developmental potentials

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Nasser Ghanem Osman Mohamed

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Korreferent:	Prof. Dr. B. Petersen
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Dedicated to my parents, my wife and my son

Molekulargenetische Analyse von Rinderoocyten mit unterschiedlichen Entwicklungspotentialen

Ein geringes Entwicklungspotential von Oozyten führt zu einer verringerten Fruchtbarkeit in der Rinderhaltung. Gleichzeitig erschwert es moderne biotechnologie Zuchtmethoden, wie z.B. Embryotransfer, die für die schnelle Verbreitung von positiven Genotypen genutzt werden können. Allerdings sind die molekularen Mechanismen, die das Oozytenentwicklungspotential regulieren, bisher nur wenig bekannt. Ziel dieser Arbeit war es demzufolge, unterschiedlich regulierte Gene in Oozyten mit unterschiedlichem Entwicklungspotential zu identifizieren, die mit der Entwicklungskompetenz der Oozyten assoziiert sein könnten. Dafür wurden Oozyten auf Grund ihres follikularen Umsatzes (Wachstum gegenüber Dominanzverhalten) (Modell 1) bzw. auf Grund von BCB Färbung (BCB^+ gegen BCB^-) (Modell 2), ausgewählt. Für jedes Modell wurden sechs Pools von Oocyten für die mRNA-Isolierung und RNA-Amplifikation verwendet. Ein speziell angefertigter cDNA Array mit ~ 2000 Klonen wurde genutzt, um Genexpressionsprofile von kompetenten und inkompetenten Oocyten zu vergleichen. Das Programm Significance Analysis of Microarray (SAM) wurde für die Datenauswertung herangezogen.

Insgesamt konnten 51 bzw. 185 unterschiedlich exprimierte Gene für die Oozyten mit unterschiedlichem follikularem Wachstum bzw. mit unterschiedlicher BCB Färbung identifiziert werden. Für die weiteren Untersuchungen wurden Gene, die in der Proteinbiosynthese als struktureller Bestandteil des Ribosoms vorkommen (RPL24, ARL6IP, RPS14 und RPS15), ein Translations-Elongations-Faktor (EEF1A1), ein Gen für Chromosomenorganisation und Biogenese (H2AFZ), sowie ein Gen für die Signal Transduktion (GNB2L1) ausgewählt. Diese Gene waren in kompetenten Oozyten höher exprimiert als in inkompetenten Oozyten. Des weiteren wurden ein Gen für die Transkriptionsregulierung (PTTG1) sowie ein Gen für die Wachstumsfaktor Aktivität (BMP15) ausgesucht, welche wiederum in inkompetenten Oozyten hochreguliert waren. Mittels quantitativer Real-time PCR wurde die relative Abundanz aus der Microarray Analyse bei acht von zehn Genen (Modell 1) bzw. neun von zehn Genen (Modell 2) bestätigt.

Insgesamt beschreibt diese Arbeit ein genomweites Expressionsprofil von Genen, die mit der Entwicklungskompetenz von Rinderoozyten assoziiert werden können. Darüber hinaus könnten weitere funktionelle Analysen, basierend auf unseren Ergebnissen, helfen, die exakten regulierenden Schlüsselgene zu finden, welche die Oozytenqualität steuern, und die zukünftig als Biomarker verwendet werden könnten.

Molecular genetic analysis of bovine oocytes with different developmental potentials

Poor oocyte developmental potential contributes to reduced fertility in livestock species and hampers the application of biotechnology techniques in cattle industry that could be used for spreading certain genotypes with production advantages. However, the molecular mechanisms regulating oocyte developmental potential are generally poorly understood. Therefore, the objective of this study was to identify differentially regulated genes in oocytes with different developmental potential that could be associated with their competence. For this, oocytes were selected based on phase of follicular turnover (growth vs. dominance) and BCB staining (BCB+ vs. BCB-) as two independent models for screening of oocyte competence. For each model, six pools of oocytes were used for mRNA isolation and subsequent RNA amplification. A custom-made cDNA array with ~ 2000 clones was used to compare the gene expression profiles of competent versus incompetent oocytes of each model. The Significance Analysis of Microarray (SAM) has been used for data generation. A total of 51 and 185 genes to be differentially expressed have been identified between the oocytes derived from growth compared to dominance follicular phase and BCB+ compared to BCB- ones, respectively. Based on biological process annotation, genes involved in protein biosynthesis as structural constituent of ribosome (RPL24, ARL6IP, RPS14 and RPS15), translation elongation factor activity (EEF1A1), chromosome organization and biogenesis (H2AFZ) and signal transduction (GNB2L1) were commonly up-regulated in competent compared to incompetent oocytes of both models. On the other hand, incompetent oocytes from both models were enriched with transcripts regulating transcription (PTTG1) and growth factor activity (BMP 15). Quantitative real-time PCR has confirmed the relative abundance of 8 out of 10 and 9 out of 10 genes to be in accordance with microarray analysis for follicular phase and BCB staining models, respectively. Overall, this study provides a genome-wide expression profiling of genes that could be associated with developmental competence of bovine oocytes. However, further functional investigations based on this data could help to define the exact key regulatory genes controlling oocyte quality that could be considered as good biomarkers.

Contents

List of abbreviations

A	: Adenine
A260	: Absorbance at 260 nm wavelength (UV light)
aRNA	: Amplified RNA
ATP	: Adenosine triphosphate
BCB+	: Brilliant Cresyl Blue positive oocytes
BCB-	: Brilliant Cresyl Blue negative oocytes
BMP	: Bone morphogenic protein
bp	: Base pairs
BSA	: Bovine serum albumin
C	: Cytosine
cDNA	: Complementary deoxyribonucleic acid
CL	: Corpus luteum
COC	: Cumulus oocyte complex
CPE	: Cytoplasmic polyadenylation element
CT	: Threshold cycle
Cy3	: Cyanine 3 fluorescent dye
Cy5	: Cyanine 5 fluorescent dye
ddH2O	: Distilled and deionised water
DEPC	: Diethylpyrocarbonate
DF	: Dominant follicle
dH2O	: Deionised or distilled water
DMF	: Dimethylformamide
DMSO	: Dimethyl sulfoxide
DNA	: Deoxynucleic acid
dNTP	: Deoxyribonucleoside triphosphate (Usually one of dATP, dTTP, dCTP and dGTP)
DOP	: Degenerate oligonucleotid primer
D/R	: Dominance/regression phase
DTT	: Dithiothreitol
EC	: Early cleavage
E.coli	: Escherichia coli

EDTA : Ethylenediaminetetraacetic acid (powder is a disodium salt)
EtBr : Ethidium bromide
EtOH : Ethanol
FSH : Follicle stimulating hormone
G : Guanine
G6PDH : Glucose-6-phosphate dehydrogenase
GC : Granulosa cells
GDF9 : Growth differentiation factor
GnRH : Gonadotropins releasing hormone
G/S : Growth/stagnation phase
GSH : Glutathione
GV : Germinal vesicle
GVBD : Germinal vesicle break down
HAS2 : Hyaluronan synthase 2
IGF2 : Insulin-like growth factor
i.m : intramuscularly
IPTG : Isopropyl ß-D-thiogalactopyranoside
IVC : In vitro culture
IVF : In vitro fertilization
IVM : In vitro maturation
IVP : In vitro production
ISH : In situ hybridization
LH : Luteinizing hormone
MgCl2 : Magnesium Chloride
MI : Metaphase I
MII : Metaphase II
mRNA : Messenger RNA
MSX1 : Msh homeobox 1
mtDNA : Mitochondrial DNA
MW : Molecular weight
NaCl : Sodium chloride
NEC : Non early cleave
OD260 : Optical density at 260 nm wavelength (UV light); = A260

OPU : Ovum pick up
PBS : Phosphate buffered saline
PCR : Polymerase chain reaction
PFA : Paraformaldehyde
PG : Prostaglandin (PGF2α)
PGCs : Primordial germ cells
POD : Peroxidase, commonly horseradish peroxidase
RAMP1 : Receptor activity-modifying protein 1
RNA : Ribonucleic acid
RT-PCR : Reverse transcriptase-polymerase chain reaction
SDS : Sodium dodecyl sulfate
SSC : Sodium chloride – sodium citrate buffer
SNP : Single nucleotide polymorphism
SSH : Suppression subtractive hybridization
TAE : Tris-acetate buffer
TBE : Tris- borate buffer
TE : Tris- EDTA buffer
TEA : Triethanolamine
Thr : Threonine
TNF : Total number of fiber
tRNA : Transfer RNA
TSA : Tyramide signal amplification
UTR : Untranslated region
UV : Ultra-violet light
V/V : Volume per volume
W : Watts
W/V : Weight per volume
X-gal : 5-Bromo-4-chloro-3-indolyl-beta-D-galactoside

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1 Introduction

A prerequisite for good lactation performance, during a cow's life span, is the production of offspring at regular intervals. Therefore, concerns for reproductive efficiency are worldwide, as fertility influences average daily milk production, average days in milk, number of calves born per year, the generation interval and ultimately the farmer's livelihood (Leroy et al. 2008, Johnson and Gentry 2000, Royal et al. 2000). However, there is a continuous declining in fertility of dairy cows with increasing milk production leading to higher economic loss during the last four decades (Macmillan et al. 1996). Lucy (2001) estimated the decline of first-service conception rate from approximately 65% in 1951 to 40% in 1996. The decline in fertility can be explained by management changes within the dairy industry and the negative genetic correlations between milk production and reproduction. Fertility decline was characterized by low fertilization rates and reduced embryonic survival (Moore and Thatcher 2006). One of the primary mechanisms that may be responsible for such abnormal pre- and post-implantation embryo development in lactating cows is poor oocyte quality (Krisher 2004, Lucy 2007, Snijders et al. 2000).

Oocyte quality has impact not only during in vivo embryo development but also it has great contribution to in vitro embryo development. In vitro maturation of bovine oocytes followed by in vitro fertilization (IVF) and culture is rapidly increasing around the world for the production of cows with certain genotypes. Thousands of IVF-generated embryos are transferred to recipient cows annually to generate offspring of higher genetic merit. In addition, it has become a useful tool not only for research but also for the production of transgenic offspring. Until now, there are distinct problems associated with in vitro production (IVP) of bovine embryos. These problems include or are derived from the fact that the origin of the oocytes used (stage of estrous cycle, stage of follicular wave, etc.) is unknown and therefore oocyte quality is very variable (Lonergan and Fair 2008). Therefore, IVP embryos perform considerably poorer than in vivo derived embryos and have frequently been associated with fetal and neonatal abnormalities after transfer (Farin et al. 2006). One of the main factors affecting the embryo yield, the implantation rate and the rate of healthy survivals, is the intrinsic quality of the oocyte (Krisher 2004, Rizos et al. 2002). The influence of the oocyte

quality on the developmental potential of the embryo has been recognized in the cow more clearly than that in any other species (Sirard et al. 2006). Thus, the lack of reliable and objective predictors of oocyte developmental competence for oocyte selection during in vitro fertilization hampers the effectiveness of this technology.

The oocyte is the gamete that contributes not only half of the genetic material but also all of the cytoplasm to the zygote, supplying the transcripts and proteins necessary for early embryonic development (Schultz 2002). This cytoplasmic environment offers the correct conditions so that the embryonic genome can be activated, and the embryo could be able to continue its development. Several studies have shown that maternal transcripts participate in embryonic genome activation, thus influencing the moment that this activation occurs (Memili et al. 1998, Misirlioglu et al. 2006, Vigneault et al. 2004). Therefore, it is obvious that oocyte quality becomes essential to embryonic development before and after genome activation considering the fact that the appropriate embryonic genome activation is a fundamental key for the subsequent embryo development. However, until now there is no a reliable non-invasive method for oocyte selection. Among the various criteria used for oocyte quality evaluation is the assessment of morphological features such as thickness, compactness of the cumulus investment and the homogeneity of the ooplasm (Gordon 2003) which is relatively popular and convenient. However, results derived from this non-invasive approach are often conflicting largely due to subjectivity and inaccuracy. Additionally, the morphological evaluation alone is insufficient to distinguish competent oocytes that have the ability to bring about full-term pregnancy (Coticchio et al. 2004, Krisher 2004, Lonergan et al. 2003a). With the urgent need for establishing a non-invasive and non-perturbing method for oocyte selection, investigating the molecular characteristics of oocytes with different developmental potential is critical to form a foundation for the development of future classification criteria for the selection of oocytes with superior developmental capacity. So, in this study we have analyzed gene expression profile of immature oocytes derived from two different well-established models of oocyte selection in two different experiments. The first model is based on retrieval of the oocyte at different stages of ovarian follicular turnover which have been previously shown that development of early embryos to the blastocyst stage was greater when oocytes are obtained during follicular growth/stagnation phase (G/S) than in the dominance/regression phase (D/R) (Hagemann et al. 1998, Hagemann 1999,

Machatkova et al. 2004). In the second model, oocytes were screened based on BCB staining which have also been shown to be different in their developmental potential to reach blastocyst stage (Alm et al. 2005, Bhojwani et al. 2007).

From the first model, the transcription factor gene MSX1 (msh homeobox 1) was selected for further analysis in the ovaries and early cleavage stages embryos due to the following reasons: i) it has also been identified as differentially regulated transcript between biopsies derived from blastocysts resulted in pregnancy and no pregnancy in our previous study (El-Sayed et al. 2006) ii) results from previous studies (Satokata and Maas 1994, Park et al. 2001) suggest a physiologically optimal level of MSX1 expression is vital for normal cellular function, iii) as transcription factor gene its differential regulation may be correlated with the control of the expression of other developmentally relevant genes. Therefore the objectives of the present study were:

-To compare gene expression profile of bovine oocytes with different developmental competence [retrieved from 3 to 5 mm size follicles at growth versus dominance phase of the first follicular wave in addition to BCB staining oocytes (BCB^+ versus BCB^-)] using custom cDNA microarray.

-To identify differences in mRNA transcript abundance of some candidate transcripts in cumulus cells derived from oocytes retrieved at growth versus dominance phases using quantitative real-time PCR.

-Further characterization of selected candidate gene (MSX1) at mRNA and protein level.

2 Literature review

Investigation on the molecular characteristics of oocytes of poor developmental competence is critical to form a foundation for the development of future classification criteria for the selection of oocytes with superior developmental capacity (Patel et al. 2007). Furthermore, it has been shown that the molecular causes for poor developmental capacity of bovine oocytes may be highly complex and may be reliant on many small changes in the RNA levels of many genes (Donnison and Pfeffer 2004). However, our understanding of composition of the oocyte transcriptome and the identity of key oocyte-expressed genes with important regulatory roles in folliculogenesis and early embryonic development is far from complete (Yao et al. 2004). Thus, our study mainly focuses on identifications of differences in mRNA transcript abundance that may be associated with developmental competence of bovine oocytes using cDNA microarray as a functional genomics approach.

2.1 Early oogenesis and folliculogenesis

Oogenesis is defined as the process by which the oocyte is formed. While, folliculogenesis is the developmental process in which an activated primordial follicle develops to a preovulatory size following the growth and differentiation of the oocyte and its surrounding granulosa cells (Gougeon 1996, Knight and Glister 2001, Senger 1997). During folliculogenesis, a follicle may be classified as primordial, primary, secondary or tertiary (Pedersen and Peters 1968). This classification is based on the size of oocyte, the morphology of granulosa cells, and the number of granulosa cell layers surrounding the oocyte (Lussier et al. 1987, Braw-Tal and Yossefi 1997). In all mammalian species, follicle and oocyte development follow a characteristic sequence of events that commences with the establishment of the ovary shortly after pregnancy during fetal life and terminates with the ovulation of metaphase II oocyte during postnatal life (Figure 1). The total amount of oocytes present in the adult ovary originates from a definite number of primordial germ cells (PGCs) that are formed in the endoderm of the embryonic yolk sac epithelium. Oocytes originate as primordial germ cells from the endoderm of the embryonic yolk sac, and migrate by amoeboid

movement via the dorsal mesentery of the hindgut to the gonadal ridge of the mesonephros of the early embryo (Smitz and Cortvrindt 2002) by day 35 of gestation in

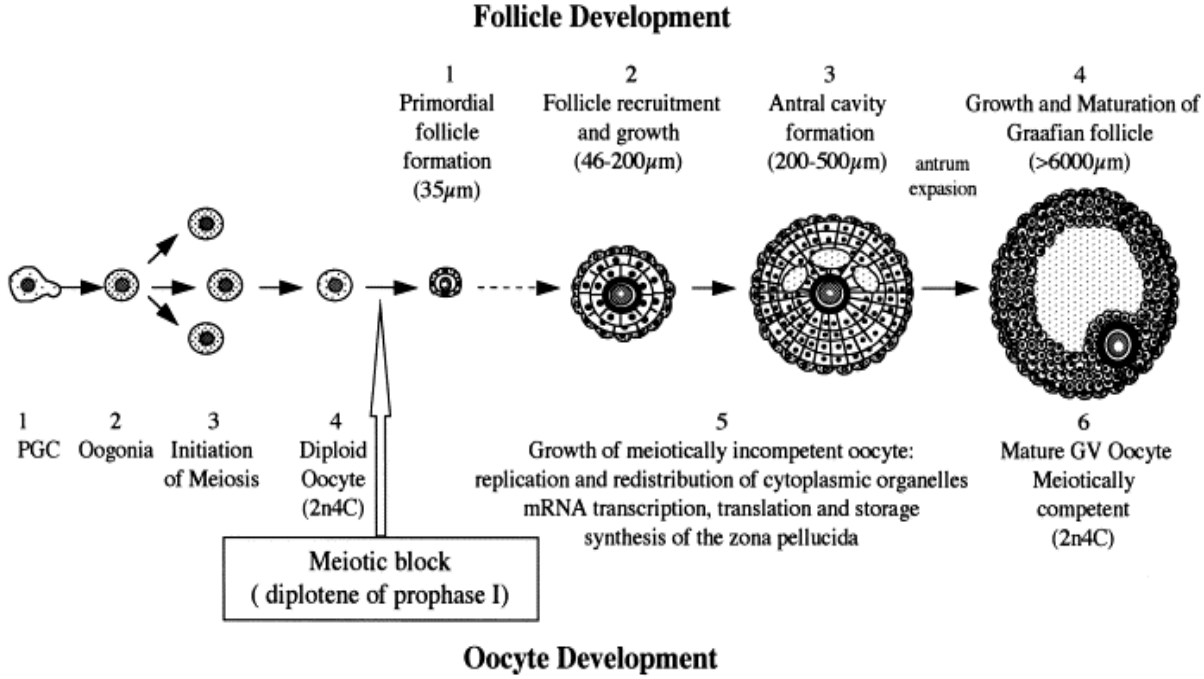


Figure 1: Stages of mammalian oogenesis and folliculogenesis from the development of primordial germ cells (PGC) to the production of a mature germinal vesicle (GV) stage oocyte in a Graafian follicle. Follicular diameter, and chromosomal (n) and DNA content (C) of the oocyte are shown (Picton et al. 1998)

cattle (Byskov and Hoyer 1994, Erickson 1966). Once PGCs have reached the developing ovary, they begin to differentiate into oogonia. The population of oogonia goes through a species specific number of mitotic divisions until the cells enter meiosis and become oocytes (Gosden and Bownes 1995). In mammalian species, several rounds of mitotic divisions occur until shortly before birth. When proliferation is concluded germ cells begin the process of meiosis however, oogonia now are termed primary oocytes and do not complete meiosis but progress only through the dictyate stage (Picton 2001). The oocytes progress into the first meiotic prophase, the onset of which occurs between days 75 and 80-post conception in cattle (Erickson 1966). The meiotic prophase is composed of several transitory stages: preleptotene, leptotene, zygotene, pachytene and the diplotene stage in which the first meiotic division is arrested. Each stage is characterized by a particular chromosome configuration (Baker and Franchi

1967). Once reaching the diplotene stage of meiosis, which occurs around day 170-post conception in cattle (Baker and Hunter 1978), the oocyte becomes surrounded by a single layer of 4–8 pre-granulosa cells and an intact basal lamina, effectively forming the first category of follicle, the resting primordial follicle. The precursors of the granulosa cells are believed to be of either mesonephric or mesothelial origin or both (Byskov and Hoyer 1994). All oocytes that do not become incorporated into primordial follicles will degenerate. Primordial follicles constitute the store of germ cells in the postnatal ovary and their numbers vary with species and age (Picton 2001). As soon as the primordial follicle store is established, follicle recruitment begins and it continues without halting for the rest of life or until the ovary is depleted. Initiation of follicular growth (activation) begins with the transformation of the flattened pre-granulosa cells of the primordial follicle into a single layer of cuboidal granulosa cells that called primary follicle (Braw-Tal and Yossefi 1997). Proliferation of granulosa cells results in an increase from 2 to 6 layers around the oocyte (secondary follicle) and continued increase up to >6 layers of granulosa cells which is the first fluid-filled antrum follicle (tertiary or antral follicle) (Braw-Tal and Yossefi 1997, Lussier et al. 1987). During oogenesis, the oocyte accumulates an extensive collection of RNAs, proteins, and organelles, such as cortical granules, Golgi complexes, ribosomes, and mitochondria (Wessel et al. 2001).

During postnatal life, ovarian follicles continue to grow, mature and either ovulate or regress. The follicle population in the cow is divided more or less evenly between each of the two ovaries. In monotocous species, the follicle selected to ovulate is the fastest growing functional unit in the body of the adult female mammal. The bovine follicle grows 300 to 400 fold in diameter from the primary (50 μm) to the preovulatory (15-20 mm) stage (Rajakoski 1960). The entire process of follicular growth takes approximately 180 days in cattle (Lussier et al. 1987).

There are some changes also in the oocytes during the postnatal life. The chromosomes, during the interruption of the meiosis are relaxed and a nuclear structure known as germinal vesicle (GV) is formed. The interruption of meiosis persists until the puberty, when one or more oocytes restarts the reductive division, the GV in these oocytes disappears, the chromatin is recondensed, the pairs of homologous chromosomes are separated and half of them are expelled forming the first polar body. At this point, the meiosis is interrupted again (in metaphase II). These events, started by the GV breaking

and completed by the formation of the first polar body, lead to the production of a mature and fertile oocyte (Homa 1995) that are able to be fertilized by sperm.

2.2 Follicular dynamics during the estrous cycle of cattle

Studies using ultrasonic imaging to monitor follicle populations in different size categories or to monitor individually identified follicle have convincingly documented that follicular growth in cattle occurs in a wave-like fashion (Figure 2) and the majority of estrous cycles in cattle are comprised of two or three waves (Adams 1999, Ginther et al. 1989a, b, Knopf et al. 1989, Pierson and Ginther 1987a, b, Pierson and Ginther 1988, Savio et al., 1988, Sirois and Fortune 1988).

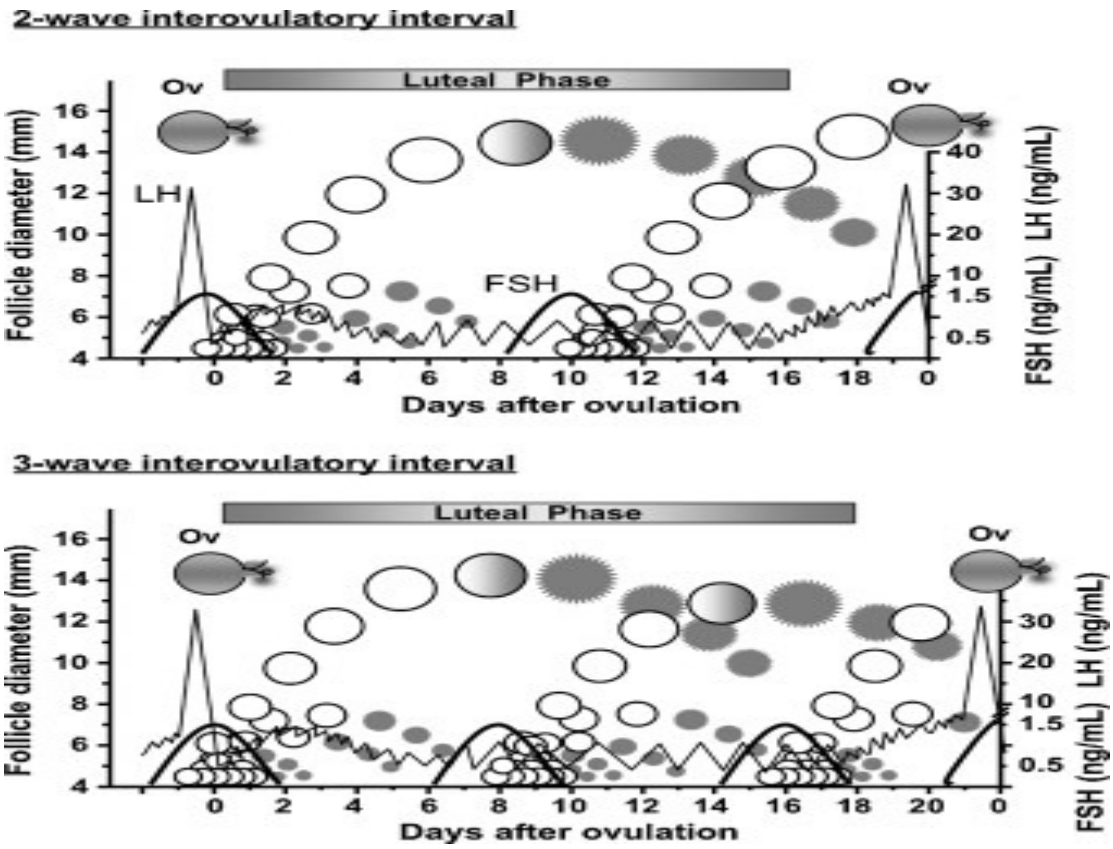


Figure 2: Dynamics of ovarian follicular development and gonadotropin secretion during two- and three-wave estrous cycles in cattle. Dominant and subordinate follicles are indicated as open (viable) or shaded (atretic) circles. A surge in circulating FSH concentrations (thick line) precedes emergence of each wave. A surge in circulating LH concentrations (thin line) precedes ovulation. The LH surge is preceded and succeeded by a period of high-LH pulse frequency as a result of low-circulating progesterone concentrations (Adams et al. 2008)

Some authors reported a preponderance (>80%) of the 2-wave pattern (Ahmad et al. 1997, Ginther et al., 1989a, Rajamahendran and Taylor 1990) while others have reported a preponderance (>80%) of the 3-wave pattern (Celik et al. 2005, Noseir 2003, Sirois and Fortune 1988) and still others have reported a more even distribution of 2- and 3-wave patterns (Savio et al. 1990, Price and Carriere 2004). Although the subject has not been studied extensively, there appears to be no breed, or age specific predilection for a given wave pattern in *Bos taurus* (Driancourt 2001). However, an increase in the proportion of 3-wave patterns has been associated with a low plane of nutrition (Murphy et al. 1991) and heat stress (Badinga et al. 1993, Wolfenson et al. 1995). In *Bos indicus*, no seasonal effect on wave pattern was detected (Zeitoun et al. 1996), but the pattern was influenced by parity. The majority of Nelore heifers (65%) exhibited a 3-wave pattern, whereas the majority of cows (83%) exhibited a 2-wave pattern (Figueiredo et al. 1997). Others have reported that up to 27% of estrous cycles in *Bos indicus* cows consist of 4 waves of follicular development, compared to only 7% in heifers (Bo et al. 2003).

Follicular wave emergence in cattle is characterized by the sudden (within 2–3 days) growth of 8–14 small follicles that are initially detected by ultrasonography at a diameter of 3–4 mm (Adams et al. 2008). The pattern of 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 in FSH (Adams et al. 1992) to stimulate them to start to grow (Figure 2).

In cattle, which have three follicular waves, the first, second and third waves were detected on days (0 to 2), (9) and (15 to 16) of the estrous cycle, respectively. Cows having two-wave pattern showed the first and second follicular waves on days (0 to 2) and (8 to12) of the cycle (Figueiredo et al. 1997, Ginther et al. 1989a, b, Savio et al. 1988, Sirois and Fortune 1988, Taylor and Rajamahendran 1991). In alternative pattern, cattle have showed four waves per cycle, the waves began on days 2, 8, 14 and 17, respectively (Sirois and Fortune 1988). Each wave involves follicular growth, stagnation, dominance and regression phases. In the growth phase a cohort of 20–24 follicles, at least 4 mm in diameter, begins to grow beyond this diameter during each wave. One follicle of the cohort is selected to continue growth (dominant) while its sister follicles (subordinates) plateau in growth and then regress around day 5 of the

follicular wave. The dominant follicle produces factors that inhibit its counterparts in the same wave and suppress their growth (Ginther et al. 1996). This effect is more apparent in 6–8 mm follicles than in smaller follicles (Hendriksen et al. 2000). Selection of the dominant follicle is associated with decreasing blood FSH concentration during the first 3 days of the wave. The dominant follicle acquires more LH receptors on its granulosa cells than its subordinates and therefore is able to shift its gonadotropin dependence to LH during the FSH nadir, and continue to grow while the subordinates regress. In non-ovulatory waves, all follicles, including dominant one(s), become atretic and undergo apoptosis (Hendriksen et al. 2000, Mihm and Austin 2002) while in the last wave the dominant follicle ovulates to give mature oocytes. Ginther et al. (1989c) demonstrated that the dominant follicle suppresses the growth of subordinates and the emergence of new wave via systemic (endocrine) rather than local channels. Only one follicle from the pair of ovaries is selected to become dominant, the side of dominant follicle development was random, and the dominant follicle was equally likely to reside in the same or contra lateral ovary to that of the largest subordinate follicle. The side of the CL or dominant follicle of a previous wave had no effect on the side of the ovulatory follicle.

2.3 Oocyte developmental competence

The term “oocyte competence“ is the final measurement of its quality. Sirard et al. 2006 have defined the five levels of oocyte competence that represent the key steps that characterize developmental competence:

(1) Ability to resume meiosis.

(2) Ability to cleave following fertilization.

(3) Ability to develop to the blastocyst stage.

(4) Ability to induce a pregnancy and bring it to term.

(5) Ability to develop to term in good health

There are a lot of criteria (morphological, cellular, molecular) have been proposed to evaluate oocyte competence or quality (Wang and Sun 2007). Generally, oocytes are evaluated based on the morphological appearance like cytoplasm darkness, number of cumulus layers, extrusion of polar body and spindle formation (Gordon 2003, Wang and Sun 2007). However, this type of oocyte selection is inconsistent and sometimes is

controversial (Gordon 2003, Wang and Sun 2007), but it could be useful as a pre-selection parameters for the other methods. On the other hand, cellular organelles like mitochondria (El-Shourbagy et al. 2006) or protein activity such as glucose-6-phosphate dehydrogenase (Alm et al. 2005) have been shown to be good predictors of oocyte quality. Gene expression profiling of cumulus and the oocytes were considered as the molecular markers of the oocyte quality (Assidi et al. 2008, Bettegowda et al. 2008, Patel et al. 2007, Torner et al 2007). Some other researchers used different criteria for oocyte selection (Dinnyes et al. 1999, Lonergan et al. 1999) who indicated that there is a positive relationship between the time of first cleavage post-insemination in vitro and developmental competence, with those oocytes cleaving earliest after IVF being more likely to reach the blastocyst stage than their later cleaving counterparts. Some of these previously mentioned criteria of oocyte quality will be described in more details in this section or in the discussion part in particular the molecular predictors.

2.4 Acquisition of oocyte developmental competence

Acquisition of oocyte developmental competence is a continuous process throughout folliculogenesis (Figure 3).

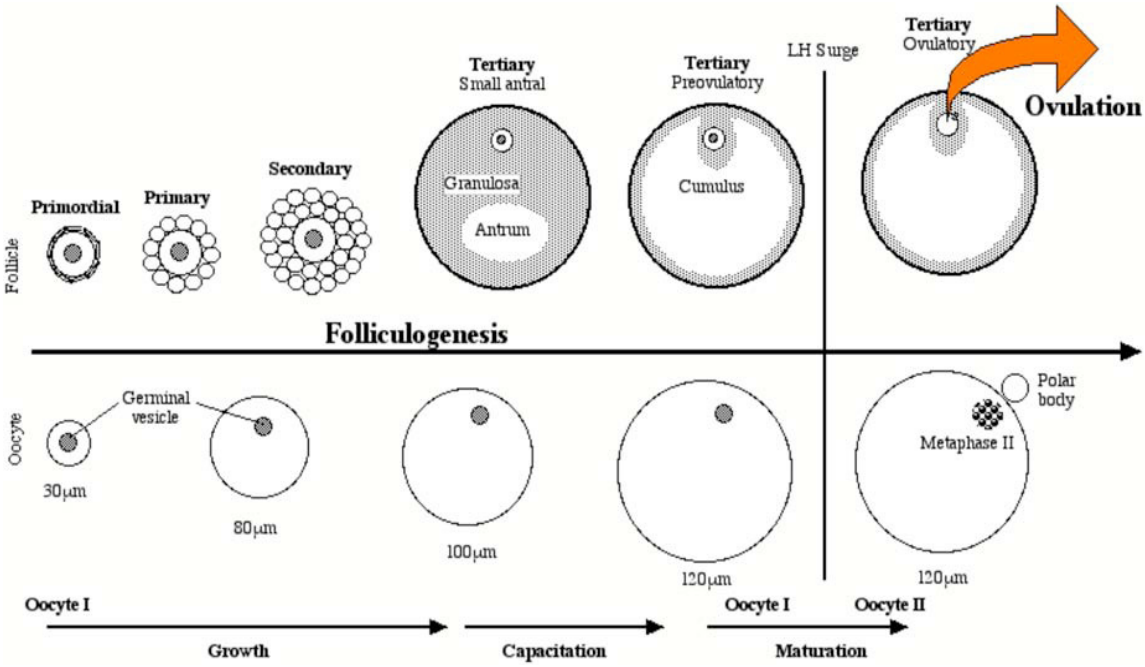
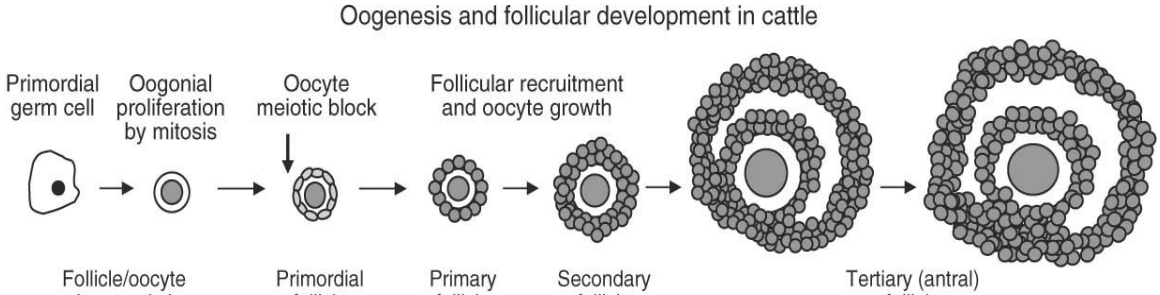


Figure 3: Schematic representation of oocyte growth, capacitation and maturation along the axis of folliculogenesis (Mermillod et al. 1999)

This acquisition could be divided into three separate parts defined by special physiological events (Mermillod et al. 1999): (1) oocyte growth, taking place mainly during the beginning of follicular emergence (primary and secondary pre-antral follicles); (2) oocyte capacitation, starting at the end of oocyte growth in antral (tertiary) follicles; and (3) oocyte maturation, starting after LH surge in preovulatory follicles or after removal of oocyte from the follicular environment that inhibiting meiotic resumption. In bovine oocytes, acquisition of meiotic competence does not occur until the antral follicle stage, when the oocyte diameter greater than 100µm (Fair et al. 1995). As the oocyte grows it gains meiotic competence in three stages (Figure 4). The oocyte first acquires the capacity to undergo germinal vesicle break down (GVBD) then it becomes capable of progressing to metaphase I (MI) and finally it gains the ability to progress to metaphaseII (MII) (Fair et al. 1995). At a diameter of 110µm, the majority of bovine oocytes exhibit full meiotic competence and can reach MII (Fair et al. 1995). As the follicular diameter increases to approximately 2mm and the oocytes increase in diameter from 110 to120µm, developmental competency is acquired and the majority of oocytes become capable of supporting fertilization and embryonic development (Hyttel et al. 1997).



Follicle/oocyte characteristic	Primordial follicle	Primary follicle	Secondary follicle	Tertiary (antral) follicle		
Follicular diameter (mm)	0.03–0.04	0.04–0.10	0.10–0.60	0.60–1.5	1.5–2	>2
Oocyte diameter (µm)	30	30	30–95	95–100	100–110	110–120
GV → GVBD	–	–	–/+	+	+	+
GVBD → MI	–	–	–	+	+	+
MI → MII	–	–	–	–	+	+
Developmental capacity	–	–	–	–	–	+

Figure 4: Summary of oogenesis and folliculogenesis in cattle. Acquisition of meiotic and developmental competence increase as the diameter of the oocyte and follicle increase. +, present; -. Absent; GV, germinal vesicle; GVBD, germinal vesicle break down; MI, MII, metaphase I and II, respectively adapted from (Rodriguez and Farin 2004)

2.5 Oocyte origin and its developmental competence

Several groups of investigators compared the effects of replacing the successive in vivo steps in the development process by in vitro conditions on early embryonic development (Figure 5). From these studies it is evident that, besides the process of fertilization, growth and maturation of the oocyte is the most critical factor influencing the outcome of embryo technologies (Merton et al. 2003).

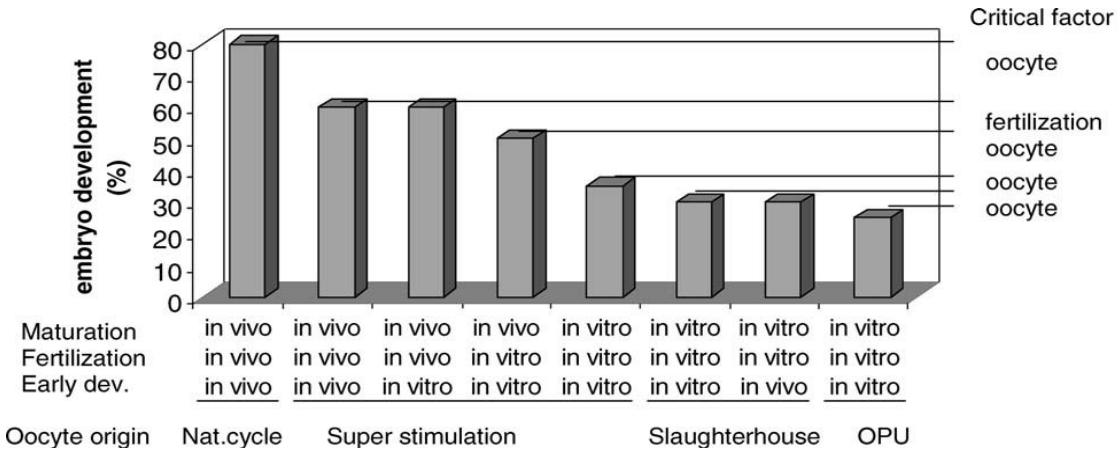


Figure 5: Effect on embryo production of the origin of the oocyte and the successive steps of maturation, fertilization and early development performed in vivo and/or in vitro (Merton et al. 2003)

In the natural cycle, after fertilization, approximately 80% of the ovulated oocytes will develop in vivo to the embryo stage. Super stimulation is intensively used to increase the number of good quality oocytes. In a study of Besenfelder et al. (2008), 1098 out of 1411 collected complexes (78%) were assessed as viable embryos that had developed to at least the morula stage after superovulation. A relatively large decrease in embryo production rate occurs when superovulation-derived oocytes are matured in vitro in standard medium compared to in vivo maturation (Hendriksen et al. 2000, Rizos et al. 2002, Wurth et al. 1994). Due to the ovulation window with superovulation (Callesen et al. 1986, Dieleman and Bevers 1987), some oocytes collected from superstimulation may not have completed maturation at the time of collection (Takagi et al. 2001). Furthermore, when comparing oocytes matured in vivo versus oocytes matured in vitro, no apparent differences are evident in the level of nuclear maturation, in the rates of in

vitro fertilization or cleavage, but rather in the in vitro developmental competence of the oocytes (Rizos et al. 2002, Sirard and Blondin 1996). Oocytes matured in vivo have a higher blastocyst rate than that matured in vitro (Humblot et al. 2005, Rizos et al. 2002). Bovine oocytes resuming meiosis in vivo originate from dominant follicles about 15 mm that have been grown from 4 to 15 mm in approximately 5 days (Pavlok et al. 1992). In contrast, oocytes for in vitro maturation (IVM) are usually retrieved from 2 to 6 mm follicles that are between 4 and 10 days from potential ovulation and the IVM period lasts only 24 h (Sirard et al. 1992). Thus, it is possible that oocytes matured in vitro have not acquired maximal developmental competence due to incomplete oocyte capacitation, a process during which RNA, proteins, and other molecules are synthesized (Hyttel et al. 1997). This accentuates the importance of the oocyte and its process of growth and maturation. Noteworthy also are the differences between the in vitro embryo production rate of immature oocytes derived either after superovulation, from slaughterhouse ovaries or by OPU, indicating the importance of oocyte-related processes even before maturation. In general, it can be inferred that the treatment and technique of oocyte retrieval are crucial in determining the outcome of embryo production in the commercial application of embryo technologies (Merton et al. 2003).

2.6 Follicular environment and oocyte competence

There is growing evidence supporting the idea that the follicle differentiation influences the ability of the oocyte to become an embryo (Mermillod et al. 1999).When oocytes are obtained for in vitro maturation and fertilization (IVM-IVF), not all of them have the ability to develop into an embryo. The incapability to sustain further development may be associated with incomplete maturation of the oocyte during folliculogenesis (Fulka et al. 1998, Moor et al. 1998). It is still not known at which size or step of follicular development the oocyte becomes competent. Some oocytes collected from large follicles are still not able to reach the blastocyst stage, whereas others coming from smaller follicles are fully competent (Blondin and Sirard 1995, Mermillod et al. 1999). In a recent study, it was demonstrated that the developmental capacity of oocytes obtained from follicles of the same size category, with the same grade of COC morphology and cumulus expansion, may vary significantly according to levels of GC apoptosis. Conversely, at the same level of atresia, oocytes may differ in development

when they were from follicles of different sizes, or with different grades of cumulus expansion (Feng et al. 2007). It was inferred that the developmental potential of an oocyte is determined by multifactor interactions, and that multiple factors must be considered simultaneously to accurately predict the quality of an oocyte. So, some of the factors that considered having profound influence on oocyte competence will be described.

2.6.1 Follicular wave and oocyte competence

There is a general agreement that the stage of the follicular wave affects the developmental competence of bovine oocytes after fertilization due to the changes of follicles from which the oocytes are obtained (de Wit et al. 2000, Hagemann et al. 1999, Hendriksen et al. 2000, Machatkova et al. 1996). During the estrous cycle, the follicle population changes under the influence of follicular waves. The interactions between the follicles throughout each follicular wave affect oocyte quality (Figure 6, 7).

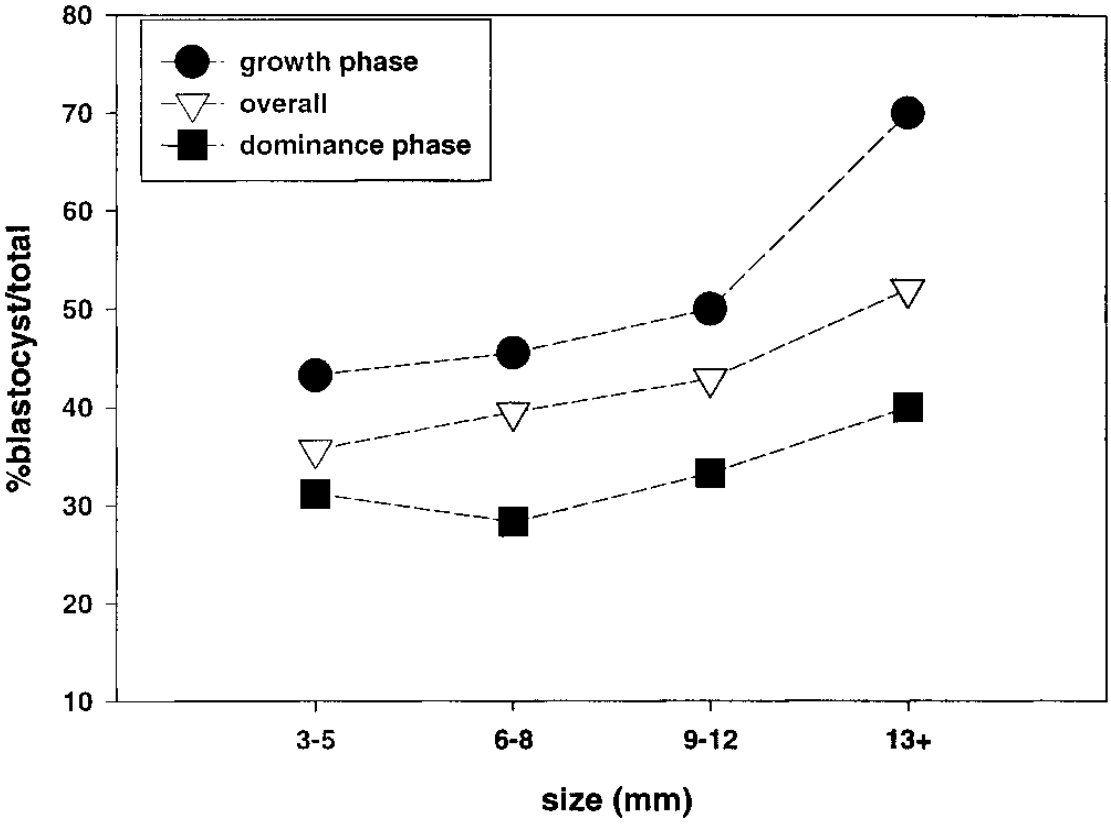


Figure 6: Oocyte development to blastocyst of all grades on day 8 of culture. Significant effects of cycle phase and follicle size (Hagemann et al. 1998, Hagemann et al. 1999)

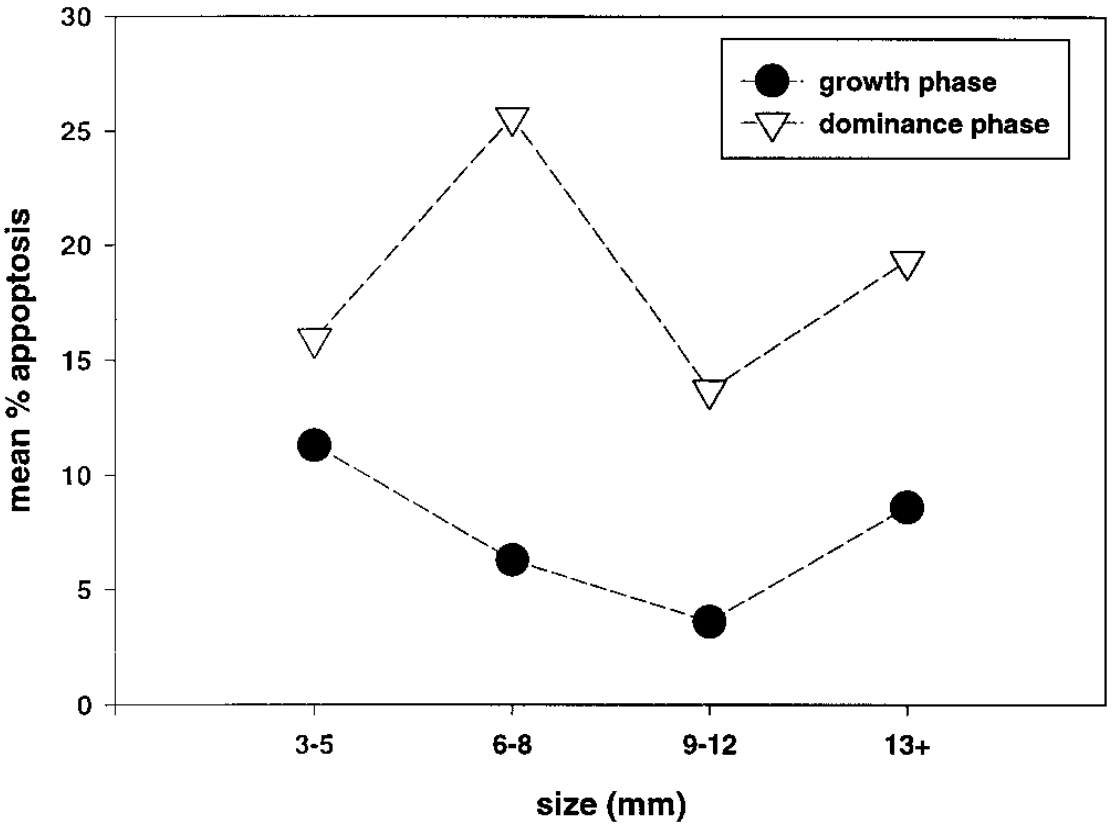


Figure 7: Mean percent apoptotic cells in follicles from which the oocyte developed to morula or blastocyst by day 8 in culture. Significant effects of cycle phase and follicle size (Hagemann et al. 1998, Hagemann et al. 1999)

The earliest studies (Hagemann et al. 1998, Hagemann et al. 1999) has shown that oocytes from ≥3 mm size follicles collected during days 2 and 10, presumably at emergence of a follicular wave, produced significantly more (44.7% vs. 31.3% total blastocysts) and higher quality (36.0% vs. 24.9% Grade l-2 blastocysts) embryos than those collected during dominance phase (day 7 and 15). In addition, the proportion of apoptotic cells was higher during dominance than during growth phase. Machatkova et al. (2000) induced estrus by PG injection and collected oocytes by ovum pick up (OPU) at either day 1, 2 or 3 of the cycle (day 0 was the day of observed estrus). The blastocyst rate was significantly lower at day 1 (12.8%) than at days 2 and 3 (27.8 and 27.5%, respectively). In another study, oocytes obtained by OPU at either day 2, 5 or 8 of the follicular wave, in which the start of the follicular wave was induced by ultrasound-guided aspiration of all large follicles. The proportion of suitable COCs was significantly higher at days 2 and 5 (85, 96%, respectively) than at day 8 (68%) of the follicular wave. Moreover, the blastocyst rates were 27, 29 and 15% for days 2, 5 and 8,

respectively (on the basis of 95, 55 and 64 COCs), indicating impaired oocyte competence during the late phase of dominance (day 8) and not during the early phase of dominance (day 5) and phase of growth (day 2). This idea was supported by the study done by Hendriksen et al. (2004). In which the proportion of oocytes with three or more layers of non-expanded cumulus cells was higher for day 5 than day 8, while days 2 and 5 did not significantly differ from each other (85, 96 and 68% of 113, 60 and 101 oocytes for days 2, 5 and 8, respectively). The proportion of oocytes competent to develop a blastocyst in vitro was higher for days 2 and 5 than for day 8 being 27, 29 and 15% for the oocytes with fair to good cumulus investment and 23, 27 and 11%, respectively, when all oocytes were taken in account. This indicates that the dominant follicle reduces the developmental competence of oocytes from subordinate follicles at a relatively late stage of dominance. In a study conducted to look at the interaction between the stage of follicular development and the follicle size, in relation to the efficiency of embryo production (Machatkova et al. 2004). In this study, mean numbers of useable oocytes per donor were similar in both follicular phases, but the mean number of embryos per donor and the development rate of oocytes into blastocysts were higher in the growth phase than in the dominance phase which was two-fold when oocytes were collected in the growth versus the dominance phase (30.3% versus 14.9%). In that regard, the number of medium follicles on ovaries increased in the growth phase and therefore a higher proportion of oocytes were derived from them. On the contrary, in the dominance phase, the number of medium follicles decreased and embryos were derived predominantly from oocytes recovered from small follicles which had lower developmental potential than oocytes from small follicles in the growth phase. Regarding the last ovulatory follicular wave, Assey et al. (1994) reported that bovine oocytes aspirated from dominant follicles before the LH surge display alterations in their nuclear and cytoplasmic morphology, which, according to the authors, are a prerequisite for the acquisition of full developmental competence. This would indicate that not only final oocyte maturation (i.e., the processes occurring between LH surge and ovulation) is significant, but also the period preceding the LH surge may be important for the establishment of developmental competence (Hyttel et al. 1997). From all these previously described studies it was concluded that the oocyte competence is relatively low at day 1 of the follicular wave, is high during days 2–5 and is decreased again at days 7 and 8, while the ovulatory follicle at 21 is most competent one. This

indicates that although the dominant follicle (DF) is formed at days 3–5, its negative effect on the competence of the oocytes of the subordinate follicles does not become apparent before days 6 or 7, when the subordinate follicles reach more advanced stages of atresia. Apparently, beginning follicular atresia is not detrimental for oocyte competence but, in contrast, is even beneficial for it. This conclusion has consequences for the practice when cows are undergoing OPU only once or very irregularly. In that case, the yield of embryos can be improved by performing the OPU during the days 2–5 of the cycle or to perform a DF ablation 2–5 days prior to the OPU session. Indeed lower blastocyst rates have also been reported when OPU was performed once a week in comparison with OPU every 3 to 4 days (Goodhand et al. 1999, Hanenberg and van Wagtendonk-de Leeuw 1997) and this presumably due to that the higher frequency of OPU prevents the establishment of a DF.

2.6.2 Follicle health

Moor et al. (1980) has noticed that an oocyte requires a specific follicular steroid environment to attain complete maturation and normal fertilization, which suggests that the constantly changing microenvironment of the oocyte throughout follicular growth (whether the follicle is healthy or atretic) might be related to its acquisition of developmental competence. In fact, 85% of follicles found in an ovary at any time in the estrous cycle are atretic (Kruip and Dielman 1982). This appears normal, as most follicles do not ovulate, and enter a phase of regression and become atretic (Quirk et al. 1986). The first signs of atresia are manifested by degeneration of the granulosa cells, whereas the oocyte is affected in the very last stages of atresia (Kruip and Dielman 1982). While the detection of granulosa cells (GCs) apoptosis has been widely used to identify atretic follicles, very little evidence for its use as a marker of oocyte quality exists. Blondin and Sirard (1995) demonstrated that, developmental competences of oocytes from nonatretic, intermediate and slightly atretic follicles were the same. The percentage of embryos produced from oocytes with signs of increasing atresia was even higher, except for oocytes that were derived from heavily atretic follicles (de Wit et al. 2000, Jewgenow et al. 1999). Moor et al. (1996) have demonstrated that a low level of atresia tended to improve the in vitro competence of bovine oocytes. It has been proposed that, presence of a dominant follicle has a negative effect on the IVF-produced

bovine embryos (Varisanga et al. 1998, Machatkova et al. 2004). This is due to the direct inhibitory effect of the dominant follicle on the development of subordinate follicles which causing them to undergo atresia (Wolfsdorf et al. 1997) leading to lower in vitro developmental competence compared to their counterparts at growth phase (Hagemann et al. 1999).

2.6.3 Effect of follicle size and oocyte diameter

In cattle, the oocyte and follicle continue to grow in parallel until the follicle reaches a diameter of 3 mm; thereafter, the oocyte diameter plateaus at about 120–130 µm, while the follicle can grow up to 15–20 mm in diameter before ovulation (Fair 2003). In a study (Otoi et al. 1997) conducted to determine the diameter of bovine oocytes that were able to attain their full developmental competence to blastocysts.

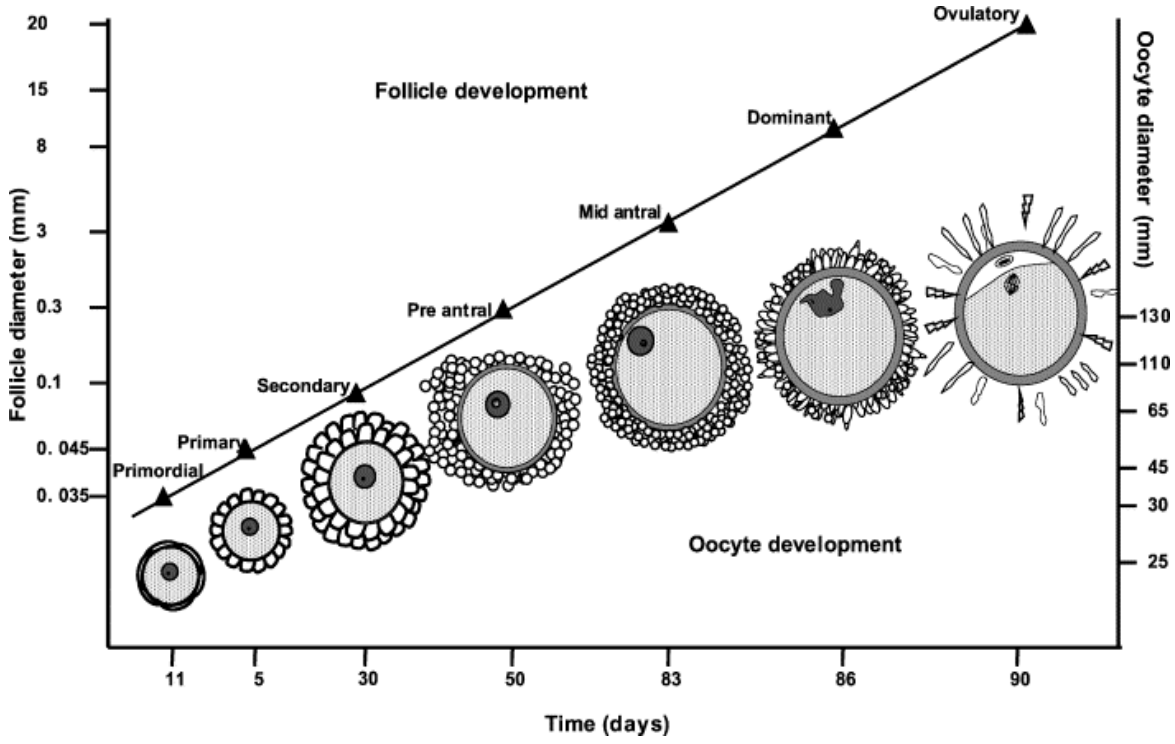


Figure 8: The relationship between follicle development and oocyte development in cattle. The growth of oocytes (surrounded by granulosa cells) is shown schematically in relation to follicle diameter, oocyte diameter and estimated duration of stage of folliculogenesis from the primordial to preovulatory stage. Based on data from Hulshof et al. (1992) and Lussier et al. (1987)

The authors have recovered the oocytes from slaughter house ovaries by aspiration of surface-visible follicles from 1 to 7 mm and categorized the oocyte diameter into < 110 μm, 110 to < 115 μm, 115 to < 120 μm, 120 to < 125 μm, 125 to < 130 μm and ≥ 130 μm. The oocytes displayed size-related ability to undergo meiotic maturation, to cleavage and to develop into blastocyst stage. Finally, it was suggested that bovine oocytes have acquired full meiotic competence at a diameter of 115 μm but only attained full developmental competence to blastocysts when oocytes reach the diameter of 120 μm (Otoi et al. 1997).

The follicle size from which the oocytes are obtained characterizes the developmental stage of the follicle and the maturational stages of the oocyte within that follicle (Blondin and Sirard 1995, Lequarre et al. 2005, Lonergan et al. 1994). Initial studies have highlighted that developmental competency is acquired progressively during folliculogenesis in oocyte populations (Eppig 1996). Thus oocytes from preantral follicles are unable to resume meiosis after arresting in prophase I, whereas those from very small antral follicles (in the bovine <0.9 mm) are competent to progress to metaphase I, and those from larger antral follicles to metaphase II and beyond (Fair et al. 1995, Otoi et al. 1997). However, morphologically indistinguishable oocytes from larger antral follicles, which are competent to complete nuclear maturation (meiosis) and fertilization, show differences in their ability to develop to the blastocyst stage (Lonergan et al. 1994, Fair et al. 1995, Otoi et al. 1997, Blondin and Sirard 1995). Pavlok et al. (1992) did not observe a difference in the developmental competence between oocytes coming from 2 to 4 mm follicles or from 4 to 8 mm. However, oocytes from 1- to 2-mm follicles have shown a significantly lower competence to undergo in vitro maturation, fertilization and completely lack the capability to cleave beyond the 8-cell stage. In another study done by Lonergan et al. (1994), blastocyst yield from oocytes originating from follicles >6 mm was double (66%) than that of oocytes from 2 to 6 mm (34%). Oocytes derived from follicles <3 mm had a lower developmental competence but they could not show differences between sizes of 3-5 and >5 mm (Blondin and Sirard 1995), this similar to results from Hagemann et al. (1999). In a study done in three different laboratories, they have confirmed that oocytes derived from follicles with a diameter ≥6 mm have a higher developmental competence than oocytes from follicles <4 mm (Lequarre et al. 2005). From all above mentioned results, it is generally admitted that follicle size and oocyte diameter are closely related, and as

both increase the developmental potential of the oocyte also increase (Figure 8) (Albertini et al. 2003, Arlotto et al. 1996, Gandolfi et al. 2005).

2.6.4 Effect of oocyte morphology

The possibility of selecting bovine oocytes based on visual assessment of the morphological features was first examined by Leibfried and First (1979). Since then, many reports have proposed classification schemes based on the compactness and number of layers of cumulus cells surrounding the oocyte (Hazeleger and Stubbings 1992, Laurincik et al. 1992, Yang and Lu 1990) and on the appearance of the oocyte itself (de Loos 1989, Younis et al. 1989). Oocytes with the highest developmental competence have been reported to have an even, smooth, finely granulated cytoplasm, and are surrounded by less than three compact layers of cumulus cells. A positive correlation between COC morphology and blastocyst yield after in vitro culture has been reported (Hazeleger et al. 1995). Madison et al. (1992) have indicate that light microscopic assessment of oocytes based on the compactness and quantity of follicle cells (corona radiata and cumulus cells), could be used to select immature oocytes for their potential to develop into blastocysts in vitro. In this study, oocytes of category 1 (oocytes surrounded by compact, multi-layers of follicle cells) developed to the blastocyst stage at a significantly greater frequency than oocytes of category 3 (oocytes with less compact and fewer layers of follicle cells; 24.5% and 14.9%, respectively). However, Vassena et al (2003) have reported that morphologic characteristics of oocyte quality were not predictive in identifying competent oocytes. Cumulus oocyte complexes morphology and their developmental competence change during growing, static and regressing phases of subordinate follicle development (Salamone et al. 1999). Greater proportion of cumulus–oocyte complexes collected from subordinate follicles during the early regressing phase were expanded and a significantly greater proportion of oocytes showed evidence of nuclear maturation than those collected during the growing and early static phases. Furthermore, oocytes obtained from early regressing subordinate follicles (day 5 of follicular wave) were more likely to develop into embryos in vitro than those obtained from growing (day 2), early static (day 3) or late-regressing (day 7) subordinate follicles (Adams et al. 2008, Salamone et al. 1999).

2.7 Effect of animal age

Age of oocyte donor is a significant factor influencing oocyte competence and thus the efficiency of in vitro embryo production (Armstrong 2001). Antral follicles with fully grown oocytes are present in calves at or prior to birth but overall success of pregnancies from in vivo and in vitro produced embryos derived from oocytes of prepubertal animals is low (Armstrong 2001, Gandolfi et al. 1998, Khatir et al. 1996, Khatir et al. 1998, Revel et al. 1995) In cattle, although prepubertal calf oocytes are capable of undergoing nuclear maturation and fertilization at similar rates to cow oocytes, blastocyst yields from such oocytes are significantly reduced compared with that of adult oocytes (Damiani et al. 1996, Khatir et al. 1996, Khatir et al. 1998). Similar trend was also observed in other species like sheep (Ledda et al. 1997) and pigs (Archipong et al. 1987). Oocytes collected from prepubertal animals display reduced activity of maturation promoting factors, mitogen-activated protein kinase and cyclin B, altered protein synthesis, aberrant energy metabolism, less Ca2+ influx at fertilization, and an overall reduced embryo survival post-fertilization (Armstrong 2001, Gandolfi et al. 1998, Khatir et al. 1996, Khatir et al. 1998, Levesque and Sirard 1994, Revel et al. 1995). Reduced developmental capacity of oocytes from calves might be caused by an abnormal cytoplasmic maturation of these oocytes. Thus, calf oocytes represent a good negative model for attempting to understand the mechanisms involved in acquisition of developmental competence (Bettegowda et al. 2008, Patel et al. 2007). Other studies on the effect of age (Mermillod et al. 1992) showed a higher blastocyst yield from animals between 1 and 3 years of age than older animals. Moreover, Rizos et al. (2005) have clearly shown that higher proportion of presumptive zygotes derived from abattoir-derived cow oocytes reached the blastocyst stage following culture in vivo in the ewe oviduct than those derived from heifer oocytes (53.1% versus 25.2% for cow and heifer oocytes, respectively). The previous authors have concluded that the origin of the oocyte has a significant impact on its subsequent developmental potential and cow oocytes should be preferentially used over those from heifers in order to maximize blastocyst development.

2.8 Effect of nutrition

Nutrition is one of the main reasons for the decline in fertility in the modern dairy industry. During early lactation, high-yielding dairy cows are typically in a state of negative energy balance because the amount of energy required for maintenance both of metabolic function and milk production exceeds the amount of energy cows consume. Insufficient energy supply results in poor reproductive performance, which includes a delay in the onset of estrous cycles postpartum (Butler 2000, Butler and Smith 1989, Reist et al. 2000, Staples et al. 1990) and a reduction in oocyte quality (Snijders et al. 2000, Walters et al. 2002), resulting in low conception rates and a high rate of early embryonic death (Lucy 2001). Cows fed high energy diets produced more good oocytes than did cows fed low energy diets (Kendrick et al. 1999). Consistent with this, Fouladi-Nashta et al. (2007) have shown that higher level of dietary fat significantly increased rate of blastocyst production from both IVF oocytes and cleaved embryos. In addition, the higher level of dietary fat improved embryo quality through increases in total and trophectoderm cell numbers in blastocysts. In addition, short-term changes in plan of nutrition have been shown to have a direct effect on ovarian follicular dynamics in cattle, without any changes in circulating concentrations of 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 granulosa-oocyte interactions (Figure 9) and these 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 and Boland 1999, O'Callaghan et al. 2000). On the other hand, Armstrong et al. (2001) and Sinclair et al. (2000) have reported a reduction in oocyte quality when dietary protein was increased. In the high-yielding dairy cow, supplementary dietary carbohydrates can reduce the quality of oocytes and the development of embryos, resulting in fewer blastocyst cells and a lower rate of blastocyst production (Fouladi-Nashta et al. 2005). Oocyte quality is also affected by an interaction between feeding level and body condition: a high level of feeding is beneficial to oocytes from animals of low body condition, but detrimental to oocytes from animals of moderate to high body condition (Adamiak et al. 2005).

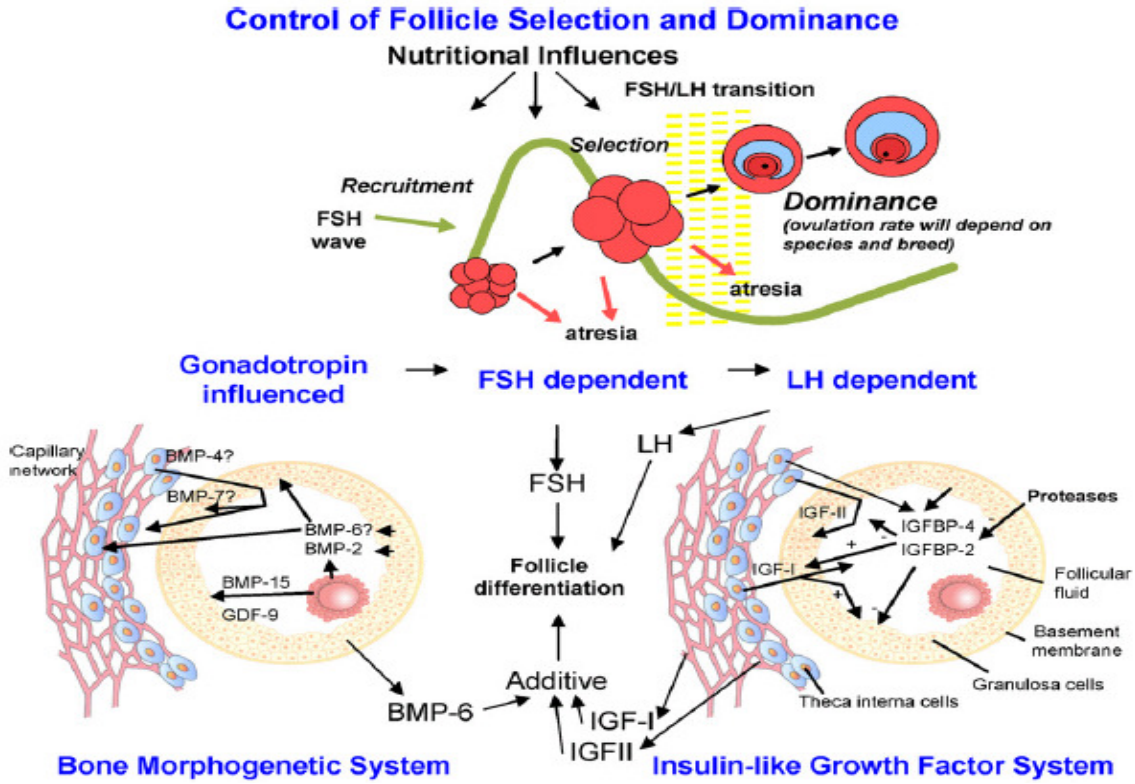


Figure 9: Diagram showing the influence of nutrition, the role of gonadotropins and the interaction with the intra-follicular IGF and BMP systems on antral follicle development in both mono- and poly-ovulatory farm animal species. The top section describes a follicular wave and when the dominant follicle(s) transfers its dependence from FSH to LH. The bottom two sections illustrate some of the key members of two local growth factor systems (IGFs and BMPs) shown to be important in follicular development. It also highlights the additive effect of the IGF and BMP systems on FSH and LH stimulated follicular development (Adapted from Campbell et al. 2006, Webb et al. 2003, Webb et al 2007)

2.9 Genetic influence

In the last 4 decades milk yield in dairy cows has increased significantly as a consequence of genetic selection. However, this improvement has been associated with reduced fertility. Few studies have reported influence of genetic merit for milk production on oocyte quality. In a large embryo transfer study, the maternal genetic ability to respond to superovulation was demonstrated (Govignon et al., 2000), as well

as the contribution of recipient cattle to fertility after embryo transfer (McMillan and Donnison 1999). Oocytes from high genetic merit cows yielded fewer blastocysts and had lower cleavage and blastocyst formation rates than oocytes from medium genetic merit cows (Snijders et al. 2000). Tamassia et al. (2003) showed a distinctive variation in number of oocytes recovered by ovum pick-up per cow allowing the classification of some cows as systematically 'good' or 'bad' oocyte producers. The same effect was also observed for blastocyst production, with cows showing a phenotype of 'good' or 'bad' blastocyst producer. The semen used did not influence extreme phenotypes for blastocyst rate, revealing a maternal influence on bovine embryo development. At this point, it is not possible to identify the origin of this maternal effect as it could be of genetic, nuclear (Picton 2001, Watson et al. 1999) or cytoplasmic origin (Cummins 2001, Duranthon and Renard 2003).

2.10 Activity of glucose-6-phosphate based on Brilliant Cresyl Blue staining

Immature oocytes are known to synthesize a variety of proteins, among them, glucose-6-phosphate dehydrogenase (G6PDH) (Wassarman 1988). G6PDH is synthesized and accumulates during the oocyte growth phase (Mangia and Epstein 1975). The activity of this protein is decreased once this phase has been completed and oocytes are then likely to have achieved developmental competence (Tian et al. 1998, Wassarman 1988). Brilliant Cresyl Blue (BCB) is a dye that can be broken down by G6PDH (Ericsson et al. 1993, Tian et al. 1998) thus, oocytes that have finished their growth phase show a decreased G6PDH activity (Figure 10) and exhibit a cytoplasm with a blue coloration (BCB^+) while, growing oocytes are expected to have a high level of active G6PDH (Figure 10) that resulted in colorless cytoplasm (BCB^-). The BCB test has been successful in selecting pig oocytes for IVM–IVF (Ericsson et al. 1993). In prepubertal goat oocytes, Rodríguez-González et al. (2002) showed that the BCB test permitted the selection of oocytes with larger diameters, higher percentages reaching Metaphase II, higher percentages of IVM–IVF oocytes with two pronuclei (normally fertilized) and higher embryo development up to the morula plus blastocyst stages compared to oocytes selected exclusively by morphological criteria.

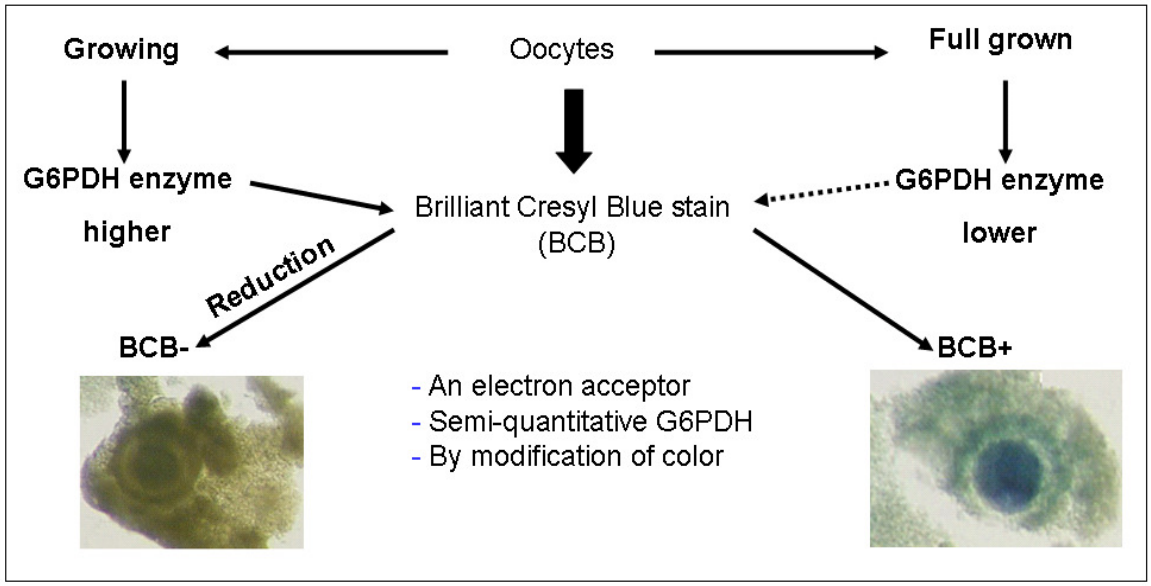


Figure 10: Differentially stained bovine COCs after exposure to BCB stain (BCB^+, blue-colored; BCB^-, unstained) based on data from Mangia and Epstein 1975, Wassarman 1988, Bhojwani et al. 2007)

The BCB test selects larger and more competent heifer oocytes for in vitro embryo production than conventional morphological criteria (Pujol et al. 2004). However, the percentage of blastocysts obtained was still lower than that obtained from cow oocytes. In addition, it has been shown that oocytes screened based on BCB staining differ in their developmental potential to reach blastocyst stage (Alm et al. 2005) and efficiency in utilization for somatic cell nuclear transfer (Bhojwani et al. 2007). The staining of buffalo oocytes with BCB stain before IVM identified developmentally competent oocytes for IVP of embryos (Manjunatha et al. 2007). In mice, BCB staining was used efficiently for oocyte selection; however the competence of the BCB^+ oocytes may vary with oocyte diameter, animal sexual maturity and gonadotropin stimulation (Wu et al. 2007).

Regarding the molecular and subcellular characterization of oocytes screened with BCB staining, it was reported that oocytes selected based on this method also different in various oocyte quality markers like cytoplasmic volume and mitochondria DNA copy number (El-Shourbagy et al. 2006). In a recent study, incompetent (BCB^-) oocytes exhibited a delay in mtDNA replication due to the delayed onset of expression of their nuclear-encoded replication factors and the oocyte attempts to rescue this during the final stages of maturation. Consequently, oocyte competence in terms of mtDNA

replication and composition is not fully synchronized and will result in either failed fertilization or developmental arrest (Spikings et al. 2007).

2.11 Contribution of cumulus to oocyte competence

Cumulus cells which surround oocytes during follicular development and ovulation play essential role in controlling oocyte growth, maturation, fertilization and its ability to support further embryonic development (Gandolfi et al. 2005, Tanghe et al. 2002). Oocytes extracted from small follicles must maintain contact with granulosa cells in order to grow, even though they remain alive in a nutrient culture medium for several days (Gosden 2002). The two cell types are metabolically coupled to each other (de Loos et al. 1991, Sugiura et al. 2005) and this union becoming much more intimate after follicle growth is initiated. Granulosa cells are also coupled to each other via gap junctions (Anderson and Albertini 1976). Pores in conventional gap junctions permit molecular exchange by diffusion of substances, including sugars, amino acids, lipid precursors and nucleotides (Brower and Schultz 1982).

It is well established that the presence of cumulus cells in the course of maturation is prerequisite for oocyte to acquire full cytoplasmic maturation. This notion is supported by previous studies done in bovine (Fatehi et al. 2002, Fukui and Sakuma 1980) and porcine (Maedomari et al. 2007, Wongsrikeao et al. 2005) showing that, removal of cumulus cells either at the beginning of in vitro maturation or shortly before in vitro fertilization adversely reduced oocyte developmental potential. The cumulus cells not only contribute to cytoplasmic maturation, but they may also involve in the maintenance of meiotic arrest (Aktas et al. 2003). It has been also proposed that cumulus cells contribute to oocyte maturation by protecting the oocyte against oxidative stress through enhancement of the cytoplasmic glutathione (GSH) synthesis (Luciano et al. 2005, Tatemoto et al. 2000, Yoshida 1993) and protein synthesis capability of the oocyte in addition to provide thermoprotection (Edwards and Hansen 1997). In addition, the transcriptional activity of the oocyte genome is modulated through the dialog between oocytes and somatic cells during follicular development (De La Fuente and Eppig 2001). In turn, oocyte secreted factors regulate a variety of cumulus cell functions (Figure 11) including proliferation, expansion, differentiation, metabolism and gene expression (Gilchrist et al. 2008, Matzuk et al. 2002, Sugiura et al. 2005, Vanderhyden

et al. 1992). A study from the Gilchrist group (Hussein et al. 2005) demonstrated that oocyte-secreted factors, especially BMP15 and BMP6, protect the cumulus cells from undergoing apoptosis by establishing a morphogenic paracrine gradient of BMPs. The bidirectional communication between cumulus cells and oocyte via gap junction is crucial in maturation regulation and chromatin remodelling of bovine oocytes (Lodde et al. 2007, Thomas et al. 2004).

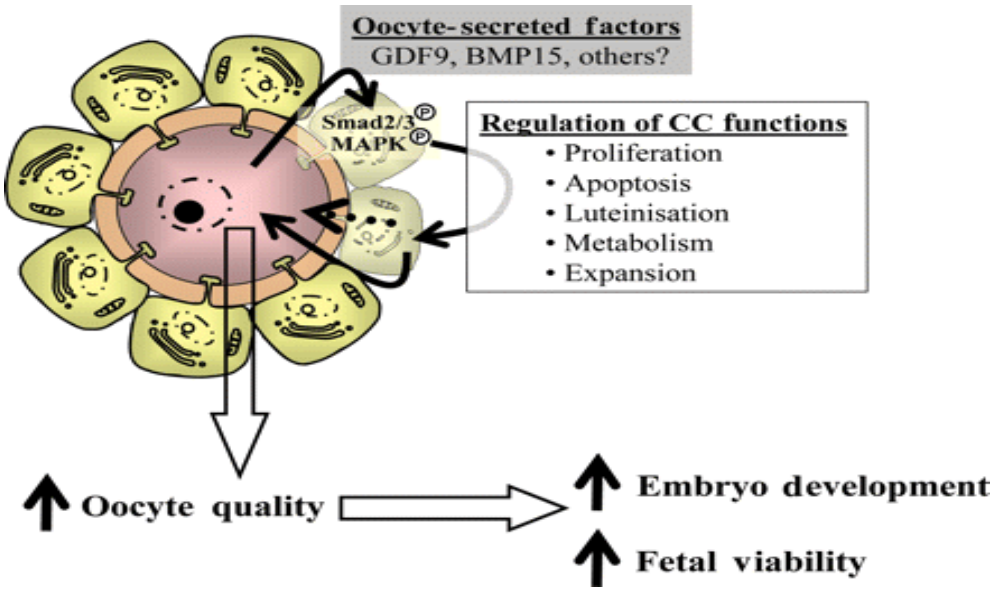


Figure 11: oocyte secreted factors (OSF) regulation of cumulus cells (CC) function and oocyte quality

2.12 Gene expression of cumulus cells as an indicator of oocyte quality

From all above mentioned facts, it is evident that coordinated bilateral dialogue between the cumulus cells and its enclosed oocyte is crucial for achievement of full competence of both cell types to support early embryogenesis. Thus, the messages stored in cumulus cells may be direct indicators of the further developmental fate of the oocytes until term. Many experiments have been recently conducted using both animal models and clinical patients to evaluate the granulosa cell gene signature(s) as molecular predictors of oocyte competence (Assidi et al. 2008, Bettegowda et al. 2008, Cillo et al. 2007, Feuerstein et al. 2007, Hamel et al. 2008, McKenzie et al. 2004, Zhang et al. 2005).By using suppressive subtractive cDNA hybridization and microarray technologies, the

Sirard group (Assidi et al. 2008) identified several potential cumulus cell markers of bovine oocyte competence including several growth differentiation factor GDF9 target genes [i.e. HAS2, TNFAIP6, PTGS2 and gremlin 1 (GREM1)]. Other candidates identified are inhibin βA (INHBA), epidermal growth factor receptor (EGFR), betacellulin (BTC) and CD44 molecule (Assidi et al. 2008). Another recent study using prepubertal calf oocytes as a model of poor oocyte competence; microarray analysis found that the transcript abundance of genes encoding the cathepsin family of cysteine proteinases (CTSB, CTSS and CTSZ) is negatively associated with bovine oocyte competence (Bettegowda et al. 2008). In search of potential human granulosa cell markers to complement the morphological criteria toward oocyte/embryo selection, it was reported that the quality of human oocytes is correlated with transcript abundance for specific GDF9 targets (i.e. HAS2, PTGS2 and GREM1) in the cumulus cell compartment (McKenzie et al. 2004). A subsequent study supported the idea of using HAS2 and GREM1 as candidate cumulus markers predictive of oocyte competence (Cillo et al. 2007). However, there is some controversy regarding the association of cumulus pentraxin (PTX3) transcript abundance with oocyte quality (Cillo et al. 2007, McKenzie et al. 2004, Zhang et al. 2005). Feuerstein et al. (2007) reported that a number of genes including prostaglandin-endoperoxide synthase 2 (PTGS2), steroidogenic acute protein (STAR), amphiregulin (AREG), stearoyl-co-enzyme A desaturase 1 and 5 (SCD1 and SCD5) are associated with oocyte nuclear maturation and their transcript levels are elevated after meiosis resumption. Interestingly, lower cumulus mRNA abundance of the aforementioned genes as well as connexin 43 (CX43) is present in MII oocytes that develop to blastocysts (Feuerstein et al. 2007).

Microarray technology is now being applied to define the gene expression profiles of human ovarian somatic cells in correlation with oocyte developmental competence (Hamel et al. 2008, van Montfoort et al. 2008). One study compared granulosa cell (mainly mural granulosa cell) gene expression profiles between follicles associated with successful pregnancy and those associated with arrested embryo development during IVF (Hamel et al. 2008). Identified candidates that positively correlate with oocyte development potential include, but not limited to, genes associated with steroidogenesis [hydroxy-delta-5-steroid dehydrogenase 3 beta- and steroid delta-isomerase 1 (HSD3B1), ferredoxin 1 (FDX1) and cytochrome P450 (CYP19A1)] and genes with potential involvement in apoptosis [serpin peptidase inhibitor clade E member 2

(SERPINE2) and cell division cycle 42 (CDC42)] (Hamel et al. 2008). Another study attempted to identify differentially expressed genes between cumulus cells derived from oocytes that develop to early cleavage (EC) embryos and cumulus cells from oocytes that fail to develop into EC (NEC) embryos (van Montfoort et al. 2008). The transcripts of genes increased in NEC samples [glutathione peroxidase 3 (GPX3), chemokine receptor 4 (CXCR4), stress-induced apoptosis inhibitor (HSPB1), cyclin D2 (CCND2), 7-dehydrocholesterol reductase (DHCR7), etc.] are reflective of a potentially hypoxic state of the cumulus cell microenvironment or delayed maturation of the oocytes (van Montfoort et al. 2008). Most recently, Assou et al. (2008) have proposed that the gene expression profiling of human cumulus cells genes involved in apoptotic (BCL2L11), metabolic (PCK1) and transcription (NFIB) pathways could be used as biomarkers for predicting embryo potential and pregnancy outcome.

2.13 Oocyte transcriptional activity

In bovine oocytes, transcriptional activity has been reported as early as the secondary follicle stage, when both heterogeneous nuclear RNA (hnRNA, the precursor of messenger RNA) and ribosomal RNA are synthesized. Such activity progresses until the oocyte reaches a diameter of 110-120 µm while it is enclosed in 3 mm follicle (Hyttel et al. 1997). Once a follicle reaches this diameter, the oocyte has achieved maximum size, and the transcription essentially ceases (Crozet et al. 1986). When a bovine oocyte is aspirated from an antral follicle with a diameter >3 mm, spontaneous meiotic resumption occurs, but it requires a short burst of transcription in the cumulus-oocyte-complex during the initial hours of maturation (Kastrop et al. 1991). Once germinal vesicle break down (GVBD) is occurred the transcription activity is no longer detectable (Memili et al. 1998). This transcription seems functionally important because its inhibition by α-amanitin (an RNA polymerase II inhibitor) impairs the maturation process (Kastrop et al. 1991).

2.14 Oocyte transcriptomics

The importance of oocyte quality cannot be overstated, because it affects all events during preimplantation development of the embryo, the fetus and even the resulting

offspring as reviewed by (Krisher 2004, Sirard et al. 2006, Van Soom et al. 2007). These events are mediated by a rich array of maternal mRNAs and proteins deposited in the oocyte during oogenesis. Maternal mRNAs are stored in an inactive, masked form, and recruited for translation in a stage-specific manner during oocyte maturation or early embryogenesis (Bachvarova 1992, Davidson 1986). The relative abundance of molecules stored in an oocyte differs between species and accounts for the differences in time post-insemination and stage of preimplantation development when zygotic gene expression is triggered and directs early development and differentiation. For example, in the mouse progression from 2-cell is dependent on zygotic gene activation, while it is 4-cell stage in the pig, and between 4- to 8-cell stage in humans, and 8- to 16-cell stage in cattle and sheep embryos where transition from maternal to zygotic expression occurs (Telford et al. 1990). During the maternal-zygotic transition, embryonic transcription is initiated and many maternal RNAs are degraded. Oocytes display remarkable post-transcriptional regulatory mechanisms that control mRNA stability and translation. The regulatory mechanisms are generally negative, and target mRNAs are either subjected to degradation or repressed from undergoing translation until specifically activated. Such negative regulatory mechanisms generally are mediated by transcript deadenylation (shortening of mRNA polyA tail), interaction of transcripts with RNA-binding proteins in a nonspecific or sequence-specific fashion, and/or potentially via actions of microRNA and repeat associated small interfering RNA, which degrade maternal RNA transcripts as reviewed by (Bettegowda and Smith 2007, Schier 2007). At the time when the protein is required, during maturation, fertilization or the early stages of embryo development, such dormant transcripts can undergo cytoplasmic polyadenylation and support translation, then are rapidly degraded. The underlying molecular mechanisms were shown to involve cis-regulatory elements within the 3' untranslated region (Bettegowda and Smith 2007, Brevini et al. 2007), including cytoplasmic polyadenylation elements (CPE). Interestingly, a possible relationship between the extent of polyadenylation, mRNA stability and developmental competence during oocyte maturation was indicated by (Brevini-Gandolfi et al. 1999, Brevini-Gandolfi et al. 2002). In this previous study, it was demonstrated that an appropriate length of the poly(A) tail at the 3_ end must be present, at least in the transcripts that we examined, in order to ensure the correct concentration of the encoded protein and adequately support meiosis and early embryo development.

Different experiments have been conducted to look at the developmental competence of bovine oocytes at the molecular level (Donnison and Rodriguez-Zas et al. 2008, Pfeffer et al. 2007, Wrenzycki et al. 2007). Using candidate gene approach, mRNA transcript abundance of panels of various genes has been analyzed either during IVM (Calder et al. 2005, Humblot et al. 2005, Lonergan et al. 2003b, Watson et al. 2000) or at the blastocyst stage (Knijn et al. 2002, Nemcova et al. 2006). Most of these candidate genes have been selected due to their potential roles or functions in early development, such as metabolism, regulation of gene and protein expression, intercellular communication, protection from oxidative stress (Calder et al. 2005, Knijn et al. 2002, Lonergan et al. 2003b). For example, the relative mRNA transcript abundance of Cx43 was significantly higher in blastocysts developed from oocytes collected from both medium and small follicles in the G/S stage (greater developmental competence) compared with those in the D/R stage (Nemcova et al. 2006). Interestingly, the transcript abundance of Hsp 70 gene was found to be reduced in in vivo matured oocytes collected shortly before ovulation compared to in vitro matured ones (Humblot et al. 2005). Recently, the higher mRNA expression of genes related to microtubule molecular motors like DYNLL1 (cytoplasmic dynein light chain LC8), and (cytoplasmic dynein 1 intermediate chain) and the diverging dynamics of DYNLL1, DYNC1I1, and proteosome such as PMSB1 (proteasome beta subunit 1) associated with the developmental capacity of oocytes to reach blastocyst stage (Racedo et al. 2008). With the advent of the functional genomics era, it has become possible to identify the transcriptome of oocytes using high throughput technology such as microarray. Evidence supporting oocyte cell gene markers as predictors of oocyte competence is now emerging. One of the earliest studies (Robert et al. 2000) performed to compares the entire mRNA content of oocytes (mRNA content of oocytes from follicles 3-5 mm where considered to be more competent compared to the content of oocytes from follicles $\leq$2 mm follicle size) using a novel PCR-based suppressive subtractive hybridization technique and compares it to differential display. Several known genes like cyclin B1, splicing factor ccl.4, cytochrome c oxidase and mineralocorticoid receptor were differentially identified in two oocyte populations. By using linear amplification with subtractive suppression hybridization (SSH); set of 10 maternally expressed genes identified as being enriched in competent oocytes included genes coding for transcription factors, namely the homeodomain containing oct4 and Msx1

and Zinc-finger domain Znf198 proteins, the histone stem-loop binding protein SLBP, Cyclin A, the heat-shock protein 40/DNA-J like (Dja4), NEDD4-interacting protein 1 (NDFIP1), trafficking protein particle complex 3 (Trappc), the TGFβ superfamily member GDF9 and unknown clone 1166 which appears to be the trailer for a Ras-related GTP-binding C transcript (Donnison and Pfeffer 2004). Using SSH combined with microarray approach (Mourot et al. 2006), candidate genes regulating 6 functional gene categories: cell cycle (PTTG1, CDC5L, CKS1B, CCNB2) cell metabolism (PSMB2, PRDX1), cell signalling (RGS16), gene expression (SKIIP), and chromatin support (H2A) showed a significant quantitative association with oocyte competence according to follicle size and time of first cleavage. On the other hand, genes related to growth factors (BMP15, GDF9), and other members of cell cycle (CCNB1, STK6) did not show differential expression according to follicle sizes nor to the time of first cleavage (Mourot et al. 2006).

Global gene expression analysis using microarray allows us to establish a molecular transcriptome blueprint of oocytes during maturation (Fair et al. 2007), to better understand oogenesis, folliculogenesis and the critical events occurring during preimplantation period (Kocabas et al. 2006, Misirlioglu et al. 2006, Yao et al. 2004) in addition to develop precise criterion for assessing oocyte quality (Patel et al. 2007) and the health of the early embryos (Watson et al. 1999). In bovine, different approaches and microarray platforms have been used to examine RNA abundance at a global level and to identify downstream genes in a particular pathway or genes mis-regulated under specific experimental conditions. Bovine oocyte-specific cDNA microarray over 2000 clones (Yao et al. 2004) has identified six transcripts [ribosomal protein L7a, dynein light chain, Doc2α, calmodulin, leucine-rich protein, and the novel gene clone (Begg20_H6) in fetal ovary (enriched source of oocytes) as regulatory genes in follicular development and early embryogenesis. Using Affymetrix microarrays, Pan et al. (2005) have characterized the global changes in gene expression of mice oocytes derived from primordial, primary, secondary, small antral, and large antral follicles and used Expression Analysis Systematic Explorer (EASE) to identify biological and molecular processes that accompany these transitions and likely underpin acquisition of meiotic and developmental competence. Consistent with growth and initiation of acquisition of meiotic competence occurring between the primary and secondary follicle stages is the over-representation of genes in cell cycle, biosynthesis, and

macromolecular metabolism In addition, there was progressive increase in transcript abundance for a number of genes involved in maintenance of genomic integrity (DNA replication and DNA repair).

A recent functional genomics study using bovine cDNA array containing ESTs representing approximately 15,200 unique genes Patel et al. (2007) has revealed reduced follistatin transcript abundance to be associated with poor oocyte developmental competence. Similarly, microarray analysis has been used to investigate the gene expression profiles of oocytes recovered from aging and young female mice (Hamatani et al. 2004). Interestingly, this study showed differences only in approximately 500 genes, of which genes involved in oxidative stress and mitochondrial function were up-regulated, whereas genes involved in cell cycle and DNA stability and multiple oocyte-specific genes, including several transcription factors, were down-regulated with maternal aging. Consistent with this, variety of major functional categories including cell cycle regulation, cytoskeleton structure, energy pathways, transcription control, and stress responses, are influenced by maternal age of human oocytes (Steuerwald et al. 2007).

Microarrays also have enabled researchers to assess global gene expression changes during oocyte maturation in several species, including human, mouse and bovine. In an experiment conducted using heterologous human cDNA array (Dalbiès-Tran and Mermillod 2003) approximately 300 genes of which 70 were differentially expressed during meiotic maturation and were associated with cell cycle regulation (CCNB1 and CDC2), DNA transcription (TIF1 and GTF2H) and apoptosis regulation (DAD1, CASP4, FASTK and BCL2L1). Moreover, genes controlling DNA methylation (DNMT1 and DNMT2), transport (IGF2R, VDP and ATP2B1) and metabolism (HSD11B2, MUT, SLC3A2 and PLCG1) were up-regulated in matured oocytes compared to 8-cell stage embryos using Affymetrix bovine-specific DNA array (Misirlioglu et al. 2006). Recently, gene expression analysis of bovine oocytes pre- and post-resumption of meiotic maturation was investigated using Affymetrix gene-chip Bovine Genome Array (Fair et al. 2007). Approximately 54% of the probe sets representing 23,000 transcripts were detected in bovine oocytes and many genes related to CPE-dependent polyadenylation complex machinery were found to be differentially expressed during pre- and post-resumption of meiotic maturation. Furthermore, the majority of these genes underline the tight temporal control of protein synthesis

required for oocyte maturation and in preparation of subsequent fertilization and early embryo development. Similar studies have been conducted in mouse (Cui et al. 2007, Su et al. 2007) using oligonucleotide arrays. Genes specifically up-regulated in mature oocytes were more likely to be involved in DNA replication, amino acid metabolism, G-protein coupled receptors and signalling molecules (Cui et al. 2007). On the other hand, transcripts involved in processes that are associated with meiotic arrest at the germinal vesicle stage and the progression of oocyte maturation, such as oxidative phosphorylation, energy production, and protein synthesis and metabolism, were dramatically degraded (Su et al. 2007).

Comparison of gene expression profiles of human germinal vesicle oocytes (hGVO), human embryonic stem cells (hESC) and human foreskin fibroblasts was performed to identify genes that are expressed differentially during final oocyte maturation (Zhang et al. 2007) using Affymetrix platform of >47,000 transcripts. In this study, known components of 4 signalling pathways (MOS-MPF, transforming growth factor-beta, WNT, and NOTCH) were found expressed in hGVO. Furthermore, mature human oocytes markedly over-expressed genes involved in meiosis process such as maturation-promoting factor (MPF), anaphase-promoting complex (APC/C), spindle checkpoint complexes (Assou et al. 2006), RNA metabolism, DNA metabolism and chromatin modification (Kocabas et al. 2006). New potential regulators and marker genes related to DNA repair (BARD1) and cell cycle checkpoint (BUB3 and BUB1B) were expressed highly during oocyte maturation (Gasca et al. 2007) using Affymetrix arrays contain 54,675 sets of oligonucleotide probes which represent ~30.000 unique human genes or predicted genes. Significant differences were observed between in vivo and in vitro matured oocytes in global gene expression profile of human (Jones et al. 2008, Wells and Patrizio 2008). Most recently, Assou et al (2009) compared the transcriptome of human mature MII oocytes and embryonic stem (ES) cell and identified genes commonly expressed between these two types of cells, which included a strong cell cycle signature, genes associated with pluripotency such as LIN28 and TDGF1, a large chromatin remodelling network (TOP2A, DNMT3B, JARID2, SMARCA5, CBX1, CBX5), 18 different zinc finger transcription factors, including ZNF84, and several still poorly annotated genes such as KLHL7, MRS2, or the Selenophosphate synthetase 1 (SEPHS1). Interestingly, a large set of genes was also found to code for proteins involved in the ubiquitination and proteasome pathway.

Cross-species gene-expression comparison is a powerful tool for the discovery of evolutionarily conserved mechanisms and pathways of expression control and better understanding of the molecular mechanisms related to the unique functions found in the oocyte. In comparative analysis of gene expression conducted on cDNA microarray of bovine, mouse and Xenopus laevis, genes namely small fragment nuclease (SMFN), spindlin (Spin), and protein arginine methyltransferase 1 (PRMT1) were present in oocytes and conserved in three evolutionarily distant species (Vallée et al. 2006). Recently, the previous research group have analyzed the global gene expression profiles of oocytes in same previous species using mouse microarray that consists of 60-mer oligonucleotide probes representing more than 20,000 mouse transcripts derived from stem cell, oocyte, and early embryo cDNA libraries (Vallée et al. 2008). Interestingly, a substantial proportion of the genes expressed in mouse oocytes is conserved between the three species (74%, 7275 genes). Moreover, functional annotation of these conserved oocyte-expressed genes confirmed that certain functions are conserved among the three species. RNA metabolism and cell cycle were among the over-represented Gene Ontology terms in the biological process category. Finally, a pattern-matching analysis identified 208 conserved maternally expressed genes. This comparative analysis of oocyte transcript profiles revealed a high degree of conservation among species.

In our institute, we have performed different experiments in bovine (Salilew-Wondim et al. 2007, Ghanem et al. 2007, Torner et al. 2008) and also in buffalo oocytes (Kandil et al. 2008) to dissect the molecular mechanisms regulating oocyte developmental competence using the BlueChip cDNA microarray. Using novel non-invasive approach to identifying developmentally competent and incompetent bovine oocytes and zygotes (Salilew-Wondim et al. 2007), bovine oocytes and zygotes were dielectrophoretically separated according to their speed of migration to the opposite electrode. The authors reported that the majority of differentially regulated genes were more abundant in the very fast moving oocytes and zygotes, and fell mainly under functional categories associated with regulation of protein biosynthesis and metabolic regulation. Among these transcripts, RPL2, RPL8, RPL35 and RPLP0 were more abundant in the very fast moving developmentally superior oocytes. Regarding transcriptional analysis of buffalo oocyte, a total of 104 transcripts were differentially expressed between immature and matured oocytes (Kandil et al. 2008). Among these, transcription factors (ZFP91), M-

phase mitotic cell cycle (MPHOSPH9), growth factor (BMP15) and DNA binding (HMGN2) were found to be up-regulated in immature oocytes. Similarly, matured oocytes were found to be enriched with genes involved in cytoskeleton (ACTB), hydrogen ion transporting (ATP6V1C2) and structural constituent of ribosome (RPS27A).

The other two published studies regarding bovine oocyte transcriptome analysis are the whole work of the present thesis (Ghanem et al. 2007, Torner et al. 2008).

3 Materials and methods

3.1 Materials

In this section, materials used in this study such as animals, all other biological materials, chemicals, kits, reagents, media, softwares, equipments and their sources are mentioned.
The experimental protocol was carried out according to the rules and regulations of the German law of animal protection.

3.1.1 Animals

For OPU procedure, Sixty Simmental cyclic heifers 24 to 30 months old were selected based on general clinical examination and normal ovarian cyclicity as determined by ultrasound scanning. The animals were housed as one group and were fed grass silage ad libitum.
Eight Simmental cyclic heifers, two of each at day of estrus (day 0), day of ovulation (day 1), growth phase (day 3) and dominance phase (day 7) were slaughtered to obtain ovaries for MSX1 protein localization using immunohistochemistery and mRNA localization via in situ hybridization.

3.1.2 Materials for laboratory analysis

3.1.2.1 Chemicals, kits, biological and other materials

Amersham Biosciences (Freiburg): CyScribe™ GFX™ purification kit, CyScribe post-labelling kit

Applichem (Darmstadt, Germany): 50x Denhardt's solution

Applied Biosystems (Foster City): SYBR® Green Universal PCR Master Mix

Beckman Coulter (Krefeld): CEQ™ 8000 Genetic Analysis System, Dye Terminator Cycle Sequencing (DTCS), Glycogen

Biomol (Hamburg): Phenol, Phenol/ Chlorophorm/Isoamyl alcohol (25:24:1), Lambda DNA Eco9lI (BstE II) and Lambda DNA HindIJJ

Biozym Diagnostic (Epicentre Technologies) (Hessisch-Oldendorf): Sequagel XR Sequencing Gel (National Diagnostics), SequiTherm EXCELTM II DNA Sequencing kit-LC, AmpliScribe^{TM} T7 transcription kit Corning (Amsterdam): GAPS II coated slides

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DYNAL Biotech (Hamburg): Dynabeads oligo (dT)25

Eppendorf (Hamburg): 2.5x RealMasterMix/ 20x SYBR Solution

Invitrogen Life Technologies (Karlsruhe): DTT, SuperScript^{TM} II RNase H^{-} Reverse Transcriptase, 5 X first strand buffer, Random Primers

MBI Fermentas (St. Leon-Rot): Glycogen

MWG Biotech (Eberberg, Germany): Oligonucleotide primers

Promega (Mannheim): Random primer, BSA, pGEM®-T vector, RQ1 RNase-free DNase. RNasin (Ribonuclease inhibitor), 2X rapid ligation buffer, T4 DNA ligase, Pronto!^{TM} Plus systems

Qiagen (Hilden): RNeasy® Mini kit, QIAquick PCR Purification Kit, Mini Elute^{TM} Reaction Cleanup Kit

Roche (Mannheim, Germany): Fish sperm DNA, Horseradish peroxidase anti-digoxigenin antibody

Roth (Karlsruhe): Acetic acid, Agar, Ampicillin, Bromophenol blue, Dimethyl sulfoxide (DMSO), Ethylenediaminetetraacetic acid (EDTA), Ethanol, Ethidium bromide, Hydrochloric acid, Isopropyl -D-thiogalactoside (IPTG), Kohrso1in FF, Nitric acid, Peptone, Potassium dihydrogen phosphate, 2- Propanol, Silver nitrate, Sodium acetate, Sodium carbonate, Sodium chloride, Sodium hydroxide, Trichloromethane/chlorophorm, Tris, X-Gal (5 -bromo-4-chloro-3-indolylbeta-D-galactopyranoside), Yeast extract

Sigma-Aldrich Chemie GmbH (Munich): Acetic anhydride, Agarose, Ammonium acetate, Calcium chloride, Calcium chloride dihydrate, Calcium lactate, Dulbecco's phosphate buffered saline (D-PBS), Formaldehyde, GenEluteTM plasmid Miniprep kit, Heparin, Hepes, Hydroxylamin, Hypotaurin, Igepal, Isopropanol, Magnesium chloride, Magnesium chloride hexahydrate, Medium 199, 2-Mercaptoethanol, Mineral oil, Oligonucleotide primers, Penicillin,

Phenol red solution, 10 X PCR reaction buffer, Potassium chloride, Sodium dodecyl sulfate (SDS), Sodium hydrogen carbonate, Sodium hydrogen phosphate, Sodium hydrogen sulfate, Sodium lactate solution (60%), Sodium pyruvate, Streptomycin sulfate, Taq DNA polymerase, yeast tRNA

STARLAB GmbH (Ahrensburg): Rigid thin wall 96 X 0.2 ml skirted microplates for real-time PCR.

Stratagene (Amsterdam): 5 α DH *Escherichia coli* competent cells

USB (Ohio): ExoSAP-IT

3.1.2.2 Reagents and media

All solutions used in this investigation were prepared using deionized Millipore water (ddH_2O) and pH was adjusted with sodium hydroxide (NaOH) or hydrochloric acid (HCl).

General reagents

DEPC-treated water:	DEPC	1.0
	Water added to	1,000.0
	Incubate overnight at 37˚C and heat inactivate by autoclaving	
dd H_2O:	Deionised millipore water	
LB-agar plate	Sodium chloride	8.0 g
	Peptone	8.0 g
	Yeast extract	4.0 g
	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
TBE (10x) buffer	Tris	108.0 g
	Boric acid	55.0 g
	EDTA (0.5 M)	40.0 ml
	ddH_2O added to	1000.0 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
10x FA buffer, pH 7.0	MOPS	41.8 g
	Sodium acetate	4.1 g
	EDTA (0.5M)	20.0 ml
	ddH_2O added to	1000.0 ml
1.2% FA gel	Agarose	1.2 g
	10 x FA buffer	10.0 ml
	RNase free H_2O	90.0 ml
	Ethidium bromide	2.0 µl
	Formaldehyde (37%)	1.8 ml
Agarose loading buffer	Bromophenol blue	0.0625 g
	Xylencyanol	0.0625 g

	Glycerol	7.5 ml
	ddH_2O added to	25 ml
Digestion buffer	NaCl	100 mM
	Tris-HCl	50 mM
	EDTA pH 8.0	1mM
SDS solution	Sodium dodecylsulfat in ddH_2O	10% (w/v)
Proteinase K solution	Protein K in 1 x TE buffer	2% (w/v)
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
3M 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)

Reagents for microarray

Binding buffer:	Binding buffer:	1.0 ml
	5 M LiCl	10.0 ml

	5 mM MEDTA pH 8.0	20.0 ml
	Water added to	50.0 ml
Washing buffer:	1 M Tris HCl pH 7.5	0.5 ml
	5 M LiCl	1.5 ml
	5 mM EDTA	10.0 ml
	Water added to	50.0 ml
Lysis buffer:	Igepal	0.8 µl
	RNasin	5.0 µl
	DTT	5.0 µl
	Water added to	100.0 µl
Prehybridization buffer:	BSA	0.5 g
	10 % SDS	0.5 ml
	20 % SSC	7.5 ml
	water added to	50.0 ml
Hybridization buffer:	Hybridization solution	15 µl
	100% Formamid	30 µl
	DEPC water	11 µl

Reagents for Immunohistochemistery

0.2% Triton-X100:	Triton	2 ml
	10x PBS : added to	1,000.0 ml
0.3% BSA in PBS	PSA	3 g
	10x PBS : added to	1,000.0 ml
3% BSA in PBS	PSA	30 g
	10x PBS : added to	1,000.0 ml

Reagents for in situ hybridization

10x PBS:	NaCl	8.77	g
	Na_2HPO_4	1.50	g
	NaH_2PO_4	2.04	g
	Water added to	1,000.0	ml
1x PBS:	10x PBS	100.0	ml
	Water added to	1,000.0	ml
	For in situ hybridisation treat with 1 ml DEPC, incubate overnight at 37oc and heat inactivate by autoclaving.		
1x PBS-Tween (PBST):	1x PBS	999.50	ml
	Tween®20	0.50	ml
Tris-HCl (1M):	Tris	121.14	g
	Water added to	1,000.00	ml
	Adjust pH to the required pH.		
EDTA (0.5M, pH 8.0):	Ethylendiamin tetra acetic acid disodium salt	186.12	g
	Water added to	1,000.00	ml

In situ hybridisation

50% Dextran sulphate:	Dextran sulphate	2.00	g
	DEPC-treated H_2O	2.50	ml
	Leave to dissolve overnight. Adjust to 4.0 ml with DEPC-treated H_2O. Prepare always fresh in 25.0 ml measuring cylinder one day prior to preparation of hybridisation buffer.		
70% DMF:	Dimethylformamide	3.50	ml
	DEPC-treated H_2O	1.50	ml
	Prepare fresh		
Hybridisation buffer:	50% Dextran sulphate	4.00	ml
	NaCl (2.5 M)	1.20	ml
	Formamide, deionised	10.00	ml
	20x SSC	2.00	ml
	Yeast tRNA (10 mg/ml)	0.40	ml

	50x Denhardt's solution	0.40	ml
	Fish sperm DNA (10 mg/ml)	2.00	ml
	Mix thoroughly, wait until air bubbles have disappeared, and store 1 ml aliquots at -20˚C		
NaCl (2.5 M):	NaCl	14.61	g
	Water added to	100.00	ml
	Treat with 0.1% (100 µl) DEPC. Incubate overnight at 37˚C and heat inactivate by autoclaving.		
4% PFA (pH 7.3):	Paraformaldehyde	10.00	g
	1 x PBS added to	250.00	ml
	Bring to 65˚C under ventilation hood. Add 5 µl of 5 M NaOH for solution to become clear. Store protected from light and use within 2 weeks.		
20x SSC (pH 7.0):	NaCl	87.66	g
	Tri-Na-citrate*2 H_2O	44.12	g
	Water added to	500.0	ml
	Treat with 1% (500 µl) DEPC. Incubate overnight at 37˚C and heat inactivate by autoclaving.		
2 x SSC:	20 x SSC	100.00	ml
	DEPC-treated water	900.00	ml
0.1 M TEA buffer	Triethanolamine	13.20	ml
(pH 8.0):	DEPC-treated water added to	1,000.00	ml
	Autoclave. Store at RT protected from light.		
TNB blocking buffer:	Blocking reagent	0.50	g
	TN buffer	100.00	ml
	Add blocking reagent slowly in small increments to TN buffer while stirring, if necessary heat to 60˚C to dissolve completely. Store aliquots at -20˚C.		
TN buffer (pH 7.5):	Tris	12.11	g
	NaCl	8.77	g
	dd H_2O added to	1,000.00	ml
TNT wash buffer:	TN buffer	995.00	ml
	10% Tween 20	5.00	ml

10% Tween 20:	DEPC-treated H_2O	45.00	ml
	Tween®20	5.00	ml
Yeast tRNA (10 mg/ml):	Yeast tRNA	25.00	mg
	DEPC-treated H_2O	2.50	ml
	Dissolve by incubating on ice for at least 1 h.		
	Store aliquots at -20˚C.		

3.1.3 Used softwares

BLAST program	http://www.ncbi.nlm.nih.gov/BLAST
Entrez Gene	www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
Gene Ontology	http://www.geneontology.org
GenePix Pro Version 4.0	Axon Instruments, Foster city, CA
GPRocESSOR	http://bioinformatics.med.yale.edu/softwarelist.html
PermutMatrix	http://www.lirmm.fr/~caraux/PermutMatrix/EN/index.html
Multiple Sequence Alignment	http://searchlauncher.bcm.tmc.edu/ multialign/Options/clustalw.html
Primer Express® software	Applied Biosystems, Foster city, CA, USA Primer Express. Software v2.0
Significant Analysis of Microarray	http//www-stst.stanford.edu/tips/SAM
SAS (version 8.02)	SAS Institute Inc., NC, USA

3.1.4 Equipment

ABI PRISM® 7000 SDS	Applied Biosystems, Foster city, USA
Centrifuge	Hermle, Wehingen
CEQ™ 8000 Genetic Analysis System	Beckman Coulter GmbH, Krefeld
Electrophoresis (for agarose gels)	BioRad, Munich
Electrophoresis (vertical apparatus)	Consort, Turnhout
Fluorescence microscope (DM-IRB)	Leica, Bensheim, Germany
GFL 7601 hybridization chamber	Fisher scientific, Leicestershire, UK

GenePix 4000A scanner	Axon Instruments, Foster City, USA
HERA safe Bioflow safety hood	Heraeus Instruments, Meckenheim
Hybridization cassettes	TeleChem International, Sunnyvale
Icycler	Bio-Rad Laboratories, München, Germany
Incubator	Heraeus, Hanau
Millipore apparatus	Millipore corporation, USA
PCR thermocycler (PTC100)	MJ Research, USA & BioRad, Germany
pH meter	Kohermann
Savant SpeedVac®	TeleChem International, Sunnyvale
Power supply PAC 3000	Biorad, Munich
Spectrophotometer, Ultrospec™ 2100 *pro* UV/Visible	Amersham Bioscience, Munich
Thermalshake Gerhardt	John Morris scientific, Melbourne
Tuttnauer autoclave	Connections unlimited, Wettenberg
Ultra low freezer (-80˚C)	Labotect GmbH, Gottingen
UV Transilluminator (Uvi-tec)	Uni Equip, Martinsried, Germany
Spectrophotometer (DU-62)	Beckman, Unterschleissheim-Lohhof
GenePix 4000B Microarray Scanner	Axon Instruments, Foster city, CA

3.2 Methods

In this section, the basic molecular genetics methods used in this study are described first, followed by microarray hybridizations and data analysis. Further more, the methodologies used for comparative sequencing, SNP screening and genotyping of candidate genes are given. The overview of the present work is illustrated in Figure 12.

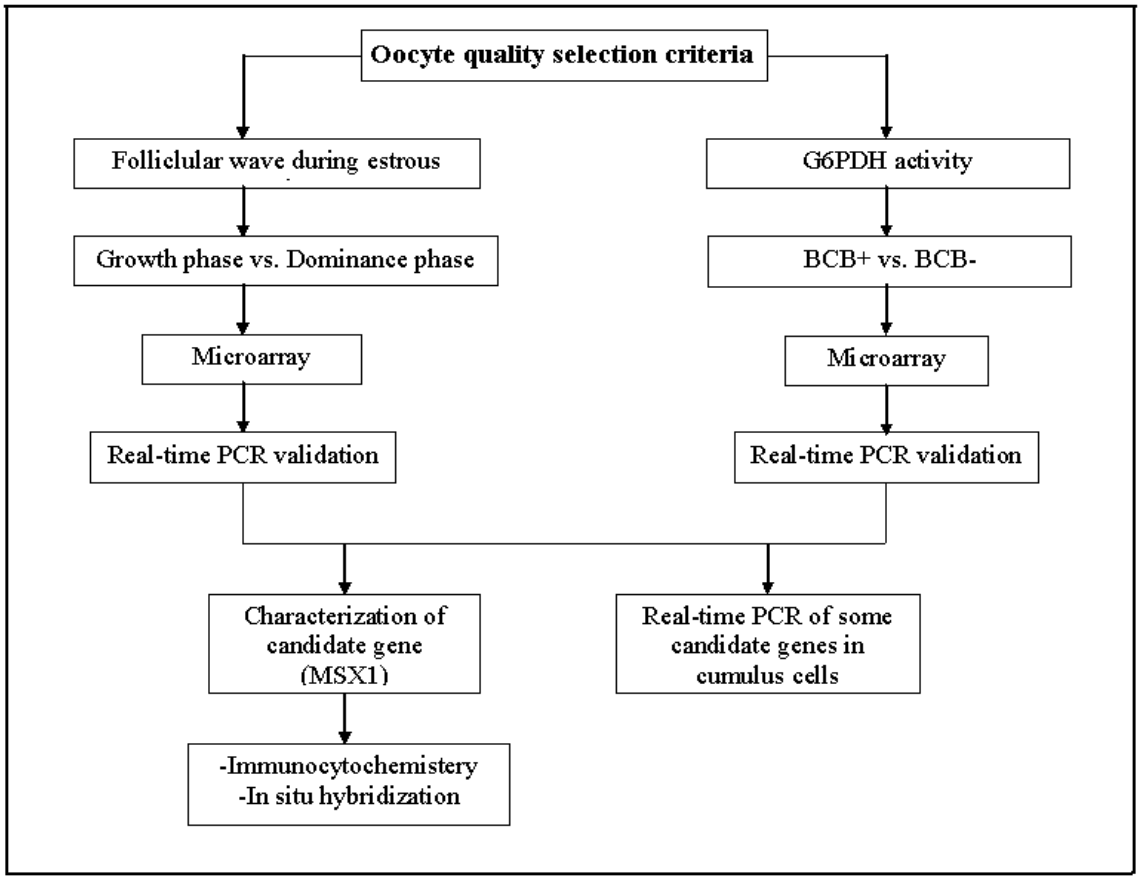

Figure 12: Overview of the present study

3.2.1 Estrus synchronization and detection

Estrus and subsequent ovulation were synchronized by two administrations of prostaglandin PGF2α (Estrumate, 2 ml i.m, Fa. Essex, Germany) at 11 days interval followed by GnRH injection (Receptal, 2500 IU i.m, Intervet, Unterschleissheim, Germany) on the day of estrus onset. Estrus was detected after two days of the last prostaglandin PGF2α treatment. Common signs of estrus were monitored by visual observation followed by careful palpation of ovaries for confirmation.

3.2.2 Ovum pick up

OPU was performed at two different phases of follicular development during the first follicular wave at growth (day 3 after estrus, n = 30) and dominance phase (day 7 after estrus, n = 30) for two sessions of OPU in each phase. The heifers were restrained in a

chute, and given 5 ml of epidural anaesthesia (procaine hydrochloride 2%, Selectavet, Munich, Germany). Using transvaginal ultrasound scanner (Pie Medical 400 Vet, Maastricht, Netherlands) with a 7.5 MHz sector probe the follicles were visualized on the monitor and counted. The follicles were classified according to their size into three categories: small (3–5 mm), medium (6–8 mm) and large follicles (≥ 9 mm). A 50-cm-long 18-gauge needle was passed through a needle guide along the polyethylene housing of the transducer, and carried into the fornix vagina. After fixing the ovary against the transducer, the needle was advanced to puncture the vaginal wall to enter the ovarian follicle. The needle was attached via Teflon tubing to a 50-ml Falcon tube and vacuum pressure provided with a regulated vacuum pump (K-MAR-5000B, William Cook Europe) and adjusted to create a flow rate of 16–20 ml/min. The follicular content of each heifer was aspirated individually into modified Parker maturation medium (MPMM) supplemented with 15% estrus cow serum (CS), 0.5 mM L-glutamine, 0.2 mM pyruvate, 50 µg/ml gentamycin sulphate, 10 µl/ml FSH (Folltropin, Vetrepharm, Canada) and kept at 39˚C in thermos. The follicular fluid contents were poured into a square grid dish to facilitate finding of oocytes under a stereomicroscope. The collected oocytes were kept in the same maturation medium used for collection before being separated from the surrounding cumulus cells. Oocytes from each follicular size category and phase were handled and frozen separately.

3.2.3 Oocyte recovery and Brilliant Cresyl Blue staining

Oocytes aspirated from slaughterhouse ovaries were used for BCB staining. The procedure of BCB staining was done as described in previous studies (Alm et al. 2005, Bhojwani et al. 2007). Briefly, a total of 2128 morphologically good quality compact cumulus oocytes complexes (COC) were subjected to 26 µM BCB (B-5388, Sigma-Alderich, Taufenkirchen, Germany) diluted in mDPBS for 90 min at 38.5˚C in humidified air atmosphere. After washing the stained COCs were examined under stereomicroscope and categorized into two groups according to their cytoplasm coloration: oocytes with any degree of blue coloration in the cytoplasm (BCB^+) (n = 1167) and oocytes without visual blue coloration (BCB^-).

3.2.4 Oocytes denudation and storage

Cumulus cells were removed from all oocytes mechanically in maturation medium supplemented with hyaluronidase 1 mg/ml (Sigma). Separation of cumulus cells was carefully checked under a stereomicroscope. Cumulus free oocytes and the corresponding cumulus cells of each group were washed two times in PBS (Sigma) and snap frozen separately in cryo-tubes containing 20 µl of lysis buffer [0.8 % IGEPAL (Sigma), 40 U/µl RNasin (Promega Madison WI, USA), 5 mM dithiothreitol (DTT) (Promega Madison WI, USA)]. Finally, samples were allocated according to experiment and stored at -80˚C until RNA extraction.

3.2.5 cDNA microarray procedure

3.2.5.1 RNA isolation and cDNA synthesis

Messenger RNA isolation of oocytes and cumulus cells was performed at six time points during the whole experiment. 1) A total of six pools, each containing 20 oocytes from growth and dominance phases of follicular development, were used for array analysis after amplification, 2) A total of six pools, each containing 20 oocytes from growth and dominance phases, were used for real-time validation of array results, 3) The cumulus cells detached from the oocytes at the growth and dominance phases were also used for mRNA isolation and subsequent expression analysis of selected transcripts, 4) A total of six pools, each containing 110 BCB^+ and BCB^- oocytes, was used for array analysis after amplification, 5) A total of 10 pools, each containing 25 oocytes, was used for real time validation of array results, 6) A total of 8 pools of oocytes, each containing 50 oocytes from BCB^+ and BCB^- categories were used to validate the expression profile of selected transcripts in oocytes with different developmental competences.. In all cases, mRNA isolation was performed using Dynabead oligo (dT)25 (Dynal Biotech, Oslo, Norway) according to manufacturer's instructions. Briefly, oocytes or cumulus cells in lysis buffer were mixed with 40 µl binding buffer [20 mM Tris HCl with pH 7.5, 1 M LiCl, 2 mM EDTA with pH 8.0] and incubated at 70˚C for 5 min to obtain complete lysis of and to release RNA. Ten microlitres of oligo (dT)25 attached magnetic bead suspension were added to the

samples, and incubated at room temperature for 30 min. The hybridized mRNA and magnetic beads were washed three times using washing buffer (10 mM Tris HCL with pH 7.5, 0.15 mM LiCl, 1 mM EDTA with pH 8.0). For each sample, cDNA synthesis has been performed using oligo (dT)23 primer and superscript reverse transcriptase II (Invitrogen, Karlsruhe, Germany) except for samples used in array analysis where the reverse transcription was performed using T7 promotor attached oligo d(T)21 primer (TCTAGTCGACGGCCAGTGAATTGTAATACGACTCACTATAGG-GCG(T)21).
One microlitre of oligo (dT)21 primer was added to 11µ1 mRNA sample and he mixture was incubated for 3 minutes at 70˚C and then immediately chilled on ice. Eight microlitres of the master mix containing 4 µl of 5x first strand buffer, 2 µl of 0.1 M DTT, 1 µl of dNTP (10 pmol/µl) and 0.3 µl of RNase inhibitor and 0.7 µl of SuperScriptIITM reverse transcriptase (200 unit/µl) was mixed and incubated for 90 minutes at 42˚C followed by heat inactivation for 15 min at 70˚C. The synthesized cDNA was stored at -20˚C for further use.

3.2.5.2 RNA amplification

Second strand synthesis and global PCR amplification were carried out using degenerated oligonucleotides primer (DOP) PCR master kit (Roche Diagnostics, Mannheim, Germany). For this, 20 µl of first strand cDNA product, 40 µl of 2X DOP PCR master mix, 1 µl of DOP primer (5'-CCGACTCGAG NNNNNN ATGTGG-3') (to the final concentration, 1 µl of 20µM T7 oligo (dT)21 primer, and 16 µl water were added and properly mixed. This PCR reaction was heated at 95˚C for 5 min to denature the sample and activate the polymerase, followed by cycle of denaturation at 95˚C for 30 seconds and annealing at 30˚C for 90 sec. Unspecific primer annealing was achieved through application of relatively low annealing temperature. Subsequently the temperature was increased at the rate of 0.2˚C /sec until it reaches 72˚C and incubated for 3 min at this temperature. To this end, second strand synthesis was completed and the global PCR amplification continues for the rest of 10 cycles at 94˚C for 30 sec, 60˚C for 30 sec, 72˚C for 3 min and final extension at 72˚C for 7 min. Ten amplification cycles were used during second strand synthesis as this shown to be less biased on the representativeness of the original mRNA population after in vitro transcription (Mamo et al. 2006). The cDNA was purified and used for in vitro transcription using

AmpliScribe T7 transcription kit (Epicentre technologies, Oldendorf, Germany) according to manufacturer's instruction. Briefly, 2 µl of 10 X reaction buffer, 4 µl dNTP (100 mM each of ATP, CTP, GTP and UTP), 2 µl of DTT and 2 µl of T7 RNA polymerase were added to the 10 µl of purified cDNA preparation, mixed well and incubated at 42˚C for 3 hrs. At the end of incubation, 1 µl of DNase has been added and incubated at 37˚C for 30 minutes. Then the amplified RNA (aRNA) was purified using RNeasy Mini kit (Qiagen, Hilden, Germany) following the manufacturer's recommendations. Finally the aRNA was eluted in 30 µl RNase free water from which 8 µl was taken to estimate the yield and purity of aRNA by UV absorbance reading A260/280 using Ultrospec™ 2100 pro UV/Visible Spectrophotometer (Amersham Bioscience, Freiburg, Germany)

3.2.5.3 Aminoallyl indirect labelling and dye coupling

MIAME (Minimum Information About Microarray Experiments) guidelines were adhered to the experimental design. Two independent labelling reactions were carried out per aRNA sample pertinent to each biological replicate for dye-swap hybridizations. Accordingly, 3 µg of aRNA from each oocyte pool was used as template in reverse transcription reactions incorporating amino-modified dUTPs into the cDNA using the CyScribe Post-Labelling Kit (Amersham Biosciences, Freiburg, Germany). Seven microlitres of the amplified RNA and 1.5 µl of anchored oligo(dT) and 1.5 µl of random nanomer primers were co-incubated at 70˚C for 5 min followed by 10 min incubation at room temperature. Then 10 µl reaction mix (containing 4 µl of 5 X first strand buffer, 2 µl of 0.1 M DTT, 1.5 µl of dNTP mix, 1.5 µl aminoallyl dUTP and 1µl CyScript reverse transcriptase), was added to the reaction and incubated at 42 ˚C for 90 min. At the end of this reaction, 2 µl of 2.5 M NaOH was added to the reaction to hydrolyze the mRNA template and incubated at 37 ˚C for 15 min. The aminoallyl labeled cDNA samples were purified using CyScribe™ GFX™ Purification kit (Amersham Biosciences) after adding of 10 µl of 2 M HEPES. The purified Aminoallyl labeled cDNA was then eluted in 60 µl 0.1 M sodium bicarbonate. The cDNA samples from each oocytes pool were differentially labeled indirectly using N-hydroxysuccinate-derived cyanine (Cy3 and Cy5) fluorescent dyes and incubated for 1.5 hrs at room temperature in dark. At the end of incubation, non reacting dyes were quenched by adding 15 µl of 4 M hydroxylamine

solution (Sigma) and incubated for 15 min at room temperature in dark. To avoid variation due to dye coupling, aRNA samples from the same oocytes pool were labelled reversibly either with Cy3 or Cy5 for dye swaps hybridizations. The reaction was then purified with CyScribe™ GFX™ Purification kit (Amersham Biosciences, Freiburg, Germany). Samples were finally eluted in 60 µl elution buffer.

3.2.5.4 Probe hybridization

Pre-hybridization of the slides was performed by placing the array slides into a corning GAPS II slide container and incubated in warmed (55 ˚C) [filtered pre-hybridization buffer containing 5x SSC and 0.1% SDS (Sigma) and 1% BSA (Roche, Diagnostic, Basel, Switzerland)] for 20 min. Following pre-hybridization, slides were rinsed short in boiling water to denature probes and wash unbound DNA from the slide surfaces, followed by immediate immersion in water at room temperature and isopropanol consecutively. Then the slides have been dried by centrifugation at ≥2000 rpm for 2 min. Hybridization and post-hybridization washes were carried out as previously described by Hedge et al. (2000) with slight modifications. Samples that were going to be hybridized on specific array were mixed and dried in speedvac centrifuge (GMI, Inc. Minnesota, USA) then the pellet was re-suspended in pre-warmed (42˚C) formamide based hybridization buffer [15 µl hybridization buffer (Amersham Bioscience, Freiburg, Germany), 30µl 100% Formamide, and 15µl DEPC water]. Yeast tRNA (4 mg/ml) and 2.5 µl of Cot-human DNA (1 mg/ml) (Invitrogen, Karlsruhe, Germany) were added in a volume of 2.5 µl each to avoid non specific hybridisation. The pellet was denatured at 95˚C for 5 min, centrifuged briefly and hybridized to the array. The arrays were covered with glass cover slips (ROTH, Karlsruhe, Germany) and fixed in the hybridization cassettes (TeleChem International, Inc, CA, USA.) before incubation in a hybridization chamber (GFL, Dülmen, Germany) at 42˚C for 16–20 hrs. After hybridization, slides were washed twice with 2× SSC-0.1% SDS buffer for 5 min at 42˚C, then once with 1× SSC, 0.2× SSC and 0.1× SSC for 5 min each at room temperature. Finally, the slides were rinsed in RNA free water then in isopropanol for 1 min each and centrifuged at ≥ 2000 rpm for 2 min.

3.2.5.5 Custom array characterization

Ready made bovine cDNA array (BlueChip) (Sirard et al. 2005) provided by Centre de Recherche en Biologie de la Reproduction was used in this study. The glass slide contains 4928 spots divided into two sub-arrays. Each sub-array was composed of 2304 ESTs randomly selected clones obtained from four different subtraction suppressive hybridizations (SSH) made with bovine embryos and tissues (First SSH: GV oocytes subtracted from somatic tissues, second SSH: GV oocytes subtracted from day-8 blastocysts, third SSH: day-8 blastocysts subtracted from GV oocytes and fourth SSH: day-8 blastocysts subtracted from somatic tissues). All the clones were spotted in each sub-array for a total of four replicates per slide. Eleven more samples namely vide (32 spots), alien1 (8 spots), alien2 (8 spots), GFP (4 spots), GFP1 (4 spots), GFP1/2 (4 spots), GFP1/4 (4 spots), GFP1/8 (4 spots), GFP 1/16 (4 spots) and H20/DMSO (50 spots) were spotted to be used as negative controls for determination of hybridization background during the statistical analysis. Housekeeping genes including tubulin (8 spots), ubiquitin (8 spots), β-actin (6 spots) and actin (8 spots) were also added as positive controls.

3.2.5.6 Array scanning and data analysis

The slides were scanned using Axon GenePix 4000B scanner (Axon Instruments, Foster City, CA, USA). The GenePix® Pro 4.0 software (Axon Instruments, CA, USA) was used to process the images, to find spots, to integrate robot-spotting files and finally to create reports of spot intensity data. The LOWESS normalization of microarray data was performed using GProcessor 2.0a software [Zhao's Lab of Statistical Genomics and Proteomics (http://bioinformatics.med.yale.edu/group/)]. The normalized data were used to calculate intensity ratios of all replicates and to obtain one value per clone. Ratios were finally log2 transformed and submitted to SAM analysis. Microarray data analysis was performed using SAM, free software developed at Stanford University (http://www-stat.stanford.edu/~tibs/SAM/). To get truly differentially expressed genes, the FDR was set at 5% and P-value of < 0.05. Hierarchical clustering and heatmap of log2-transformed data for up and down regulated genes were generated using

PermutMatrix (version 1.8.2) available at [Le Laboratoire d'Informatique, de Robotique et de Microélectronique de Montpellier (http://www.lirmm.fr/~caraux/PermutMatrix/)]. In addition, average linkage clustering algorithm method was employed (Eisen et al. 1998). Genes expressed equally in both samples were not included in the hierarchical clustering.

3.2.6 Plasmid DNA preparation

3.2.6.1 Primers design

All primers used in this study were designed using Primer Express® Software v2.0 (Applied Biosystems, Foster City, CA, USA) either for PCR amplification or in vitro transcription. Primer sequences, size of amplified products, annealing temperature and the GenBank accession number are shown in Table 1.

3.2.6.2 Polymerase chain reaction (PCR) and electrophoresis

The PCRs were performed in 20 µl reaction volume reaction volume containing 2 µl of 10 x PCR buffer (Sigma), 0.5 µl of each primer (10 *pmole*), 0.5 µl of dNTP (50 µM), 0.5 U of Taq DNA polymerase (Sigma), 14.4 µl Millipore H_2O which finally added to 2 µl of cDNA templates or 50 ng/µl genomic DNA as positive control and 2 µl of Millipore H_2O as negative control. The PCR reactions were carried out in a PT-100 Thermocycler (MJ Reasearch) and the thermal cycling program was set as: denaturation at 95˚C for 5 min, followed by 35 cycles at 95˚C for 30 sec, annealing at the corresponding temperature (as shown in Table1 and 2) for 30 sec and extension at 72˚C for 1 min, final extension step at 72˚C for 10 min. Finally, 2 µl of loading buffer were added to the PCR products and loaded on agarose gel (2% for cDNA checking; 0.7% for PCR product extraction) in 1X TAE buffer by staining with ethidium bromide. The amplified PCR products were electrophoresed for 30 min at 120 voltages and visualized under UV transilluminator and the gel that have the DNA fragment of interest was cut.

Table 1: Details of the primers used for real-time quantitative PCR analysis and in situ hybridization

Gene name	Gene bank accession number	Primer sequences	Annealing temperature (˚C)	Product size (bp)
BMP15 [a]	AY304484	F: 5´- CTGACGCAAGTGGACACCCTA -3´ R: 5´- GACACACGAAGCGGAGTCGTA -3´	60	396
PTTG1 [a]	NM_004219	F: 5´- GAAGAGCACCAGATTGCGC -3´ R: 5´- GTCACAGCAAACAGGTGGCA -3´	55	204
MSX1 [a]	NM_174798	F: 5´- AAGGTATCCACAGTCCCCAGC -3´ R: 5´- TCTGCCTCTCCTGCAAAGTTC -3´	55	180
PP [a]	AF170490	F: 5´- GCTGCATCCTACTTGTCGGAA-3´ R: 5´- TTCCAAACTACAACCGCCTTG -3´	55	194
S100A10 [a]	NM174681	F: 5´- GGATTTCTGAGCATATGGGACC -3´ R: 5´- GAGCAAGAGGATGCAAGCAATA -3´	55	131
ANXA2 [a]	NM_174716	F: 5´- CGTGCTCCAGCTAACAGTTCT-3´ R: 5´- GGAAAGCCAGGTAATGCGTA-3´	55	139
CKS2 [a]	NM_001827	F: 5´- CGACGACGAGGAGTTCGAGTA-3´ R: 5´- CCTGACTCTGCTGAACACCAAG-3´	55	122
RPL24 [a]	NM_174455	F: 5´- CCGTGCAGTCAAATTCCAAA-3´ R: 5´- CAACTCGAGGAGCAGAAACCTT-3	55	242
FL396 [a]	AY308068	F: 5´- CGCCAACACGTCTTATACCAAC-3´ R: 5´- CTGCGAAGAGGCTTCCAATTAG-3´	55	201
CCNB1 [a]	L26548	F: 5´- CGATATGGTGCACTTTCCTCC-3´ R: 5´- TGACCACATTCTTTGCCAGG-3´	55	145
GAPDH [a]	BC102589	F: 5´-ACCCAGAAGACTGTGGATGG-3´ R: 5´-ACGCCTGCTTCACCACCTTC-3´	60	247
MSX1 [a, b]	NM_174798	F:5´-AGAAGCAGTACCTGTCCATCG R: 5´-GGCCTTCTATGTCAGGTGGTA	55	382
MSX1 [c]	NM_174798	T7-F:5´-GTAATACGACTCACTATAGGGAGAAGCAGTACCTGTCCATCG R: 5´-GGCCTTCTATGTCAGGTGGTA	55	382
MSX1 [d]	NM_174798	SP6-R:5´-GATTTAGGTGACACTATAGAAGGCCTTCTATGTCAGGTGGTA F:5´-AGAAGCAGTACCTGTCCATCG	55	382

[a] The primer used for real-time PCR , [b] The primer used for DNA template amplification, [c] The forward primer coupled with T7 promoter (underlined) used for sense probe synthesis, [d] The reverse primer coupled with SP6 promoter (underlined) used for antisense probe synthesis

Table 1: Details of the primers used for real-time quantitative PCR analysis (Continued)

Gene name	Gene bank accession number	Primer sequences	Annealing temperature (˚C)	Product size (bp)
NASP [a]	BT006757	F: 5´- CCTAGAGCTTGCCTGGGATATG-3´ R: 5´- TCGTGGGCTTCCAGGTACTG-3´	55	198
SMARCA5 [a]	NM_003601	F: 5´- AGTGAACTTTCGCCCATCTTG-3´ R: 5´- AGGCTTGTGGATCAGAATCTG-3´	55	194
EEF1A1 [a]	AB060107	F: 5´- CCATGGCATATTAGCACTTGGTT-3´ R: 5´- GCTTACACCCTGGGTGTGA-3´	55	214
ODC1 [a]	NM_174130	F: 5´- CAAAGGCCAAGTTGGTTTTAC-3´ R: 5´- CAGAGATGGCCTGCACAAAG-3´	55	201
ATP5A1 [a]	NM_174684	F: 5´- CTCTTGAGTCGTGGTGTGCG-3´ R: 5´- CCTGATGTTGGCTGATAACGTG-3´	55	184
RPS27A [a]	AB098891	F: 5´- TGCAGATTTTCGTGAAGACCCT-3´ R: 5´- TTCTTTATCCTGGATCTTGGCC-3´	54	203

[a] The primer used for real-time PCR

3.2.6.3 PCR product extraction

The PCR product was extracted from 0.7% (W/V) agrose gel using phenol-chloroform. All centrifugation were performed at 4˚C with 12,000 x g speed. The gel that have PCR fragment was sliced into small pieces and homogenized with 0.5 ml of 1x TE buffer by repeatedly forcing through a syringe and needle till it is completely dissolved. The extraction was carried out by vigorously vortexing the gel solution with 0.6 ml of phenol-chloroform. After centrifugation for 15 min, the mixture was separated into three phases: a lower phenol-chloroform phase, an interphase of precipitated protein, and an upper aqueous phase containing the PCR product. The upper aqueous phase was transferred into a new tube, shaken vigorously with an equal volume of chloroform to remove possibly carried over phenol, and recentrifuged for another 10 min. The clear upper aqueous phase was precipitated by gentle mixing with 50 µl (or 1/10 volume) of sodium acetate solution (3 M, pH 5.3) and 1.5 ml of 100% ethanol (or 2.5 volume). Precipitation was maximized by placing at -20˚C for 2 hour. The precipitated PCR product was pelleted at 12,000 x g for 30 min at 4˚C. The supernatant was then removed and the pellet was washed with 200 µl of 70% and recentrifuged to remove the supernatant. The pellet was air dried and gently dissolved in 10 µl of ddH2O. The dissolved pellet was kept at -20˚C till ligation.

3.2.6.4 Ligation

Ligation of a PCR fragment was performed using pGEM®-T Vector System I ligation kit. According to the manufacturer instruction, ligation was performed in a 6 µl reaction mix containing 3 µl of 2X rapid ligation buffer, 0.5 µl of pGEM vector (50 ng), 0.5 µl of T4 DNA ligase (3 units/µl), and 2 µl of gel purified PCR product. The reaction was then incubated at 4 ˚C overnight.

3.2.6.5 Transformation

Transformation was performed by combining 3 µl of ligation reaction with 60 µl of competent E. coli cells DH5α in a 15 ml falcon tube and incubating on ice for 30 min. After incubation, the mixture was then heat-shocked briefly in a 42oc water bath for 90

sec and immediately returned to ice for 2 min. Then, 650 µl of LB broth medium was added to the previous mixture and all together was shaken with speed of 110 rpm at 37˚C for 90 min. LB-agar with ampicillin (5 µ1/mI) plates were prepared 30 min prior to use by spreading 20 µl of 0.5 M IPTG and 20 µ1 of 50 mg/ml X-Gal over the surface of LB-ampicillin plates with a glass spreader. At the end of incubation period, the transformation culture was spread completely on two previously prepared LB-agar plates and incubated at 37˚C overnight till the colonies become visible.

3.2.6.6 Colony screening and plasmid DNA isolation

Successful cloning of DNA insert in the pGEM-T vectors was checked based on the activity of β-galactosidase. B-galactosidase is an enzyme produced by lacZ gene in pGEM®-T vector which interacts with IPTG to produce a blue colony. On the other hand, when an insert was successfully cloned, the lacZ gene is disrupted; β-galactosidase is not produced any more which resulted in white colonies. Three white colonies in addition to one blue colony were picked up and suspended in 30 µl 1x PCR buffer for M13 reaction for further confirmation of transformation and sequencing. At the same time, colonies were also cultured in 600 µl ampicillin/LB-broth (5 mg / 100 ml) in a shaking incubator 110 rpm at 37˚C for 90 min then transferred to 15 ml tube and 5 ml of LB-broth with ampicillin were added and incubation continued overnight at the previous conditions for further plasmid isolation. On the other hand, bacteria that were suspended in 30 µl 1x PCR were lysed by heating at 95˚C for 15 minutes. The colonies were screened for the insert by performing a PCR with primers designed in M13 promoter region of the vector. 20 µl of reaction volume containing 10 µl of lysate, 0.5 µl dNTPs (10 mM), 0.5 µl of each of primer (forward: 5´-TTGAAAACGACGGCCAGT-3´, reverse: 5´-CAGGAAACAGCTATGACC-3´), 0.5 U of Taq polymerase (Sigma) in 1 x PCR buffer were amplified in PTC 100 (MJ Research) thermal cycler for 35 cycles at 95˚C denaturation, 60˚C annealing and 72˚C extension followed by 10 minutes of final extension at 72˚C. The products were electrophoresed in 2 % agarose gel. Clones having insert would be having higher molecular weight fragments than the blue clones.

3.2.6.7 Sequencing

The identity of inserts was confirmed by sequencing. Only the M13 PCR products from white colonies containing inserts were used as a template for subsequent sequencing. A volume of 5 µl of M13 products was purified by adding 1 µl of ExoSAP-IT(USB Corporation) then incubated at 37˚C for 45 min followed by enzyme inactivation step at 80˚C for 15 min. The purified DNA product (6 µl) was subsequently used as template for the sequencing PCR which contains 8 µl of millipore water, 2 µl of 1.6 pmole M13 forward or reverse primer, 4 µl of DTCS Quick Start Master Mix (Beckman Coulter). The PCR sequencing reaction was performed for 30 cycles at 96˚C for 20 sec, 50˚C for 20 sec and 60˚C for 4 min, followed by holding at 4oc. The stop solution was prepared in a volume of 2.0 µl of 3M NaOAc (pH = 5.2), 2.0 µl of 100 mM EDTA (pH = 2.0) and 1.0 µl of glycogen (20 mg/ml). The sequencing PCR product was transferred to a 1.5 ml sterile tube and mixed with 5 µl stop solution. A volume of 60 µl cold ethanol (98%) was added and mixed by vortex and then centrifuged for 15 min at 4˚C. The supernatant was removed and the pellet washed 2 times with 200 µl cold ethanol (70%) and centrifuged for 5 min at 4˚C. The pellet was dried by the speed vacuum machine at 35˚C and resuspended in 40 µl SLS (Sample loading solution). The sample was loaded in to plates and sequenced using the CEQ8000 Genetic Analysis System (Beckman Coulter). The final sequence of each DNA fragment was blast into GenBank database (http://www.ncbi.nlm.nih.gov/blast/).

3.2.6.8 Plasmid DNA isolation

Plasmid DNA was isolated by using GenEluteTM Plasmid Miniprep Kit (Sigma, Germany) based on the manufacturer's instructions. Briefly, 5 ml of bacterial culture were centrifuged at 14,000 g for 1 min for harvesting cells, the supernatant was discarded. These cells were resuspended and vortexed in 200 µl of resuspension solution before adding 200 µl of lysis solution to lyse the solution. The mixture was subsequently mixed by inversion of tubes until it became clear and viscous. After incubating at room temperature for 4 min, cell precipitation was done by adding 350 µl of neutralization buffer, mixed gently and centrifuged at 14000 rpm for 10 min. At the same time, the GeneElute Miniprep column was prepared by adding 500 µl of

preparation solution, centrifuged shortly and discarding the flow-through. After that, the clear supernatant was transferred to this binding column and centrifuged at 14000 rpm for 1 min. The flow-through was discarded and the column was washed by adding 750 µl of wash solution followed by centrifugation at 14000 g for 1 min. To elute DNA, the column was transferred to a fresh collection tube; 50 µl of ddH2O was added and centrifuged at 14000 rpm for 1 min. The column was discarded and the DNA plasmid was then collected.

For determination of plasmid size and quality, 5 µl of plasmid together with 2 µl loading buffer was checked by agarose gel electrophoresis. In addition, the quantity of plasmid was also measured by reading the absorbance at 260 nm in a spectrophotometer UV/visible light (Beckman Du® 62). An aliquot of DNA plasmid was subjected to sequence check; the rest was stored at -20˚C to be used as template for setting up the standard curve in real-time PCR.

3.2.6.9 Plasmid serial dilution

The copy number per microlitre of plasmid DNA was calculated based on the size and concentration. The plasmid serial dilution was prepared by converting concentration of plasmid (ng/µl) into numbers of molecules using the website (http://molbiol.ru/eng/scripts/01_07.html). Serial dilutions were then prepared from the concentration of 10^9 upto 10^1 copies/µl and were stored at -20˚C. PCR was performed to test whether the serial dilution could be a suitable standard curve achieved for Real-time PCR. Afterwards, the plasmid DNA serial dilutions were used as template to generate the standard curve during real time PCR analysis.

3.2.7 Quantitative real-time PCR analysis

Real-time RT-PCR was carried out in an ABI Prism® 7000 SDS instrument based on the changes in fluorescence proportional to the increase of product. SYBR® Green, which emits a fluorescent signal upon binding to double stranded DNA, was used as a detector. Fluorescence values were recorded during every cycle representing the amount of product amplified to a point known as threshold cycle (Ct). The higher the initial

transcript amount, the sooner accumulated product was detected in the PCR. Prior to quantification, the optimum primer concentration was obtained by trying different combinations from 200 nM to 600 nM. Results from these primer combinations were compared and the one with lowest threshold cycle and minimizing non-specific amplification was selected for subsequent reaction. After selection of primer concentration, a final assay consisted of 2 µl cDNA as template, up and down stream primers and SYBR Green Universal PCR Master Mix containing SYBR Green I Dye, AmpliTaq Gold DNA polymerase, dNTPs with dUTP, passive reference and optimized buffer components were performed in a total volume of 20 µl reaction. Thermal parameters used to amplify the template started with an initial denaturation at 95˚C for 10 min followed by 40 cycles of 95˚C for 15 sec denaturation and 60˚C for 1 min annealing and extension. A dissociation curve was generated at the end of the last cycle by collecting the fluorescence data at 60˚C and taking measurements every 7 sec until the temperature reached 95˚C. Final quantification analysis was done by amplifying serial dilutions of target plasmid DNA. In all cases, quantitative analysis of cDNA samples was performed in comparison with the bovine GAPDH gene as endogenous control, and was run in separate wells using ABI PRISM® 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). Finally quantitative analysis was done using the relative standard curve method and results were reported as the relative expression or fold change as compared to the calibrator after normalization of the transcript level to the endogenous control.

3.2.8 Immunohistochemistry

Ovaries were embedded in Tissue-Tek (Sakura Finetek Europe, Zoeterwoude, Netherlands) and snap-frozen in liquid nitrogen and stored at -80˚C until sectioning. Serial sections of 5µm were cut at -20˚C using rapid sectioning cryostat (Leica microsystem Nussloch GmbH, Heidelberger, Germany). The sections were mounted on poly-L-lysine coated slides (Menzel GmbH & Co. KG, Braunschweig, Germany) then washed twice in PBS for 5 min and fixed in 4% (w/v) paraformaldehyde in PBS for 45 min at room temperature. The fixed specimens were permeabilized for 5 min with 0.2% (v/v) Triton-X100 (Sigma) in PBS and washed three times with PBS. In order 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 at 37˚C. After triple washing with 0.3% (w/v) BSA in PBS slides were incubated for 15 hrs at 4˚C with anti-MSX1 primary polyclonal antibody (Sigma) with a dilution of 1:100. After three consecutive washes with 0.3% (w/v) BSA in PBS, slides were further incubated for 1 hr with 1:100 dilutions of secondary anti-rabbit IgG FITC conjugated antibody (Sigma). Negative controls were processed in the same manner by omitting the primary antibody.

In vitro produced bovine oocytes (immature, matured) and early cleavage stages embryos (zygotes, advanced zygotes, 2-cell, 4-cell, 8-cell) were used for immunohistochemical localization of MSX1 protein. Ten oocytes or embryos from each stage were processed similar to ovarian sections with some modifications. Specimens were fixed in 4% (w/v) paraformaldehyde in PBS overnight at 4˚C and permeabilized by 0.5% (v/v) Triton-X100 (Sigma) in PBS. In order to inhibit non-specific binding of the antibodies, samples were subsequently blocked in 3% (w/v) BSA in PBS for 1 hr. The oocytes and embryos were then incubated for 1 hr at 39˚C with 1:100 dilution of anti-MSX1 primary polyclonal antibody (Sigma-Aldrich, St Louis, MO, USA). Oocytes and embryos were finally counterstained with 0.5 µg/ml propidium iodide (Sigma-Aldrich, St Louis, MO, USA) for 15 min, followed by washing with PBS. Slides were mounted with Vectashield mounting medium, covered with coverslip and viewed under confocal laser scanning microscope (CLSM LSM-510, Carl Zeiss, Germany).

3.2.9 In situ hybridization

3.2.9.1 Preparation of digoxogenin-labeled RNA probes

Pair of primers was designed according to bovine cDNA sequences found in GenBank (Table 1) using Primer Express® Software v2.0 (Applied Biosystems, Foster City, CA). The first pair of these primers was used to generate PCR amplicon (382 bp) corresponding to the coding sequence and the amplified product of the expected size were cloned using pGEM®-T Vector System (Promega, Mannheim, Germany). Following transformation and growth of bacteria, plasmid DNA was purified with GenElute™ plasmid Miniprep kit (Sigma, Steinheim, Germany), recovered, sequenced, blasted in GenBank database to confirm the matching of the MSX1 clone with its

corresponding gene. Two different templates were required allowing the separate synthesis of antisense and sense probes. This was achieved by extending one of the two MSX1 primers at its 5'-end by the sequence of the T7- or SP6-RNA-Polymerase promoter as shown in Table 1. The PCR with the MSX1_for and SP6+MSX1_rev primers led to the template for the antisense probe, and with T7+MSX1_for and MSX1_rev primers led to the template for the sense probe. Both antisense and sense PCRs were programmed at 94˚C for 5 min, 40 cycles of 94˚C for 30 sec, 55˚C for 30 sec, 72˚C for 1 min followed by a final extension step at 72˚C for 5 min. PCR products were assessed on a 2% agarose gel for quality measurement. The subsequent purification of the obtained PCR products provided optimal conditions for the transcription reaction by removing contaminations of primers, buffers and enzymes, using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The concentration was measured by UV absorbance reading at 260/280A using Ultrospec™ 2100 pro UV/Visible Spectrophotometer (Amersham Bioscience, Freiburg, Germany). Finally, the PCR product was diluted to final concentration of 50 ng/µl.

Digoxigenin-labelled riboprobes (sense and antisense) were generated by in vitro transcription of linearized cDNA insert using Dig RNA labelling kit (SP6/T7) (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instruction. Briefly, 2 µl of NTP labelling mixture, 2 µl 10x Transcription buffer, 1 µl of Protector RNase inhibitor and 1 µl of RNA Polymerase SP6 or T7 were added to the 13 µl of purified cDNA (equal to 1µg), mixed well and incubated for 2 hrs at 37˚C. At the end of incubation, 2 µl of DNase has been added and incubated for 15 minutes at 37 ˚C. The reaction was stopped by adding 2 µl of 0.2 M EDTA (pH 8.0). The probes were purified by adding 2 µl (0.1 volume) of 4 M LiCl and 50 µl (2.5 volume) of 100% ice cold ethanol for overnight at -20˚C. After centrifugation at 12,000 rpm for 15 min at 4˚C, the pellets were washed in 200 µl of 70% ice cold ethanol and centrifuged for another 10 min. The pellets were finally air dried and dissolved in 20 µl of DEPC-treated H_2O. The quality of RNA probes was checked by running 2 µl of RNA plus 2 µl of loading buffer in a 1.5% FA gel. UV spectrophotometer absorbance reading at 260/280A were taken to estimate the concentration of the probes. The RNA probes were diluted in DEPC-treated H_2O to a working concentration of 50 ng/µl and stored at -80˚C.

3.2.9.2 Preparation of cryosections

The same tissue samples that have been used in immunohistochemical fluorescent staining were serially sectioned of 5 µm at -20˚C using rapid sectioning cryostat (Leica microsystem Nussloch GmbH, Heidelberger, Germany). The sections were mounted on poly-L-lysine coated slides (Menzel GmbH & Co. KG, Braunschweig, Germany) then directly fixed in 4 % (w/v) paraformaldehyde in PBS for 15 min at room temperature. The fixed specimens were washed three times in PBS for 5 min of each then, the sections were incubated in an ascending alcohol series of 50%, 70%, 90% and 100% ethanol for 5 min each (prepared fresh in DEPC-treated H_2O), respectively. These steps were followed by a descending alcohol series of 90%, 70%, 50% ethanol for 5 min each, respectively. The sections were washed in 1x PBS for 5 min, blocked in 0.6% H_2O_2 diluted in 1x PBS for 1 hour then washed twice with 1x PBS for 5 min of each. The acetylation was performed by incubation of sections in 0.1 M TEA buffer plus 0.25% acetic anhydride for 10 min. The samples were then equilibrated in 2x SSC for 10 min.

3.2.9.3 Hybridization of probe to the target RNA

The composition of the hybridization buffer was based on the protocol of Thielen (2006). In this experiment, the sense probe served as negative control, since its sequence is identical to the target mRNA and does not attach to the target mRNA. A section hybridized with the sense probe was always prepared in parallel to each section hybridized with the antisense probe.

Different levels of probe amounts (50-100 µl) were used to optimize the hybridization procedure. The RNA probes (sense or antisense) were thawed then added to the hybridization buffer [50% Dextran sulphate, 2.5M NaCL, Formamide, 20x SSC, Yeast tRNA (10mg/ml), 50x Denhardt's solution, Fish sperm DNA(10mg/ml)] to yield a total volume of 100 µl/section and the concentration of 500 ng/ml. This solution was mixed gently, exposed for 5 min at 80˚C to denature the RNA probes and then immediately placed in ice to preserve the denatured structure. Following the final step of pretreatment, the section was placed horizontally in a humid chamber filled with DEPC-

treated H_2O, carefully overlaid with 100 µl of the hybridization mixture and covered with a glass cover slip (24 mm x 24 mm) before being incubated overnight at 52˚C.

3.2.9.4 Posthybridization washes

After the hybridization step, the slips were rinsed off by flushing 2x SSC between slip and slide. The slides were washed twice in 2x SSC at 45˚C for 10 min each, twice in a mixture between formamide and 2x SSC (ratio 1:1) at 45˚C for 10 and 20 min, respectively, and finally washed twice in 0.2x SSC on an orbital shaker at speed 100 rpm for 10 min at room temperature. The sections were then subjected to RNase digestion. Two different types of enzymes, RNase A and RNase T1 were used. The sections were incubated for 30 min at 37˚C in the combination of 5 µg of RNase A and 50 units of RNase T1 (Fermentas, Steinheim, Germany) per ml of 2x SSC followed by three times washing in 2x SSC on an orbital shaker at speed 100 rpm for 10 min at room temperature.

3.2.9.5 Immunological detection

The TSA fluorophore system (PerKin Elmer, Rodgau Juegesheim, Germany) was carried out according to the manufacturer's instruction. Briefly, after posthybridisation washes the sections were incubated in Tris-sodium (TN) buffer for 5 min. The slides were placed horizontally in a humidity chamber and incubated with TN blocking buffer (TNB) for 30 min. Next, the sections were incubated in peroxidase conjugated anti-DIG diluted 1:100 in TNB buffer (200 µl/section) for 1 hour and washed with three changes of TN buffer plus 0.05% Tween 20 (TNT) on an orbital shaker at speed 100 rpm for 5 min each. Then, the fluorescent solution was added to the sections for 10 min. After another three times of washing in TNT buffer, the samples were finally counterstained in 0.5 µg/ml of propidiumiodide (diluted in TN buffer, 200µl/section) for 15 min followed by three times washing in TNT buffer on an orbital shaker at speed 100 rpm for 5 min each. A drop of Vecta shield mounting media was applied on the section; a glass cover slip was then placed over and fixed with nail polish. The sections were viewed under laser scanning confocal microscope (CLSM LSM-510, Carl Zeiss, Germany) provided with FITC green fluorophore. Negative controls were conducted by

the substitution of sense for anti-sense probes or by the omission of anti-sense probes in the hybridization solution. The sections were observed under a light microscope with a Victor TK-1270 RGB color video camera (Victor, Tokyo) and a computer system Image- Pro Plus 3.01 (Media CyberneticsTM, Maryland, USA).

3.2.10 Statistical analysis

The mRNA expression analysis for the studied genes was performed based on the relative standard curve method. The relative expression data were analyzed using General Linear Model (GLM) of the Statistical Analysis System (SAS) software package version 8.0 (SAS Institute Inc., NC, USA). Differences among the mean values were tested using ANOVA followed by a multiple pair wise comparison using *t*-test. Differences of $P \leq 0.05$ were considered to be significant.

4 Results

4.1 Follicle distribution and oocyte recovery

As shown in Table 2, ultrasound guided ovum pick up (OPU) of sixty cyclic heifers showed that ovaries of both growth and dominance phases had greater number of small follicles (3-5 mm) than medium and large follicles. Moreover, a higher number of small and medium follicles (n=333) has been found at growth phase compared to dominance phase (n=262). Consequently, the average number of oocytes collected per cow was greater during growth phase (7.7±3.5) than in the dominance phase (4.9±2.1).

Table 2: Performance of the OPU procedure at growth and dominance phases of follicular development

Phase of follicular development	Follicle size	Number of follicles aspirated	Number of oocytes retrieved	Average number of oocytes per animal	Recovery rate
Growth (n=30)	Large	-	-	7.7±3.5	69.4%
	Medium	148	88		
	Small	185	143		
Dominance (n=30)	Large	30	7	4.9±2.1	50.7%
	Medium	39	20		
	Small	223	121		

4.2 Transcript abundance of oocytes with different developmental potentials

In the two studies, a series of six hybridizations (three biological replicates with dye swaps) were conducted to minimize the false positive expression changes and to identify genes truly differentially expressed between oocytes derived from different models. After LOWESS normalization of the data, log value of Cy5 total intensities was compared with the log value of Cy3 total intensities for both the target and the

respective dye swap hybridizations. For the oocyte selected based on follicular phase, the coefficient of determination of the target (R^2=0.96) and dye swap (R^2=0.95) hybridizations were found to be relatively the same (Figure 13). The coefficient of determination was also high and consistent between target (R^2=0.98) and dye swap (R^2=0.99) hybridizations of the oocytes selected based on BCB staining (Figure 14). This data confirms that the false positive expression change was low between hybridizations of differentially labeled (Cy5 and Cy3) cDNA.

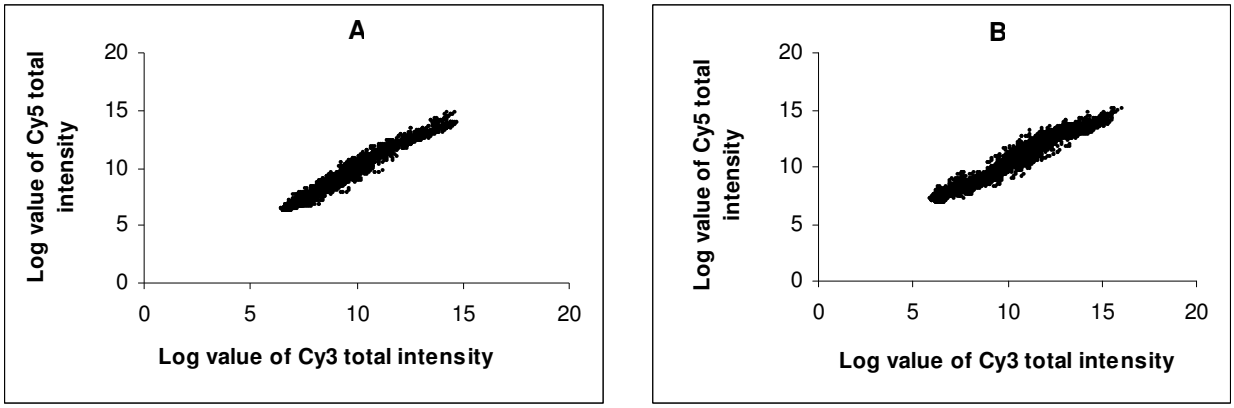

Figure 13: Scatter plot illustrating log value of Cy3 and Cy5 total intensities for the biological and dye swap (technical) replicates of the first model. Each gene is represented by a point of Cy3 and Cy5 log values, A: represents target hybridizations B: represents dye swap hybridizations

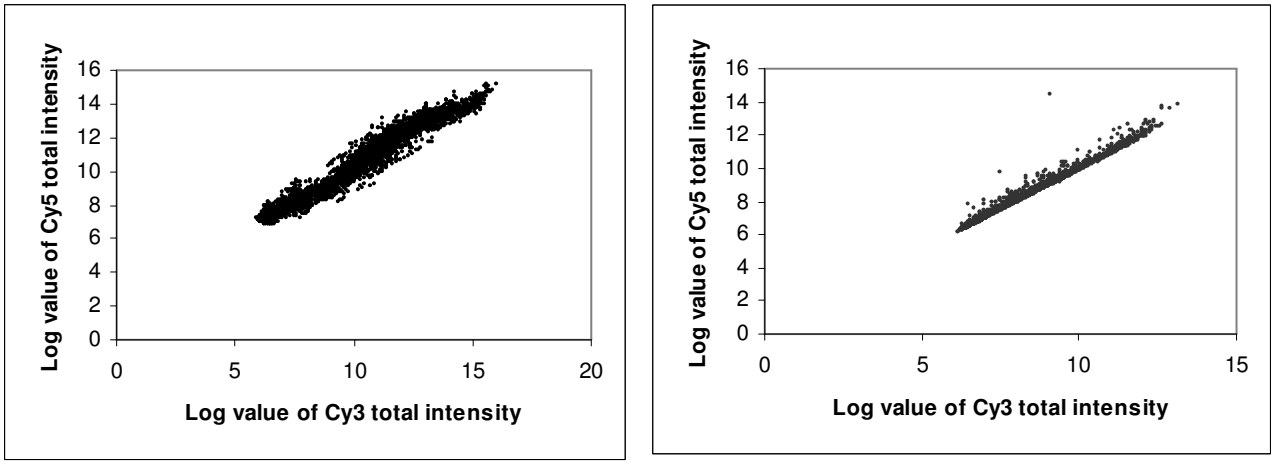

Figure 14: Scatter plot illustrating log value of Cy3 and Cy5 total intensities for the biological and dye swap (technical) replicates of the second model. Each gene is represented by a point of Cy3 and Cy5 log values, A: represents target hybridizations B: represents dye swap hybridizations.

To obtain a highly confident set of differentially expressed genes, we used a rigorous combination of *p*-values ($P \leq 0.05$) and false discovery rate (FDR $\leq$ 5%). Accordingly, the data analysis performed by SAM showed that 51 transcripts were differentially regulated between the two oocyte populations derived from growth and dominance phase. Of these, 24 genes were up-regulated (Table 3) and 27 were down-regulated (Table 4) in growth compared to dominance phase derived oocytes.

The threshold for significant variation was set at 1.5 fold change. Using this criterion, genes were sorted into two categories based on their variation factor: those for which the relative transcript abundance increased (up-regulated) or decreased (down-regulated) in growth phase versus dominance phase oocytes. The fold change was in a range of 1.5 to 5.3 for up-regulated and 1.5 to 2.6 for down-regulated genes in growth phase compared to dominance phase oocytes (Figure 15, Table 4 and 5).

Regarding the oocytes selected based on BCB staining, SAM analysis revealed that a total of 185 genes to be differentially expressed between the BCB^+ and BCB^- oocytes (with $\geq$ 1.9 fold change). Of these, 85 genes were up-regulated (Table 5) and 100 were down-regulated (Table 6) in BCB^+ compared to BCB^- oocytes. Comparative analysis of the magnitude of differential gene expression between the two oocyte groups showed that, while the up-regulated genes to be in range of 1.9 to 7.8 fold change, down-regulated genes are in range of 2.0 to 11.5 fold change in BCB^+ compared to BCB^- oocytes.

A combination of hierarchical clustering and heatmap of differentially regulated genes (Figure 16A and B) were used to show the overall expression pattern of the target genes in replicate hybridization. The average linkage clustering analysis revealed the presence of many subgroups within the up- and down-regulated-genes (or clusters) sharing similar expression pattern.

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Table 3: Transcripts up-regulated in oocytes recovered at growth phase compared to dominance phase

Gene name	Accession No in NBCI Gene bank	Fold change	Gene function
Bos taurus beta-actin mRNA complete cds (ACTB)	AY141970	2.4	Structural constituent of cytoskeleton
Bovine actin mRNA 3' end (ACT)	K00623	3.4	Structural constituent of cytoskeleton
Bovine mRNA for histone (H2AFZ)	X52318	5.3	Chromosome organization
Bovine mRNA fragment for cytokeratin A (no. 8) (KRT8)	X12877	1.8	Cytoskeleton organization and biogenesis
Bos taurus S100 calcium-binding protein A10 mRNA (S100A10)	NM_174650	3.1	Calcium ion binding
Bos taurus annexin A2 mRNA (ANXA2)	NM_174716	4.9	Calcium ion binding
Bos taurus ATP synthase, H+ transporting, mitochondrial F1 complex (ATP5A1)	NM_174684	3.2	ATP binding
Bos taurus isolate FL396 mitochondrion partial genome	AY308068	1.7	NADH dehydrogenase (ubiquinone) activity
Bos taurus isolate FL405 mitochondrion, partial genome	AY308069	1.7	NADH dehydrogenase (ubiquinone) activity
Bos taurus ribosomal protein L24 mRNA (RPL24)	NM_174455	1.8	Structural constituent of ribosome
Rattus norvegicus ribosomal protein S4, X-linked mRNA (Rps4x)	XM_343803	1.7	Structural constituent of ribosome
Homo sapiens ADP-ribosylation factor-like 6 interacting protein (ARL6IP)	BC010281	1.6	Structural constituent of ribosome
Bos taurus mRNA for similar to acidic ribosomal phosphoprotein PO (RPLP0)	AB098748	1.7	Structural constituent of ribosome
Homo sapiens ribosomal protein L8, mRNA (cDNA clone IMAGE:3504599) (RPL8)	BC000047	1.6	Structural constituent of ribosome
Bos taurus mRNA for similar to ribosomal protein S3a, partial cds (RPS3A)	AB099017	1.5	Structural constituent of ribosome
Bos taurus mRNA for similar to ribosomal protein S14, partial cds (RPS14)	AB099089	1.5	Structural constituent of ribosome
Homo sapiens ribosomal protein S15 mRNA (RPS15)	NM_001018	1.5	Structural constituent of ribosome
Bos taurus mRNA for elongation factor 1 alpha, complete cds (EF1A)	AB060107	1.5	Translation elongation factor activity
Bovine inorganic pyrophosphatase mRNA sequence (PP)	M95283	1.8	Inorganic diphosphatase, Phosphatase
Bos taurus thioredoxin mRNA complete cds (TXN)	AF104105	2.6	Thiol-disulfide exchange intermediate activity
S.scrofa mRNA encoding G-beta like protein (GNB2L1)	Z33879	1.5	Signal transduction
Homo sapiens placenta-specific 8 mRNA (PLAC8)	NM_016619	1.5	Unknown
PREDICTED: Canis familiaris similar to Placenta-specific gene 8 ((PLAC8)	XM_535633	1.5	Unknown

Differentially expressed genes were identified by SAM at a false discovery rate (FDR) of ≤ 5% and $P \leq 0.05$

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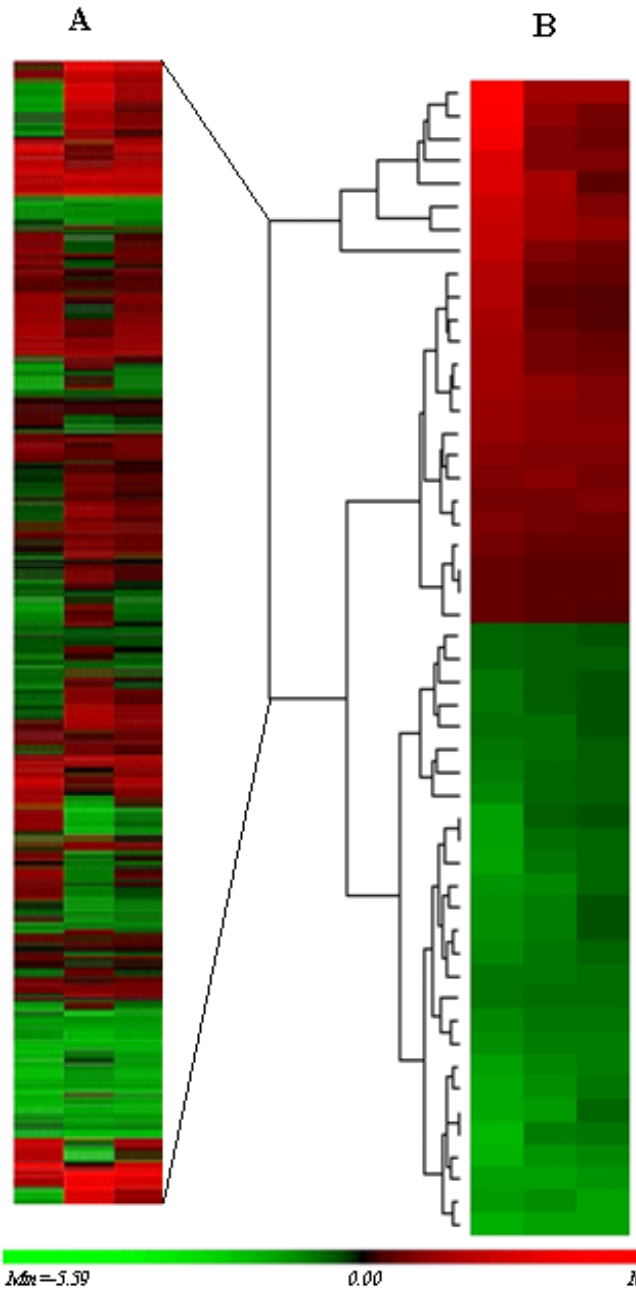
Table 4: Transcripts down-regulated in oocytes recovered at growth phase compared to dominance phase

Gene name	Accession No in NBCI Gene bank	Fold Change	Gene function
Bos taurus bone Morphogenetic protein 15 mRNA partial cds (BMP15)	AY304484	1.8	Growth factor activity
Homo sapiens bone morphogenetic protein 15 precursor gene (BMP15)	AF082350	1.7	Growth factor activity
Bos taurus msh homeo box homolog 1 (Drosophila) mRNA (MSX1)	NM_174798	1.6	Transcription factor activity
Homo sapiens pituitary tumor-transforming gene 1 mRNA (PTTG1)	NM_004219	1.7	Transcription factor activity
Homo sapiens fibronectin type 3 and ankyrin repeat domains 1 mRNA (FANK1)	BC024189	1.5	Transcription factor activity
Bos taurus fibronectin type 3 and ankyrin repeat domains 1 (FANK1)	NM_001003904	1.5	Transcription factor activity
Homo sapiens nuclear phosphoprotein similar to S. cerevisiae (PWP1)	NM_007062	1.5	Cell growth and/or transcription
Homo sapiens CDC28 protein kinase regulatory subunit 2 mRNA (CKS2)	NM_001827	1.5	Cell cycle
Bos taurus mRNA sequence (CCNB1)	L26548	1.6	Regulation of cell cycle progression
Homo sapiens centrin, EF-hand protein, 3 (CDC31 homolog, yeast)	BC005383	1.6	Mitotic centrosome separation
Homo sapiens ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog) (UBE2D3)	BC003395	1.6	Ubiquitin conjugating enzyme activity
Homo sapiens tubulin, alpha, ubiquitous, mRNA (cDNA clone MGC:4689) (K-ALPHA-1)	BC009509	1.5	Nucleotide binding
Homo sapiens tubulin alpha 6, mRNA (cDNA clone MGC:19827) (TUBA6)	BC011790	1.5	Nucleotide binding
Bovine aldose reductase mRNA 3' end (AKR1B1)	M31463	1.5	Aldehyde reductase activity
Bos taurus zona pellucida glycoprotein 4 mRNA (ZP4)	NM_173975	1.5	Binding of sperm to zona pellucida
Rattus norvegicus similar to putative nuclear protein (Loc307510)	XM_226070	1.7	Unknown
Homo sapiens genomic DNA, chromosome 11q, (clone:RP11-313I2)	AP003122	1.7	Unknown
Homo sapiens BAC clone RP11-83B6 from 2, complete sequence (RP11-83B6)	AC010872	1.5	Unknown
Homo sapiens hypothetical protein FLJ14904, mRNA (cDNA clone) (FLJ14904)	BC028595	1.5	Unknown

Differentially expressed genes were identified by SAM at a false discovery rate (FDR) of $\leq 5\%$ and $P \leq 0.05$



Gene sympol	Accession No	Fold change	q-value	SAM score
H2AFZ	X52318	5.3	0.0	1.7
ATP5A1	NM_174684	3.2	0.0	1.7
ANXA2	NM_174716	4.9	1.0	1.6
S100A10	NM_174650	3.1	0.0	1.7
ACT	K00623	3.4	0.0	1.7
FL396	AY308068	1.7	3.2	1.5
ACTB	AY141970	2.4	1.1	1.6
KRT8	X12877	1.8	2.5	1.5
RPL8	BC000047	1.6	2.0	1.6
PLAC8	NM_016619	1.5	0.0	1.7
Novel-9	–	1.7	5.3	1.4
PLAC8	XM_535633	1.5	3.9	1.5
RPS15	AB99089	1.5	7.1	1.3
RPS14	NM_001018	1.5	4.6	1.5
ARL6IP	BC010281	1.6	9.2	1.1
PP	M95283	1.8	3.4	1.6
Rps4x	XM_343803	1.7	4.2	1.6
RPL24	NM_174455	1.8	1.0	1.7
RPLP0	AB098748	1.7	3.6	1.5
FL405	AY308069	1.7	2.3	1.6
TXN	AF104105	2.6	4.7	1.5
GNB2L1	Z33879	1.5	2.2	1.6
EF1A	AB060107	1.5	5.0	1.5
RPS3A	NM_001018	1.5	3.4	1.6
K-ALPHA-1	BC009509	1.5	0.0	1.7
CCNB1	L26548	1.6	2.8	1.6
UBE2D3	BC003395	1.6	4.1	1.5
TUBA6	BC011790	1.5	3.5	1.5
Novel-8	–	1.5	9.5	1.5
ZP4	NM_173975	1.5	1.7	1.7
Novel-7	–	1.5	1.0	1.1
CKS2	NM_001827	1.5	2.3	1.6
FANK1	NM_001003904	1.5	4.2	1.6
RP11-313I2	NM_007062	1.7	3.9	1.5
FANK1	AB003122	1.5	3.1	1.6
RP11-83B6	AC010872	1.5	2.8	1.6
Novel-4	–	1.9	7.3	1.3
PWP1	BC024189	1.5	4.4	1.5
Novel-6	–	1.5	9.8	1.0
FLJ14904	BC028595	1.5	2.5	1.6
AKR1B1	M31463	1.5	4.6	1.5
CDC31	BC005383	1.6	3.1	1.6
Novel-5	–	1.7	1.5	1.7
BMP15	AY304484	1.8	4.9	1.5
BMP15	AF082350	1.7	3.3	1.6
MSX1	NM_174798	1.6	2.8	1.6
LOC307510	XM_226070	1.7	4.1	1.5
Novel-1	–	2.6	3.9	1.5
PTTG1	NM_004219	1.7	2.5	1.6
Novel-3	–	2.3	8.1	1.2
Novel-2	–	2.5	3.7	1.6

Figure 15: Hierarchical clustering and heatmap of 51 differentially expressed genes. The red blocks represent up-regulated genes while the green blocks represent down-regulated genes in oocytes recovered at growth phase

Table 5: Genes up-regulated in BCB^+ compared to BCB^- oocytes

Gene name	Accession No in gene bank	Fold change	Gene function (biological process)
Homo sapiens zinc finger protein 91 homolog (mouse) (ZFP91), transcript variant 1, mRNA	NM_053023	7.8	DNA binding (Transcription)
Homo sapiens zinc finger protein 519, mRNA, complete cds (ZNF519)	BC024227	4.4	DNA binding (Transcription)
Homo sapiens high-mobility group nucleosomal binding domain 2, mRNA, (HMGN2)	BC071707	6.3	DNA binding (Transcription)
Homo sapiens DEAD (Asp-Glu-Ala-Asp) box polypeptide 10, mRNA (DDX10)	NM_004398	5.0	RNA binding
Homo sapiens tudor and KH domain containing, mRNA, with apparent retained intron (TDRKH)	BC022467	4.4	RNA binding
TPA_exp: Mus musculus regulator of sex-limitation candidate 2, mRNA, complete cds (Rslcan2)	BK001637	4.4	Nucleic acid binding(Transcription)
Bovine mRNA for histone H2A.Z (H2AFZ)	X52318	4.4	DNA binding (chromosome organization and biogenesis)
Homo sapiens proliferation-associated 2G4, 38kDa, mRNA, complete cds (PA2G4)	BC007561	4.2	Transcription factor activity
Homo sapiens zinc finger protein 85 (HPF4, HTF1), mRNA (ZNF85)	BC047646	4.0	Transcription factor activity
Bos taurus partial stat3 gene for signal transducer and activator of transcription 3(STAT3)	AJ620667	4.0	Transcription factor activity
Bos taurus DNA (cytosine 5) methyltransferase 1, mRNA (DNMT1)	NM_182651	3.9	Transcription factor binding
Homo sapiens fibronectin type 3 and ankyrin repeat domains 1, mRNA (FANK1)	BC024189	3.9	Transcription factor binding
Homo sapiens SWI/SNF related, matrix associated, actin dependent (SMARCA5)	NM_003601	6.7	RNA polymerase II transcription factor activity
Homo sapiens ring finger protein 10, mRNA, complete cds (RNF10)	BC016622	3.2	Protein binding
Homo sapiens v-ral simian leukemia viral oncogene homolog A (ras related) (RALA)	BC039858	3.6	Protein binding (Signal transduction)
Homo sapiens related RAS viral (r-ras) oncogene homolog 2, mRNA (RRAS2)	BC013106	3.9	Protein binding (Signal transduction)
Homo sapiens cell adhesion molecule with homology to L1CAM(close homolog of L1) (CHL1)	NM_006614	3.6	Protein binding (Signal transduction)
S.scrofa mRNA encoding G-beta like protein (GNB2L1)	Z33879	2.9	Protein binding (Signal transduction)
Canine rab11 mRNA for ras-related GTP-binding protein (RAB11A)	X56388	2.8	protein binding (plasma membrane to the endosome)
Homo sapiens occluding mRNA(ocLN)	NM_002538	2.6	Protein binding (Protein complex assembly)
Canis familiaris occludin 1B mRNA, complete cds (ocLN)	AF246976	2.4	Protein binding
Homo sapiens chaperonin containing TCP1, subunit 8 (theta), mRNA (CCT8)	BC012584	2.8	unfolded protein binding (protein folding)
Homo sapiens ADP-ribosylation factor-like 6 interacting protein, (ARL6IP1)	BC010281	2.7	Protein binding (protein targeting membrane)
Homo sapiens protein regulator of cytokinesis 1, mRNA (PRC1)	BC003138	2.7	Protein binding (Cell cycle)
Homo sapiens nuclear autoantigenic sperm protein (histone-binding) mRNA, (NASP)	BT006757	2.6	Hsp90 protein binding (cell cycle, blastocyst development)
Homo sapiens mutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli), mRNA (MLH1)	NM_000249	2.4	Protein binding (Cell cycle)
Homo sapiens ubiquitin-like, containing PHD and RING finger domains,2 (UHRF2), mRNA	NM_152896	2.7	Ubiquitin-protein ligase activity (Cell cycle)
Homo sapiens ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, (UBE2D3)	BC003395	2.6	Ubiquitin-protein ligase activity (Cell cycle)
Mus musculus ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog,yeast), mRNA (Ube2d3)	NM_025356	2.6	Ubiquitin -protein ligase activity (Cell cycle)
Homo sapiens aurora kinase A, transcript variant 4, mRNA (AURKA)	NM_198435	2.2	Ubiquitin-protein ligase activity (Cell cycle)
Bos taurus mRNA sequence (CCNB1)	L26548	2.4	(Regulation of progression through cell cycle)
Homo sapiens M-phase phosphoprotein 9, mRNA (MPHOSPH9)	NM_022782	2.1	(Regulation of progression through cell cycle)

Differentially expressed genes were identified by SAM at a false discovery rate (FDR) of $\leq 5\%$ and $P<0.05$

Table 5: Genes up-regulated in BCB^+ compared to BCB^- oocytes (Continued)

Gene name	Accession No in gene bank	Fold change	Gene function (biological process)
Homo sapiens centrin, EF-hand protein, 3 (CDC31 homolog, yeast), (CETN3)	BC005383	4.1	Calcium ion binding (Cell cycle)
Bos taurus isolate Cow1 ASPM mRNA, partial cds (ASPM)	BC010658	2.2	Phosphoprotein phosphatase activity (cell cycle)
Homo sapiens nucleolar and spindle associated protein 1, mRNA (NUSAP1)	BC011008	2.5	(Establishment of mitotic spindle localization)
Homo sapiens discs, large homolog 7 (Drosophila), mRNA (DLG7)	AY485424	2.1	Calmodulin binding (cell cycle)
Homo sapiens IQ motif containing GTPase activating protein 1 (IQGAP1)	NM_003870	2.1	Calmodulin binding (Signal transducion)
Homo sapiens regulator of G-protein signalling 16, mRNA(RGS16)	NM_002928	2.6	Calmodulin binding (Signal transducion)
Homo sapiens regulator of G-protein signalling 2, 24kDa (RGS2)	NM_002923	2.2	Calmodulin binding (Signal transducion)
Bos taurus EF1A mRNA for elongation factor 1 alpha, complete cds (EEF1A1)	AB060107	2.5	Translation elongation factor activity (Translation)
Bos taurus mRNA for elongation factor 1 alpha (EEF1A1)	AJ238405	2.0	Translation elongation factor activity (Translation)
Homo sapiens ribosomal protein S15 mRNA (RPS15)	NM_001018	2.0	Structural constituent of ribosome (Translation)
Bos taurus mRNA for similar to ribosomal protein S14, partial cds, (RPS14)	AB099089	2.1	Structural constituent of ribosome(Translation)
Bos taurus mRNA for similar to ubiquitin-S27a fusion protein, (RPS27A)	AB098891	1.9	Structural constituent of ribosome
Bos taurus ribosomal protein S29, mRNA (RPS29)	NM_174804	2.4	Structural constituent of ribosome
Bos taurus ribosomal protein S29 mRNA, complete cds (RPS29)	U66372	1.9	Structural constituent of ribosome
Bos taurus mRNA for similar to ribosomal protein L18a, partial cds, (RPL18A)	AB098916	2.4	Structural constituent of ribosome
Bos taurus mRNA for similar to ribosomal protein L9, partial cds, (RP L9)	AB099048	1.9	Structural constituent of ribosome
Bos taurus ribosomal protein L24 mRNA(RPL24),	NM_174455	2.0	Structural constituent of ribosome (Translation)
Bos taurus type 4 mucus-type core 2 (GCNT3)	AY283766	2.3	Gucosyltransferase activity
Homo sapiens asparagine-linked glycosylation 6 homolog (ALG6)	BC001253	2.4	Gucosyltransferase activity (N-linked glycosylation)
Homo sapiens RIO kinase 3 (yeast) transcript variant 1, mRNA (RIOK3)	NM_003831	2.1	Transferase activity (phosphorylation)
Bos taurus S-adenosylmethionine decarboxylase 1 mRNA (AMD1),	NM_173990	2.1	Lyase activity (spermine biosynthetic process)
Homo sapiens tectorin beta mRNA, complete cds (TECTB)	AF312827	1.9	Glycosylphosphatidylinositol anchor binding
Homo sapiens galactokinase 2, mRNA(GALK2)	NM_002044	1.9	Galactokinase activity (galactose metabolic process)
Bos taurus ornithine decarboxylase (ODC1), mRNA	NM_174130	2.1	Ornithine decarboxylase activity (polyamine biosynthetic)
Bos taurus seryl-tRNA synthetase mRNA, complete cds (SARS)	AF297553	2.0	Ligase activity (seryl-tRNA aminoacylation)
Bos taurus mitochondrion, complete genome	AY526085	2.0	Oxidoreductase activity (electron transport)
Homo sapiens degenerative spermatocyte homolog, lipid desaturase (Drosophila), (DEGS1)	BC000961	2.2	Electron carrier activity (lipid metabolic process)
Homo sapiens tropomyosin 3, mRNA (TPM3)	NM_153649	2.3	Actin binding (Cell motility)
Homo sapiens cytoplasmic dynein intermediate chain mRNA, complete cds (DYNC1I2)	AY037160	2.0	Microtubule motor activity (microtubule movement)
Homo sapiens kinesin family member 20A, mRNA (KIF20A)	BC012999	2.9	Microtubule motor activity (microtubule movement)
Bos taurus zona pellucida glycoprotein 4, mRNA (ZP4)	NM_173975	2.0	Receptor activity (fertilization)
Homo sapiens tripartite motif-containing 51, mRNA (SPRYD5)	BC005014	2.0	Unknown

Differentially expressed genes were identified by SAM at a false discovery rate (FDR) of $\leq 5\%$ and $P<0.05$

Table 5: Genes up-regulated in BCB^+ compared to BCB^- oocytes (Continued)

Gene name	Accession No in gene bank	Fold change	Gene function (biological process)
Bos taurus p97 protein mRNA(CFDP2)	NM_174800	2.0	Unknown
Homo sapiens hematological and neurological expressed 1, mRNA (HN1)	BC039343	2.0	Unknown
Arabidopsis thaliana T-DNA flanking sequence, left border, clone	AJ521477	2.0	Unknown
Homo sapiens transforming, acidic coiled-coil containing protein 3 (TACC3)	NM_006342	2.3	Unknown
Homo sapiens WW domain containing adaptor with coiled-coil, mRNA (WAC)	BC004258	2.3	Unknown

Differentially expressed genes were identified by SAM at a false discovery rate (FDR) of ≤ 5% and $P \leq 0.05$

Table 6: Genes down-regulated in BCB^+ compared to BCB^- oocytes

Gene name	Accession No in gene bank	Fold change	Gene function (biological process)
Homo sapiens neuronal pentraxin II mRNA (NPTX2)	NM_002523	11.5	Calcium ion binding (synaptic transmission)
Bos taurus S100 calcium-binding protein A10 mRNA (S100A10)	NM_174650	11.2	Calcium ion binding
Homo sapiens S100 calcium binding protein A14 mRNA (S100A14)	NM_020672	10.6	Calcium ion binding
Homo sapiens S100 calcium binding protein A16, mRNA (S100A16)	BC019099	10.5	Calcium ion binding
Homo sapiens chloride intracellular channel mRNA 1 (CLIC1),	NM_001288	10.4	Chloride ion binding (chloride transport)
Human cysteine-rich intestinal protein mRNA, complete cds (CRIP1)	U58630	9.7	Metal ion binding
Homo sapiens hypothetical protein DKFZp564K0822, mRNA (ECOP)	BC016650	9.5	Signal transducer activity
Homo sapiens basigin long isoform mRNA, complete cds (BSG)	AF548371	9.8	Signal transducer (cell surface receptor linked signal transduction)
Bos taurus mRNA for similar to galactose-binding lectin, partial (LGALS1)	AB099039	6.3	Signal transducer (regulation of apoptosis)
Bos taurus T cell receptor alpha gene, J segments and C region (TCRA)	AY227782	9.1	Transferase activity (apoptosis)
Bos taurus arachidonate 15-lipoxygenase (ALOX15), mRNA	NM_174501	6.3	Lipoxygenase activity (anti-apoptosis)
Homo sapiens, clone IMAGE:4428430, mRNA (PARP12)	BC044660	6.0	Transferase activity (protein amino acid ADP-ribosylation)
Bos taurus conserved helix-loop-helix ubiquitous kinase mRNA (CHUK)	NM_174021	7.4	Transferase activity (immune response)
Homo sapiens fumarate hydratase, mRNA (cDNA clone MGC:15363 (FH)	BC017444	8.6	Fumarate hydratase activity (cell cycle)
Homo sapiens nucleophosmin (nucleolar phosphoprotein B23 numatrin), mRNA (NPM1)	BC016768	7.4	RNA binding (anti-apoptosis)
Homo sapiens poly(A) polymerase gamma (PAPOLG), mRNA	NM_022894	3.7	RNA binding (RNA polyadenylation)
Homo sapiens KIAA0020 mRNA, complete cds (KIAA0020)	D13645	5.9	RNA binding
Homo sapiens maelstrom homolog (Drosophila) mRNA (MAEL)	BC028595	7.3	DNA binding
Homo sapiens zinc finger, BED domain containing 4, mRNA (ZBED4)	NM_014838	7.2	DNA binding
Homo sapiens pituitary tumor-transforming 1, mRNA (PTTG1)	NM_004219	3.7	Transcription factor binding
Homo sapiens centromere protein F, 350/400ka (mitosin) (CENPF)	NM_016343	7.1	Chromatin binding (G2 phase of mitotic cell cycle)
Mus musculus ADP-ribosylation factor 4 mRNA (Arf4),	NM_007479	6.7	Nucleotide binding
Homo sapiens RAN, member RAS oncogene family, mRNA (RAN)	BC014901	6.7	GTP binding (DNA metabolic process)
Homo sapiens F-box only protein 5 mRNA (FBXO5)	NM_012177	5.4	protein binding (cell cycle)
Bos taurus BTAB2MDS3 beta-2-microglobulin gene, 3'UTR (B2M)	AY325771	4.9	Protein binding (immune response)
Homo sapiens NACHT, leucine rich repeat and PYD containing 2, mRNA (NLRP2)	BC001039	4.4	Protein binding (Apoptosis)
Homo sapiens chromosome 15 open reading frame 23 (C15orf23), mRNA	NM_033286	6.7	Protein binding
Homo sapiens GrpE-like 1, mitochondrial (E. coli), mRNA (GRPEL1)	BC024242	4.6	Unfolded protein binding
Homo sapiens ralA binding protein 1, mRNA (RALBP1)	BC013126	7.1	protein binding (Signal transduction)

Differentially expressed genes were identified by SAM at a false discovery rate (FDR) of ≤ 5% and $P \leq 0.05$

Table 6 Genes down-regulated in BCB^+ compared to BCB^- oocytes (Continued)

Gene name	Accession No in gene bank	Fold change	Gene function (biological process)
Homo sapiens F-box only protein 34 mRNA (FBXO34),	NM_017943	3.3	Protein transport
Bos taurus non-selenium glutathione phospholipid hydroperoxide (AOP2)	AF090194	2.3	Oxidoreductase activity (response to reactive oxygen species)
Bos taurus prostaglandin G/H synthase-2 mRNA, complete cds (PGHS-2)	AF031698	3.5	Oxidoreductase activity (prostaglandin biosynthetic process)
Homo sapiens retinol dehydrogenase 11 (all-trans and 9-cis), mRNA (RDH11)	BC026274	6.1	Oxidoreductase activity (metabolic process)
Bos taurus NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 7 (NDUFA7)	NM_176658	2.5	Oxidoreductase activity (mitochondrial electron transport)
Bos taurus cytochrome c oxidase subunit VIIa polypeptide 2 (liver) (COX7A2)	NM_175807	5.3	Cytochrome-c oxidase activity (electron transport)
Bos taurus isolate FL405 mitochondrion, partial genome (FL405)	AY308069	3.1	Oxidoreductase activity (mitochondrial electron transport)
Bos taurus ATP synthase, H+ transporting, mitochondrial F0 complex (ATP5G2)	NM_176613	2.4	Hydrogen ion transporting ATPase activity (ATP synthesis)
Bos taurus ATP synthase, H+ transporting, mitochondrial F1 complex (ATP5A1	NM_174684	3.8	Hydrogen ion transporting ATPase activity (ATP synthesis)
Homo sapiens lectin, galactoside-binding, soluble, 3 (galectin 3), (LGALS3)	BC001120	2.4	Immunoglobulin binding of the IgE isotype
Bos taurus mRNA for steroidogenic acute regulatory protein (STAR)	Y17259	2.4	Cholesterol binding (regulation of steroid biosynthetic process)
Homo sapiens bone morphogenetic protein 15 precursor gene (BMP15)	AF082350	5.1	Growth factor activity (female gamete generation)
Bos taurus bone morphogenetic protein 15 mRNA, partial cds (BMP15)	AY304484	3.9	Growth factor activity
Bos taurus partial mRNA for bone morphogenetic protein 15 (BMP15)	AJ534391	2.7	Growth factor activity
Bovine mRNA fragment for cytokeratin A (no. 8) (KRT8)	X12877	3.1	Structural molecule activity
Homo sapiens calmodulin 2 (phosphorylase kinase, delta) (CALM2),	NM_001743	2.0	Unknown
Human DNA sequence from clone RP11-146N23 on chromosome 9, complete (DENND4C)	AL161909	2.3	Unknown
Bos taurusBAC CH240-454H24 complete sequence	AC150492	2.4	Unknown
Bovine thymus satellite I (1.715 g/ml) DNA	J00037	2.5	Unknown
Bovine satellite DNA fragment	V00121	2.2	Unknown
Homo sapiens chromosome 8 clone CTC-369M3 map 8q24.3 , complete sequence	AF186190	2.2	Unknown
Homo sapiens chromosome 16 clone RP11-19H6, complete sequence	AC012175	2.4	Unknown
Dictyostelium discoideum extrachromosomal palindromic ribosomal RNA	AY171067	2.0	Unknown
Bos taurus clone rp42-194o5, complete sequence	AC098687	5.1	Unknown
Bos taurus clone RP42-351K5, complete sequence	AC092727	5.6	Unknown
Bos taurus butyrophilin gene, complete cds (BTN1A1)	AF005497	2.2	Unknown
O.aries mRNA for thyroid hormone receptor beta1 (ERBA BETA1)	Z68307	5.0	Unknown
Bos taurus DNA for SINE sequence Bov-tA	X64124	2.9	Unknown
Bos taurus X-inactivation center region, Jpx and Xist genes (XIST)	AJ421481	2.1	Unknown

Differentially expressed genes were identified by SAM at a false discovery rate (FDR) of ≤ 5% and $P \leq 0.05$

Table 6: Genes down-regulated in BCB^+ compared to BCB^- oocytes (Continued)

Gene name	Accession No in gene bank	Fold change	Gene function (biological process)
B.taurus DNA for SINE sequence Bov-2	X64125	2.5	Unknown
Bos taurus clone RP42-400M23, complete sequence	AC090976	2.2	Unknown
Bos taurus clone RP42-221D7, complete sequence	AC136966	2.0	Unknown
Bos taurus clone rp42-513g13, complete sequence	AC107065	2.0	Unknown
Homo sapiens placenta-specific 8, mRNA (PLAC8)	NM_016619	2.7	Unknown
B.taurus cosmid-derived repetitive DNA (clone IDVGA-50; subclone3Rev)	X89421	2.3	Unknown
Homo sapiens chromosome 5 clone CTC-448D22, complete sequence	AC093206	2.2	Unknown
Mouse DNA sequence from clone RP23-44F9 on chromosome 11, complete	AL935275	2.5	Unknown
Mus musculus 11 days embryo gonad cDNA, RIKEN full-length (7030402D04Rik)	AK078561	2.1	Unknown
B.primigenius mRNA for alpha-cop coat protein	X96768	2.0	Unknown
Homo sapiens G antigen, family C 1, mRNA (PAGE4)	NM_007003	2.0	Unknown
Canis beta-galactosides-binding lectin (LGALS3) mRNA, 3'end	L23429	2.4	Unknown
Gallus gallus finished cDNA, clone ChEST201k3	BX950233	2.0	Unknown

Differentially expressed genes were identified by SAM at a false discovery rate (FDR) of $\leq 5\%$ and $P \leq 0.05$

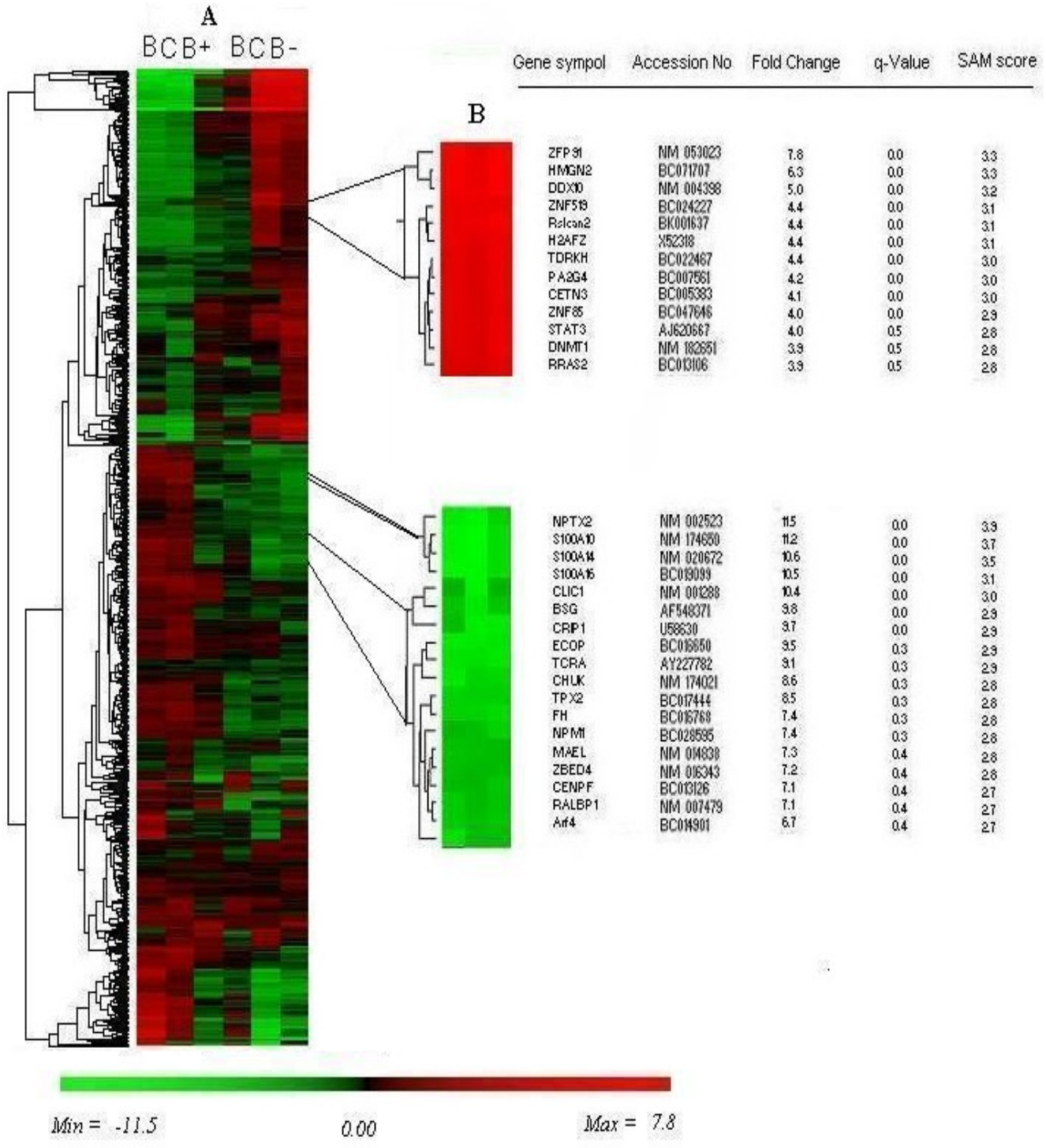


Figure 16: Hierarchical clustering and heatmap of differentially expressed genes. The red blocks represent up-regulated genes while the green blocks represent down-regulated genes in BCB^+ compared to BCB^- oocytes. Columns represent individual hybridizations, rows represent individual genes

4.3 Functional classification of differentially regulated genes

All differentially regulated transcripts were functionally classified based on the criteria of Gene Ontology Consortium classifications (http://www.geneontology.org). The resulting data were supplemented with additional information from various tools available in NCBI public database (http:www.ncbi.nlm.nih.gov/) and from NetAffx Analysis Center and CowBase at the AgBase database (www.agbase.msstate.edu). Accordingly, the differentially regulated transcripts between oocytes from growth and dominance phases represent genes with known function (36/51), genes with unknown function (6/51) and novel transcripts (9/51) (Figure 17). Regarding the molecular function, transcripts with known function showed to be involved in protein biosynthesis (18%), transcription (10%), cytoskeleton (8%), cell cycle (8%), NADH dehydrogenase activity (4%), calcium ion binding (4%), nucleotide binding (4%) and other functions (10%). Oocytes recovered at growth phase were enriched with genes regulating protein biosynthesis (RPLP0, RPL8, RPL24, ARL6IP, RpS14, RpS15, RpS4x and RPS3A), translation elongation (EF1A), ATP binding (ATP5A1), NADH dehydrogenase activity (FL396 and FL405), cytoskeleton (Actin, beta-Actin, H2AZ and KRT8), calcium ion binding (S100A10 and ANXA2), signal transduction (G-beta like protein), inorganic diphosphatase (PP) and thiol-disulfide exchange intermediate (TXN). On the other hand, oocytes recovered at dominance phase were encoded transcripts controlling transcription (MSX1, PTTG1, FANK1 and PWP1), cell cycle (CCNB1, CKS2, UBE2D3 and CDC31), aldehyde reductase activity (AKR1B1), nucleotide binding (TUBA6 and K-ALPHA-1), growth factor (BMP15) and fertilization (ZP4).

For the BCB+ versus BCB- oocytes, the differentially regulated genes between the two groups of oocytes were found to represent genes with known function [57.3% (106/185)], with unknown function [18.4% (34/185)] and novel transcripts [24.3% (45/185)]. In addition, we observed that certain functional annotations were more represented in either BCB+ or BCB- oocytes (Figure 18).

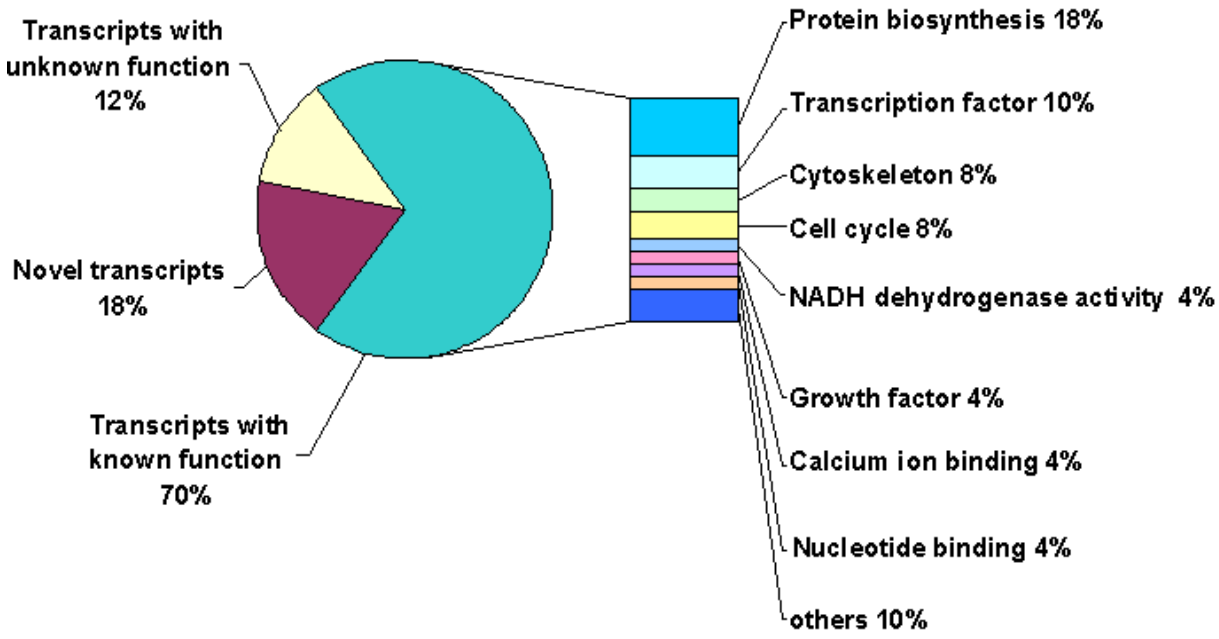


Figure 17: Differentially expressed genes in oocytes derived from growth vs. dominance phases based on the Gene Ontology Consortium classifications (http://www.geneontology.org)

The BCB+ oocytes were found to be enriched with genes related to protein binding (RALA), enzymatic activity(RIOK3), structural constituent of ribosome (RPS14), nucleic acid binding (H2AFZ), transcription (SMARCA5), ubiquitin-protein ligase activity (UHRF2), calmodulin binding (RGS16), translation elongation factor activity (EEF1A1) and microtubule motor activity (DYNC1I2). On the other hand, transcripts involved in protein binding (NLRP2), ion binding (NPTX2), nucleic acid binding (PAPOLG), oxidoreductase activity (PGHS-2), enzymatic activity (ALOX15), signal transduction (LGALS1), growth factor activity (BMP15) and hydrogen ion transporting ATPase activity (ATP5A1) were found to be highly abundant in BCB- oocytes compared to BCB+ ones.

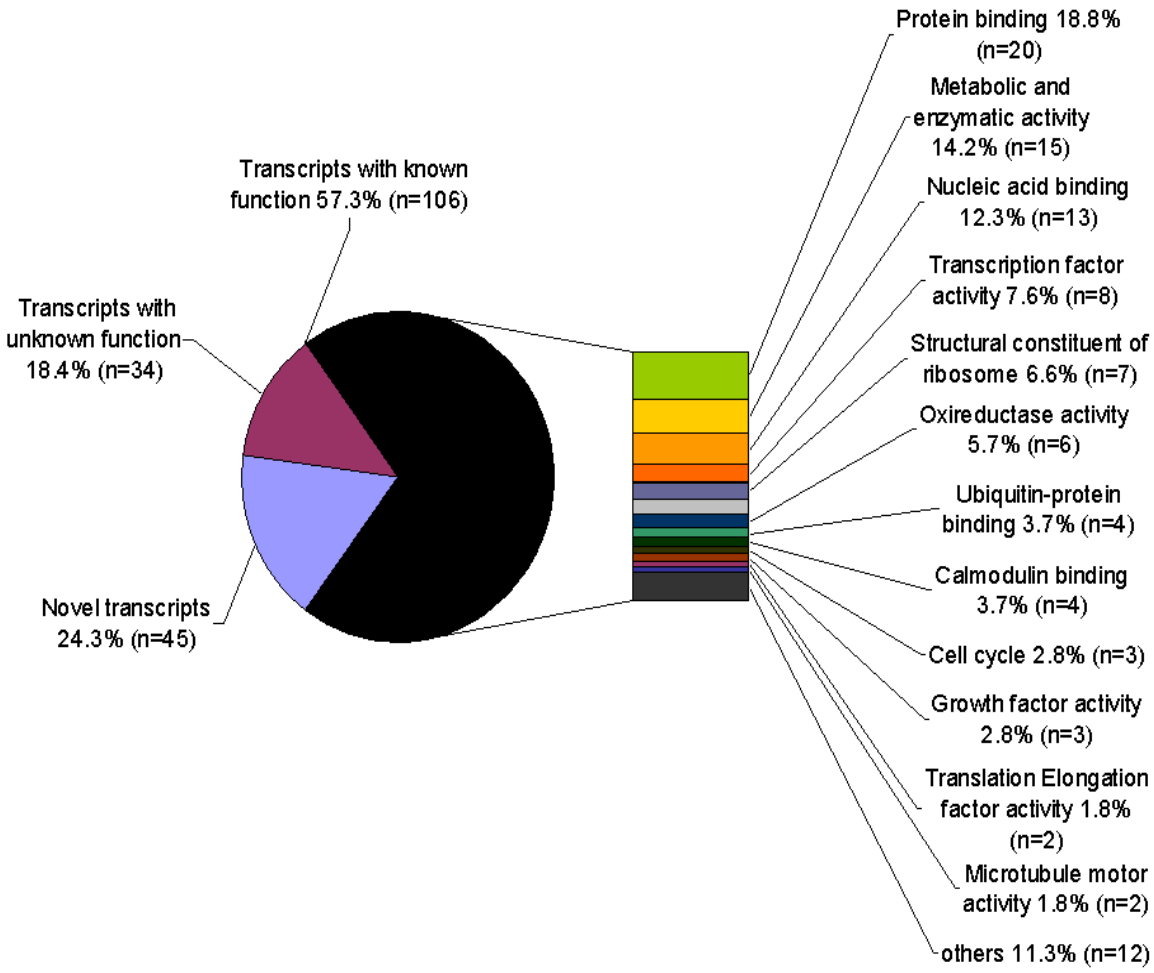


Figure 18: Differentially expressed genes in BCB^+ vs. BCB^- oocytes based on the Gene Ontology Consortium classifications (http://www.geneontology.org)

4.4 Common genes between the two experimental models

From the gene list of two experiments we have identified 20 genes were commonly differentially expressed. Out of these 20 genes, only 9 transcripts showed the same pattern of expression (up- or down-regulated) in the two experiments (Figure 19). On the contrary, 11 genes showed opposite trend from one model to the other one. Based on biological process annotation, genes involved in protein biosynthesis as structural constituent of ribosome (RPL24, ARL6IP, RPS14 and RPS15), translation elongation factor activity (EEF1A1), chromosome organization and biogenesis (H2AFZ) and signal transduction (GNB2L1) were commonly up-regulated in competent compared to incompetent oocytes of both models. On the other hand, incompetent oocytes from both

models were enriched with transcripts regulating transcription (PTTG1) and growth factor activity (BMP 15).

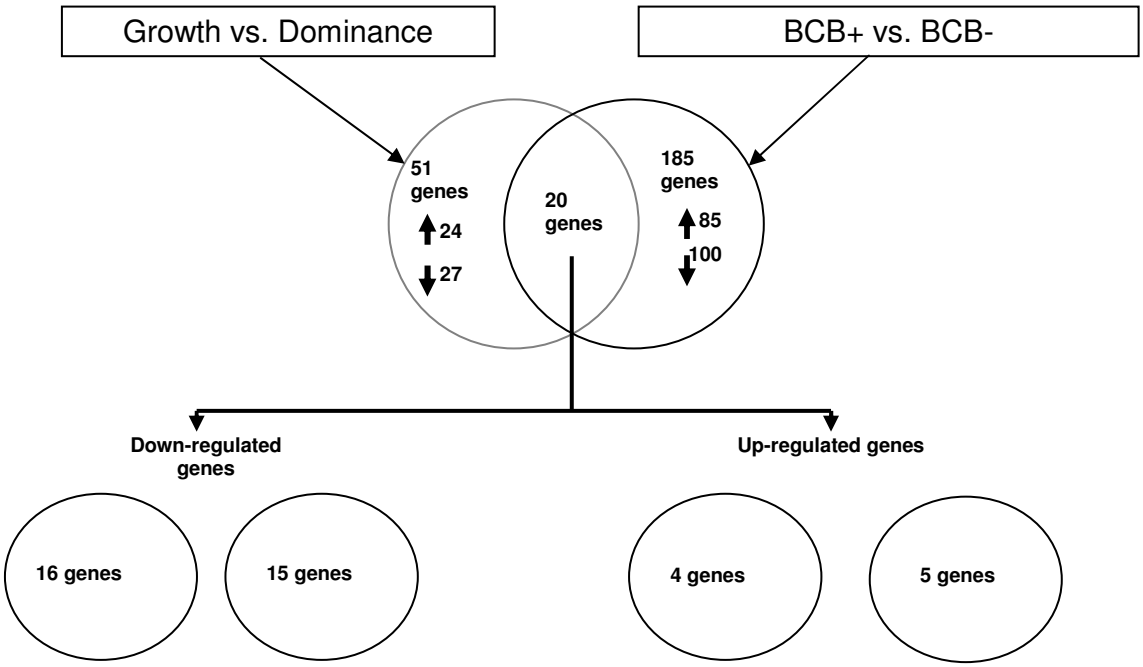


Figure19: Venn diagram of gene differentially and commonly expressed in both models

4.5 Real-time PCR Validation of oocytes derived different follicular phases

Real-time PCR analysis using two sets of oocytes different from those used for array analysis validated the expression profile of 10 differentially regulated transcripts. No differences in relative expression of internal control gene (GAPDH) were observed in all samples. Based on this, the quantitative real-time PCR has confirmed the relative abundance of 8 transcripts (80%) to be in agreement with microarray results (Figure 20A, B, C). The mRNA relative abundance of mRNAs for ANAXA2, S100A10, RPL24, and PP was significantly higher ($P \leq 0.05$) in growth phase compared to dominance phase oocytes. Greater transcript abundance for MSX1 and BMB15 was observed in dominance phase versus growth phase oocytes. However, the transcript abundance of FL396 and PTTG1 were not significantly different. The array results of CKS2 and CCNB1 could not be confirmed by real-time quantitative PCR (Figure 20A).

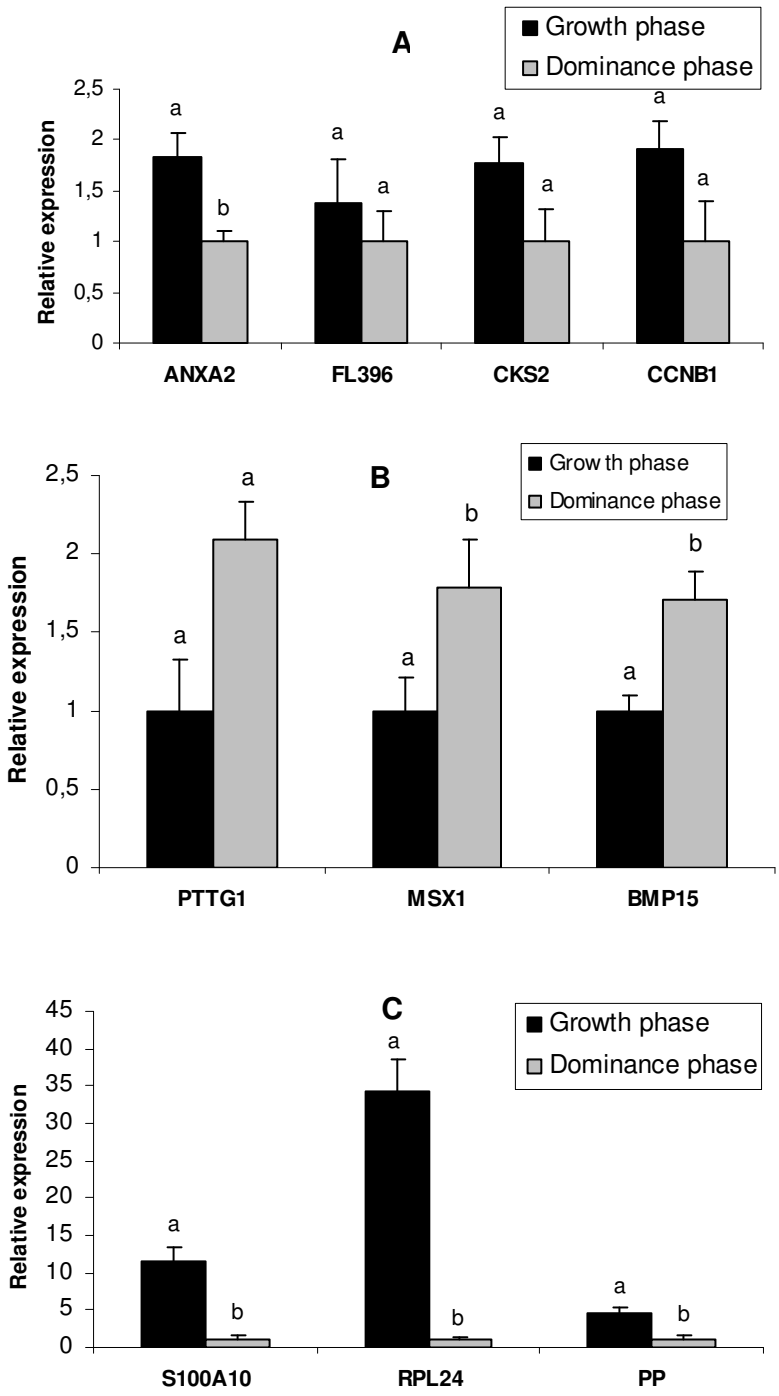


Figure 20: Quantitative real-time PCR validation of 10 differentially expressed genes in bovine oocytes recovered at growth phase (day 3) vs. dominance phase (day 7) as identified by microarray analysis (A, B, C)

4.6. Real-time PCR Validation for BCB^+ vs. BCB^- oocytes

Real-time PCR analysis using a set of samples distinct from those used in microarray experiment validated the mRNA transcript abundance of 10 genes (Figure 21). The relative abundance of GAPDH gene was tested and showed no variability between the samples under investigation. Accordingly, 5 up-regulated genes namely EEF1A1, ODC1, RPS27A, NASP and SMARCA5 showed higher transcript abundance ($P<0.05$) in BCB^+ than BCB^- oocytes as observed in array analysis. Similarly, of the down-regulated genes, the relative abundance for ATP5A1, FL405, S100A10 and PTTG1 were greater ($P<0.05$) in BCB^- than BCB^+ oocytes. The transcript abundance for BMP15 was also confirmed but the differences between the two oocyte groups were not statistically significant. These results showed faithful amplification of the original transcripts in oocytes of both groups and has validated the expression level of 90% (9/10) to be in agreement with the array analysis.

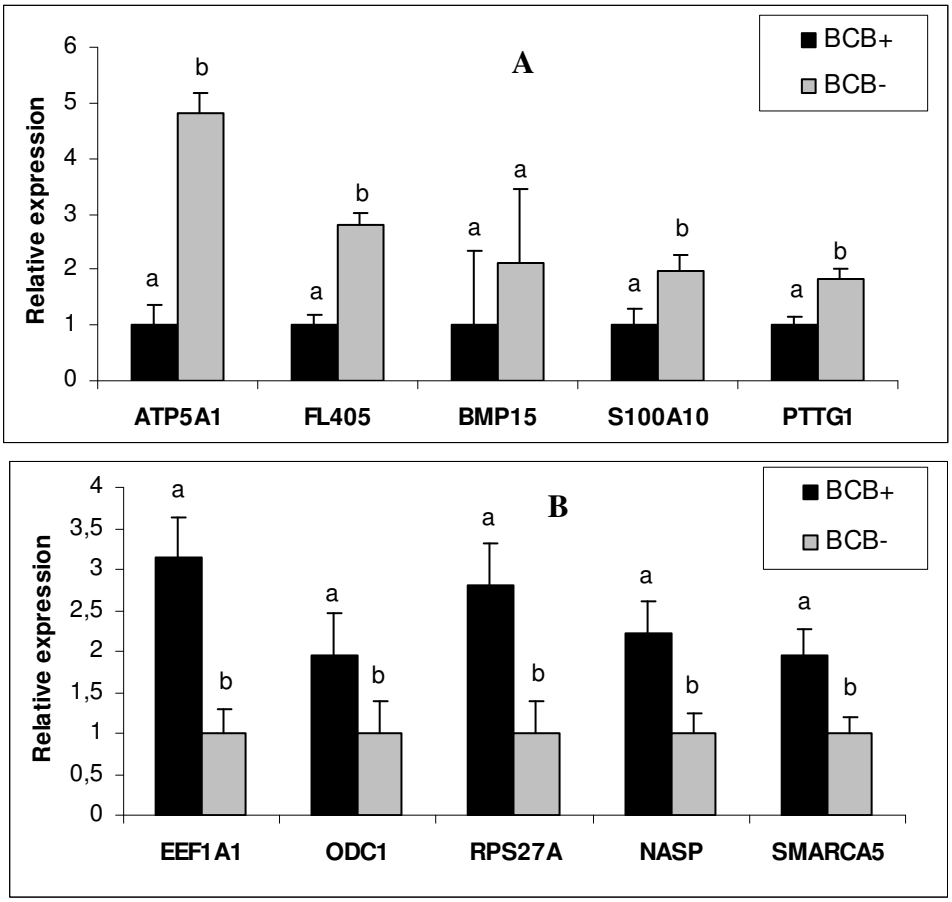


Figure 21: Quantitative real-time PCR validation of 10 differentially expressed genes in BCB^+ and BCB^- oocytes as identified by microarray analysis (A, B)

4.7. Quantitative analysis of selected transcripts in cumulus cells

To avoid any biasness in the number of cumulus cells used as an input for mRNA isolation and subsequent real-time PCR, cumulus cells were derived from equal number of cumulus oocyte complexes (COCs) with similar morphological appearance. After mRNA isolation and subsequent cDNA synthesis, the relative abundance of GAPDH gene was tested and showed no variation between these samples. This indicates that the observed differences in quantitative expression profiles of the five selected transcripts in cumulus cells are not due to variations in total RNA abundance of these samples. Two transcripts (MSX1 and FL396) were highly abundant ($P<0.05$) in cumulus cells of dominance phase versus that of growth phase. On the other hand, RPL24 and CKS2 transcripts were found to be more abundant in cumulus cells of growth phase compared to that of dominance phase (Figure 22). The relative abundance of mRNA for S100A10 was nearly the same in cumulus cells of both phases.

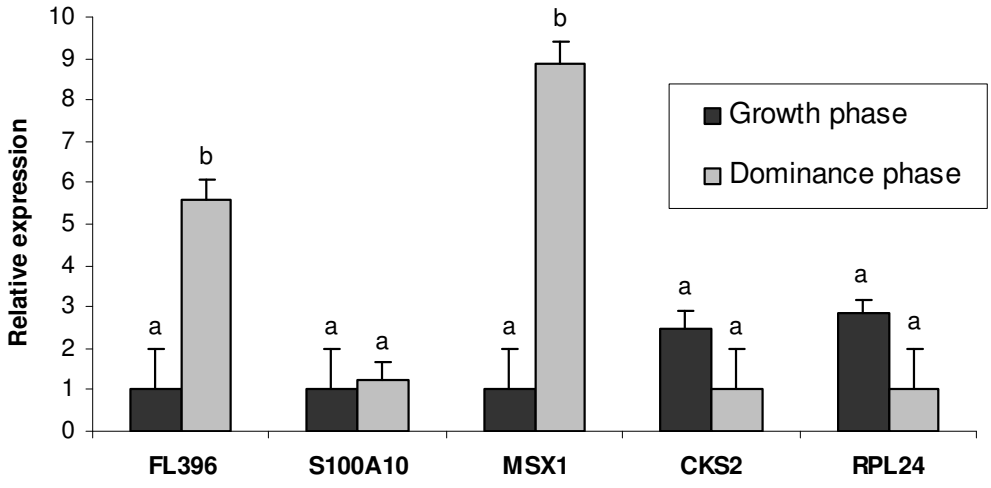


Figure 22: Quantitative real-time PCR of 5 genes in bovine cumulus cells denuded from oocytes recovered at growth phase (day 3) vs. dominance phase (day 7).

4.8. Transcript abundance of five selected genes in oocyte of both models

Immature oocytes screened for developmental competence based on Brilliant Cresyl Blue (BCB) staining were used for validation of the expression profile of five transcripts (BMP15, RPL24, PP, MSX1 and PTTG1) from the results of oocytes derived from growth versus dominance phases. The cDNA synthesized from BCB^+ and BCB^- oocytes were quantified with real-time PCR. After confirming that there were no significant differences in the relative abundance of GAPDH between the samples, all transcripts were quantified using independent real-time PCR runs. The relative abundance for MSX1 and PTTG1 were higher ($P<0.05$) in BCB^- oocytes than BCB^+ ones (Figure 23). The BMP15 was also more abundant in BCB^- than BCB^+ but the differences were not significant. On the other hand, greater mRNA abundance ($P<0.05$) for RPL24 and PP has been observed in BCB^+ versus BCB^- oocytes (Figure 23).

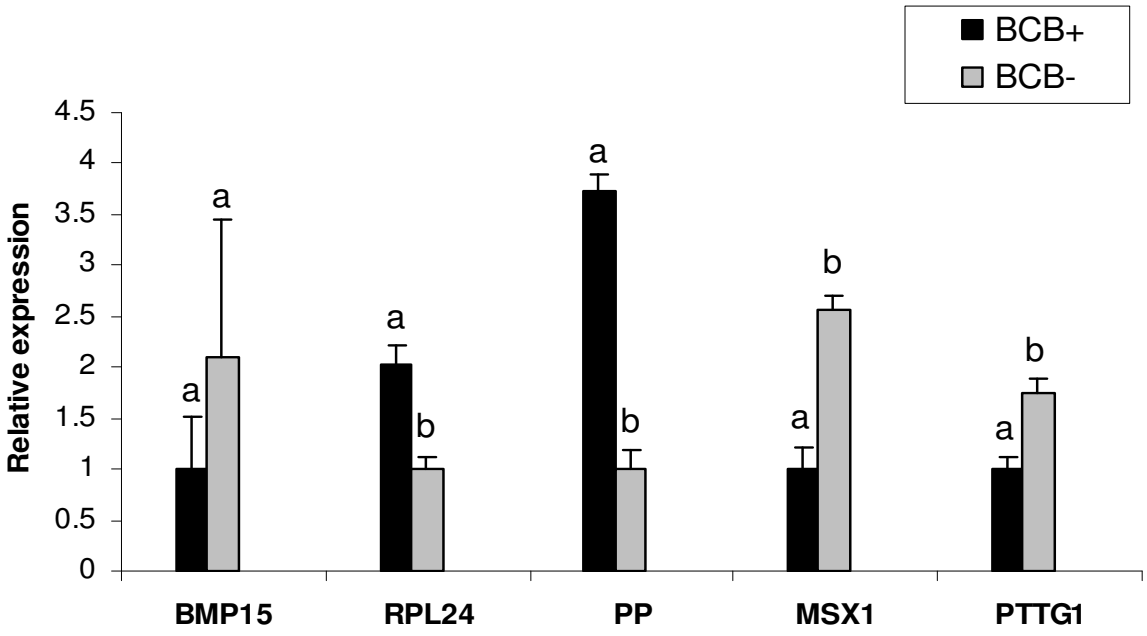


Figure 23: Quantitative real-time PCR of 5 genes in BCB^- and BCB^+ bovine oocytes

4.9 Localization of bovine MSX1 protein

Immunofluorescent labelling of MSX1 gene in ovarian samples collected at the time of estrus (day 0), ovulation (day 1), growth phase (day 3) and dominance phase (day 7) showed the presence of this protein in all stages of follicular development under investigation. More specifically, this protein was found to be more localized in the oocytes cytoplasm than the enclosed cumulus cells (Figure 24d, h, p) or other cellular layers of the growing follicle (Figure 24b, f, n) at all stages of follicular development except at growth phase (Figure 24j, l).

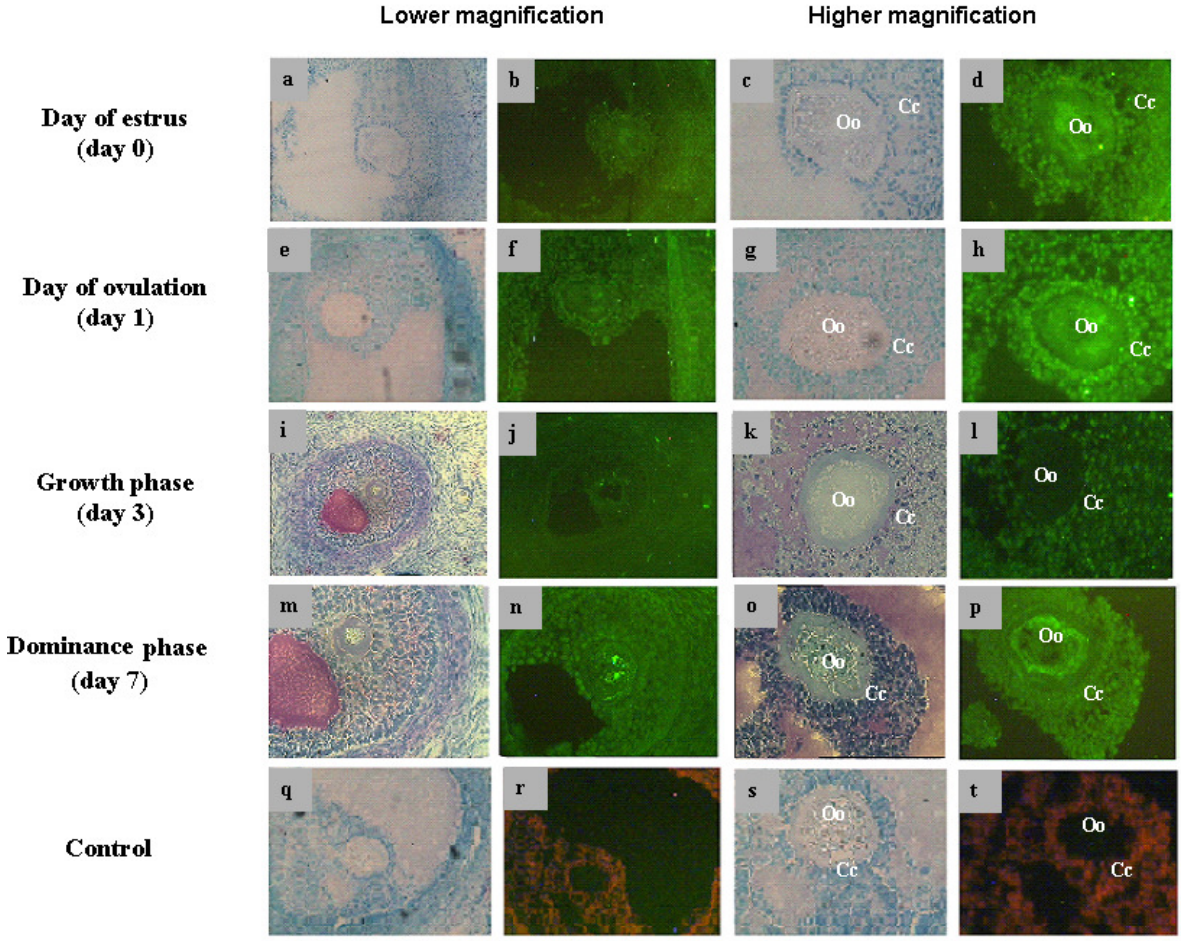


Figure 24: Immunohistochemical localization of MSX1 protein in bovine ovarian sections at day of estrus (b, d), day of ovulation (f, h), growth phase (j, l), dominance phase (m, q). Cumulus cells are marked with Cc and oocytes are marked with Oo. Negative controls were processed without addition of primary anti-MSX1 antibody (r, t). Sections were counterstained with toluidine blue (a, c, e, g, i, k, m, o, q and s). Images from the same ovarian sections were captured with lower and higher magnification

Furthermore, MSX1 protein was found to be dispersed in the cytoplasm of immature and matured oocytes and early zygote (Figure 25a, b, c) but tends to be localized around the nucleus at advanced zygote, 2-cell, 4-cell and 8-cell embryos (Figure 25d, e, f, g). Comparative analysis of protein signals between oocytes showed that green fluorescence signals were reduced after maturation (Fig 25b).

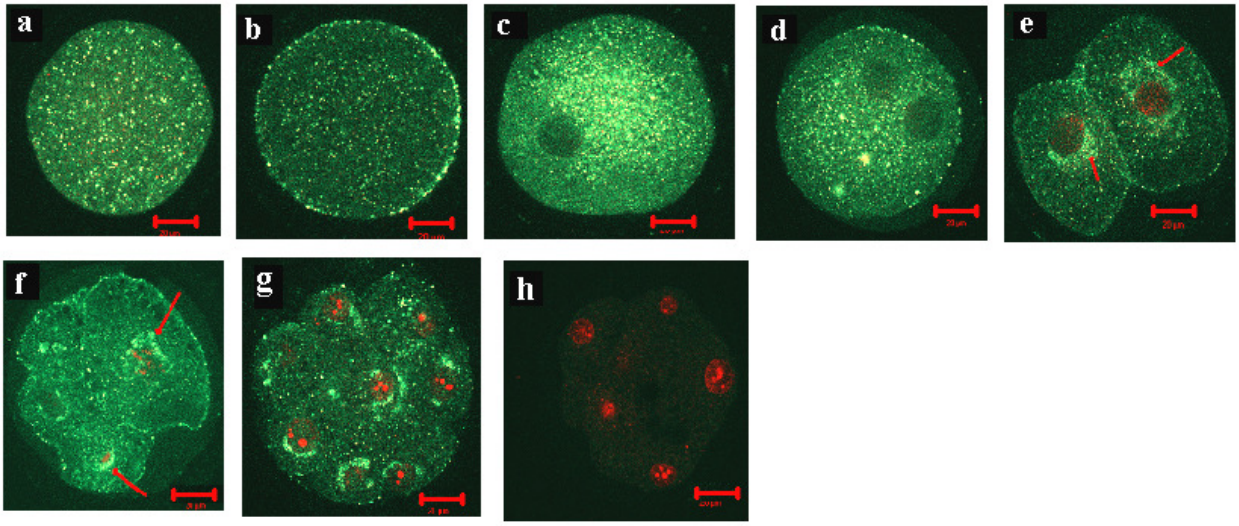


Figure 25: Subcellular localization of bovine MSX1 protein in bovine oocytes and early cleavage embryonic stages [immature oocyte (a), matured oocyte (b), zygote (c), advanced zygote (d), 2-cell (e), 4-cell (f) and 8-cell (g)]. Negative control (h) was processed without addition of primary anti-MSX1 antibody. Nuclei are stained with propidium iodide (red). Scale bars represent 20 µm

4.10 In situ hybridization for MSX1 mRNA

In situ hybridization experiment showed that MSX1 mRNA was localized in the oocytes, cumulus cells and follicular wall throughout all stages of follicular turnover (Figure 26A, B, C, D, E, F, G, H). The signal for MSX1 mRNA were more abundant in the oocytes than of the cumulus cells and in theca than granulosa cells

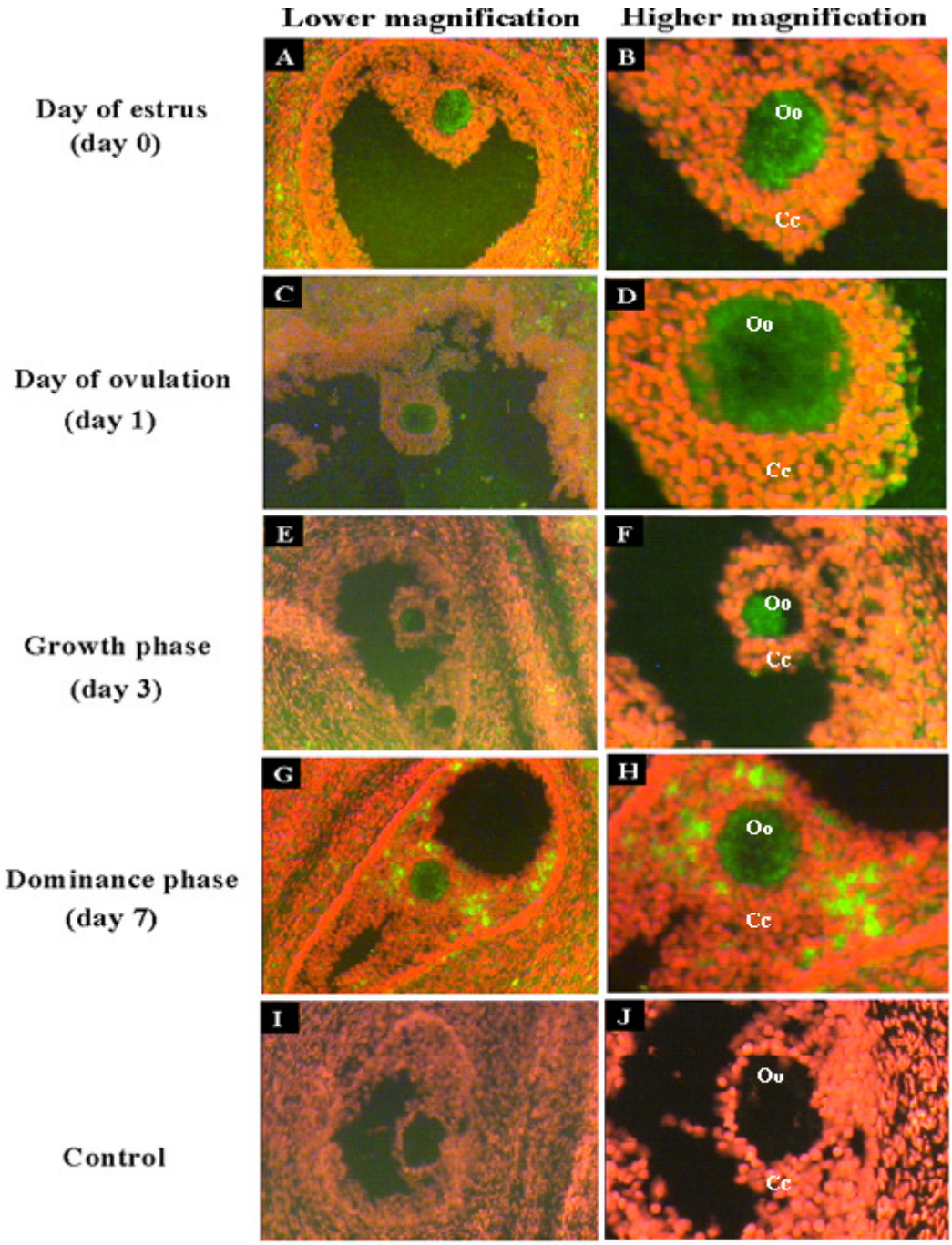


Figure 26: Fluorescent in situ hybridization of MSX1 mRNA conducted with DIG labeled RNA antisense probe in bovine ovarian sections at day of estrus (A, B), day of ovulation (B, C), growth phase (D, E), dominance phase (F, G). Cytoplasms of the oocytes (Oo) are darkly stained with green fluorescent compared to cumulus cells (Cc). Negative controls were hybridized with DIG labeled RNA sense probe (H, I). Images from the same ovarian sections were captured with lower and higher magnification

5 Discussion

Fertility in cattle is a major component of many agricultural enterprises and there is pressure to devise methods to improve it. Poor oocyte competence contributes to infertility in humans and livestock species. In addition, so far there is no accurate method to evaluate oocyte quality. Thus, a number of approaches are ongoing to understand the cellular and molecular events of the development of reproductive tissues and to use these as targets for developing new strategies. Functional genomics and proteomics are tools that promise to provide new insights into important mechanisms controlling oocyte competence (Krisher 2004). Transcriptome analysis using microarray allow us to monitor changes in gene transcription on a genome-wide basis allows identification of groups or clusters of genes that are functionally related to a cell or tissue phenotype. Indeed, Knowledge of the gene expression profiles that occur during oocyte development will provide the first glimpse at understanding the molecular basis for development of an egg of high quality, i.e., an egg that is capable of being fertilized and supporting development to term (Pan et al. 2005). In the present study, differences in RNA transcript profiles associated with developmental competence of oocytes derived from two different models of oocyte competence were examined using a functional genomics approach and novel information on oocyte transcripts potentially associated with developmental competence identified. Distinct biological themes and candidate genes involved in pathways regulating different molecular mechanisms were also identified in both models

5.1 Follicle population and oocyte recovery

In the present study, follicles of different diameters as well as populations of collected oocytes were lower in dominance phase compared to growth phase. This may be due to the influence of the dominant follicle (Machatkova et al. 2004). The number of small follicles were higher in both phases of development as it has been reported by Lucy et al. (1992), who showed that the number of follicles within discrete size classes changes during a follicular wave and the number of follicles 3-5 mm (small follicles) were higher in early and late of the first follicular wave. The average number of oocytes recovered per cow in this study was higher during growth phase compared to the

dominance phase. This was in agreement with the report by Hendriksen et al. (2004) that showed the average number of oocytes collected per cow was higher for day 2 (growth phase) (8.7±5.5) than for day 8 (dominance phase) (5.4±3.4).

5.2 Genes differentially regulated in oocytes derived from different follicular phases

Regarding the ontological functional classification, oocytes recovered at growth phase were enriched with genes regulating calcium ion binding (S100A10 and ANXA2) and cytoskeleton (Actin, beta-Actin, H2AZ and KRT8) compared to oocytes recovered at dominance phase.

The S100A10 protein is a member of the S100 family, which is also known as calpactin I or p11, and functions as one of the mediators in the calcium-dependent signalling pathway. Two copies of S100A10 could combine with two copies of annexin II, forming the annexin II heterotetramer (AIIt) and thus, the S100A10 protein was considered as the ligand of annexin II (Kassam et al. 1998, Réty et al. 1999). Annexin A2 (ANAXA2) is a multifunctional Ca^{2+}-, lipid-, and actin-binding protein implicated in a number of intracellular functions such as signal transduction, membrane trafficking and mRNA transport, as well as the regulation of membrane/cytoskeleton contacts and extracellular functions (Waisman 1995, Gerke and Moss 2002). In addition, S100A10 was the most notable clone among genes screened from the subtracted cDNA library in implantation site of the rhesus monkey (Sun et al. 2004). A quantitative real-time analysis of ANXA2 transcript in bovine oocytes and preimplantation embryos in a study conducted in our lab showed higher transcript abundance in immature oocytes, two-cell and blastocyst stages (El-Halawany et al. 2004). Up-regulation of ANXA2 was reported in pig and mice at MII oocytes stages compared with early stages of embryonic development in both species (Cui et al. 2005). S100A10 and ANXA2 were among transcripts up-regulated in growth phase recovered oocytes. Recent similar study in bovine oocytes during follicular development has shown that oocytes from larger follicles > 8mm at both GV and metaphase II stages have higher level of annexin II mRNA compared to oocytes from the smaller follicles (<3mm) (da Costa et al. 2006). These results together with ours have shown the relatively higher abundance of annexin II in developmentally competent bovine oocytes.

It is well known that normal actin distribution is essential for the polymerization of microfilaments, which play a role in many oocyte and embryo developmental events, including polar body formation and release, nuclear migration, and embryo cleavage (Maro et al. 1986, Kim et al. 1996, Kim et al. 1997, Wang et al. 2000). Mammalian histone variant, H2AZ, plays a critical role in early development and establishing the chromatin structures required for the complex patterns of gene expression essential for normal mammalian development. The lack of functional H2AZ in mice leads to embryonic lethality (Faast et al. 2001). In our study housekeeping genes (Actin, beta-actin, H2AZ) were found to be up-regulated only in oocytes collected in growth phase. A recent proteomic analysis conducted in bovine oocytes and embryos have identified various maternal housekeeping proteins including ß-actin and γ-actin as differentially regulated genes (Massicotte et al. 2006). From this study, it was suggested these maternal housekeeping proteins might be involved in oocyte maturation and further embryonic development (Massicotte et al. 2006). Abnormal actin filament distribution in pigs is one possible reason for abnormal embryo cleavage and small cell numbers in in vitro-produced embryos (Wang et al. 1999).

Various transcription factors are known to be present in the maternal mRNA store until their recruitment for translation at the time of maternal-embryonic transition (Vigneault et al. 2004). In the present study, different transcription factors (MSX1, PTTG1, FANK1 and PWP1) were found to be up-regulated in oocytes derived from the dominance phase compared to the growth phase. MSX gene families encode homeodomain transcription factors, their expression is associated with epithelio-mesenchymal interactions at many sites in vertebrate embryos such as limb buds, craniofacial bones, and tooth buds (Davidson 1995). These gene families encode proteins controlling key developmental processes such as differentiation and patterning during early development (Favier and Dolle 1997). While mice MSX1 homozygous mutants die at birth (Satokata and Maas 1994), over-expression of the MSX1 homeobox gene suppresses cell growth and cell cycle progression in human ovarian cancer cell line (OVCAR3) by regulating the expression of key cell cycle regulators (Park et al. 2001). Moreover, MSX1 has been reported to regulate the p53 tumor suppressor protein in human tumors and thereby enhances apoptosis mediated suppression of p53 (Park et al. 2005). The enrichment of dominance phase oocytes with apoptotic related genes (MSX1 and PTTG) may be related to the level of atresia in subordinate follicles as

influenced by other follicles, mainly the dominant follicle (Hagemann 1999). Consistent with this, greater transcript abundance of MSXI gene was reported in less competent oocytes as a different model of incompetent oocytes (Patel et al. 2007). In a study conducted in our laboratory, MSX1 and PTTG1 were found to be up-regulated in embryo biopsies derived from blastocysts resulted in no pregnancy after transfer to recipients (El-Sayed et al. 2006). From these facts, it is possible to conclude that a physiologically optimal level in the expression of the MSX1 gene is essential to support further embryonic development.

From previous research in other species (Satokata and Maas 1994, Park et al. 2001), optimal expression of MSX1 as transcription factor seems to be important for cellular physiology. In the present study, the MSX1 protein was detected in all follicular compartments of the growing follicle at different stages of follicular turnover and early preimplantation stages, with a slight decrease in fluorescent intensity in ovaries at growth phase. As embryonic development proceeds there is accumulation of the MSX1 protein around the nucleus of the blastomeres which is consistent with the localization of homeobox gene family namely cdx2 in mouse embryos (Deb et al. 2006). As a member of transcription factor gene, the possible role of MSX1 in regulating the expression of other developmentally relevant genes needs further investigation.

5.3 Genes differentially regulated between BCB^+ and BCB^- oocytes

In terms of biological processes, the expression profiles of BCB^+ oocytes were markedly different from those of BCB^- ones. The majority of expressed genes in BCB^+ oocytes are associated with regulation of the cell cycle (NASP, MLH1, PRC1, UHRF2, UBE2D3, CCNB1, MPHOSPH9, CETN3, ASPM, NUSAP1 and AURKA), transcription (SMARCA5, ZFP91, ZNF519, ZNF85, HMGN2, PA2G4, STAT3, DNMT1 and FANK1) and translation (EEF1A1, RPS27A, RPS14, RPS15, RPS29, RPL18A, RPL9 and RPL24); while BCB^- oocytes encoded genes controlling ATP synthesis (ATP5A1), mitochondrial electron transport (FL405) and calcium ion binding (S100A10).

Numerous factors involved in cell cycle regulation have been more recognized in BCB^+ than BCB^- oocytes. Among these cell cycle regulators, a NASP was first identified as a nuclear-associated protein in rabbit testis (Welch and O'Rand 1990, Welch et al. 1990).

This gene has high homology with Xenopus histone-binding protein, N1/N2, which is expressed in oocytes (Kleinschmidt et al. 1986, Kleinschmidt and Seiter 1988). NASP is an H1 histone-binding protein that is cell cycle regulated and occurs in two major forms: tNASP, found in gametes, embryonic cells and transformed cells; and sNASP, found in all rapidly dividing somatic cells (Richardson et al. 2000). Moreover, it was strongly expressed in mouse embryos developed under non-blocking culture conditions in which embryos do not exhibit developmental arrest at the two-cell stage; however, the function of this transcript in early embryonic development remains unknown (Minami et al. 2001). NASP was one of the genes with increased expression in very fast moving bovine oocytes, which showed higher blastocyst rate compared with the slow groups after dielectrophoretic separation (Salilew-Wondim et al. 2007).

Mitochondrial localization, structure and activity change significantly during mammalian oogenesis and early embryogenesis (Bavister and Squirrell 2000). The Preimplantation embryos are dependent on the energy produced from oocyte-inherited mitochondria. The reduced developmental capacity of early embryonic development has been associated with mitochondrial dysfunction and low ATP in mammalian oocytes and embryos (Keefe et al. 1995, Barnett et al. 1997, Van Blerkom et al. 1998, Van Blerkom 2004). In addition, inadequate distribution of mitochondrial throughout the ooplasm is a marker of cytoplasmic immaturity and is highly associated with low developmental competence (Bavister and Squirrell 2000, Sun et al. 2001). Recently, the amount of mitochondrial DNA and transcripts has been quantified in bovine oocytes and embryos (May-Panloup et al. 2005) showing that bovine oocytes that failed to cleave contained significantly lower transcripts implicated in mitochondrial biogenesis. Furthermore, mtDNA defects in oocytes have been linked with impaired oocyte quality and insufficient embryonic development rhesus macaque (Gibson et al. 2005). A global down-regulation of mitochondrial transcripts has been reported in human compromised oocytes and embryos (Hsieh et al. 2004). In the pig, competent BCB^+ oocytes contain more copies of mtDNA and are more likely to be fertilized than incompetent BCB^- oocytes (El-Shourbagy et al. 2006). However, supplementation of BCB^- oocytes with mitochondria from BCB^+ oocytes, and subsequent improved fertilization outcome, again demonstrates the association between mitochondrial number and fertilization outcome. Mouse BCB^+ oocytes gained better cytoplasmic maturity than BCB^- oocytes as determined by a higher intracellular glutathione (peroxidase 1) level, fully polarized

mitochondrial distribution (most of mitochondria aggregated in the oocyte hemisphere around the MII spindle). In this study, it is remarkable that oocytes with high G6PDH activity (BCB^-) had an increased level of mitochondrial fluorescence intensity (Torner et al. 2008) and up-regulation of mitochondrial transcripts (ATP5A1 and FL405) compared with BCB+ oocytes (Torner et al. 2008). One can speculate that the reason for the higher fluorescence intensity of labeled mitochondria in BCB^- oocytes is likely the increased respiratory activity to provide ATP for still unfinished processes in cytoplasmic maturation. In a recent study, incompetent (BCB^-) oocytes exhibited a delay in mtDNA replication due to the delayed onset of expression of their nuclear-encoded replication factors and the oocyte attempts to rescue this during the final stages of maturation. Consequently, oocyte competence in terms of mtDNA replication and composition is not fully synchronized and will result in either failed fertilization or developmental arrest (Spikings et al. 2007). In addition, it could be possible that the higher level of mitochondrial fluorescence intensity in BCB^- oocytes may be due to increased oxidative stress in these oocytes. ATP5A1 is a nuclear-encoded gene whose protein contributes to the overall function of the ATP synthase and it is the universal enzyme for cellular ATP synthesis (Pedersen 1994). It has been reported that null mutations in ε-subunit of mitochondrial ATP synthase gene in Drosophila lead to embryonic death (Kidd et al. 2005). ATP6V1E1 transcript was up-regulated at two-cell block mouse embryos (Jeong et al. 2006). From the above-mentioned facts, it is clear that alterations in mitochondrial distribution, DNA replication, copy number and transcripts may lead to overall dysfunction for the mitochondria and influence the ability of embryos to scavenge free radicals and induce an oxidative stress response, which contributes to impaired development. It seems also that the competency of oocytes is highly dependent on distinct set of genes mainly regulating transcription, translation, cell cycle, chromatin remodelling and mitochondrial machineries that may interact to fulfil this task.

5.4 Genes highly expressed in competent vs. incompetent oocytes

BCB staining was used as an independent model to screen oocytes for their developmental competence in order to validate the differential expression of candidate genes identified in array analysis of the first model (oocytes derived from growth vs.

dominance phases). Higher abundance of MSX1 and PTTG1 in dominance phase oocytes in array experiment was consistent with their higher abundant in BCB^- oocytes than in BCB^+ ones. Similarly, the relatively higher abundance of RPL24 and PP transcripts in oocytes from growth phase was consistent with their higher abundance in BCB^+ oocytes. Thus, there is a clear association of mRNA abundance for genes detected in microarray experiment and developmental competence of oocyte tested with BCB staining. The bovine RPL24 and MSX1 as members of ribosomal proteins and transcription factors gene families, respectively can be considered as good markers of oocyte developmental competence, as they showed consistent results in both models.

Interestingly, when we compared the array results of both models, some genes showed the same expression profile in two proposed models of oocyte competence. For example, we have identified genes involved in protein biosynthesis as structural constituent of ribosome (RPL24, ARL6IP, RPS14 and RPS15), translation elongation factor activity (EEF1A1) were commonly up-regulated in competent compared to incompetent oocytes of both models. Indeed growing oocytes have higher rate of transcription and translation resulting in the formation of RNAs and proteins for both oocyte growth and storage (Fair et al. 1997). The previous molecules could be redirected to fulfil new tasks as resumption of meiosis (Tatemoto and Horiuchi 1995) and regulating maternal to zygotic transition (Hyttel et al. 2001). In addition, the developmental competence of mammalian oocytes depends on high rates of RNA and protein synthesis, imprinting processes and biogenesis of organelles such as mitochondria (Eichenlaub-Ritter and Peschke 2002). Therefore, it is not surprising to find that competent oocytes of the two models were enriched with transcripts related to protein biosynthesis. Failure to detect one or all of ribosomal RNA (rRNA) genes during oocyte growth or later during interphase of the first cell cycle is an indicator of developmental incompetence (Fair et al. 2001). This may suggest that there is an optimal threshold of mRNA transcript abundance for ribosomal RNA genes should be in present below which the developmental competence of the oocytes is compromised.

Elongation factor 1α is a component of the eukaryotic translational apparatus and it is a GTP-binding protein that catalyses the binding of aminoacyl tRNAs to the ribosome (Tatsuka et al. 1992). The tRNA carries the amino acid to the ribosome, which is then used in protein synthesis, thereby inferring a crucial role for this factor in the translation process and subsequently protein biosynthesis. Acquisition of high developmental

capacity in mammalian oocytes is dependent on high rates of RNA and protein synthesis, imprinting processes and biogenesis of organelles such as mitochondria (Eichenlaub-Ritter and Peschke 2002). Consistent with this, oocytes with greater developmental potential (oocytes derived from growth phase and BCB^{+}) showed higher mRNA transcript abundance for EEF1A1 that represent members of ribosomal and translation related genes respectively. Collectively, it is possible to conclude that competent oocytes have greater stores of cell cycle, transcription and protein biosynthesis transcripts that could be used for resuming meiosis (Tatemoto and Horiuchi 1995) and supporting maternal to zygotic transition (Hyttel et al. 2001).

5.5 Quantitative analysis of selected transcripts in cumulus cells

Oocyte growth during folliculogenesis is regulated by granulosa cell derived proteins (Sterneck et al. 1997), which are in turn regulated by oocyte-derived factors (Elvin et al. 1999). In addition, cumulus cells provide nutrients for the oocyte and influence oocyte development in a paracrine fashion (Brower and Schultz 1982), and these paracrine factors contribute to induce meiotic resumption of oocytes (Downs 2001). Characterization of the signalling pathways driving changes in transcript abundance for co-regulated and differentially regulated genes in oocytes versus associated cumulus cells may lead to a better understanding of interdependent gene regulation between germ and somatic cells (Assou et al. 2006). Moreover, molecular markers of oocyte quality could also be identified in surrounding tissues, like cumulus cells. For example, the rate of apoptosis in bovine cumulus cells before (Zeuner et al. 2003, Feng et al. 2007) or during IVM (Ikeda et al. 2003) is negatively correlated to oocyte quality. In this study, compared to other candidate transcripts the bovine MSX1 was found to be higher in both oocytes and the corresponding cumulus cells at dominance phase than those from growth phase. In addition, higher abundant of MSX1 protein and mRNA was detected in oocytes than their adjacent cumulus cells of ovarian sections. Therefore, this transcript can be considered as one of the factors activated in oocyte and cumulus cells from the subordinate follicles due to the presence of the dominant follicle. Similar studies (McKenzie et al. 2004, Zhang et al. 2005) have described the expression profile of some transcripts (cyclooxygenase-2, hyaluronic acid synthase-2, gremlin, and

pentraxin-3) in the cumulus cells as potential markers of the quality of the enclosed oocyte. Recently, Assidi et al. (2008) identified several potential cumulus cell markers of bovine oocyte competence including several GDF9 target genes [i.e. HAS2, TNFAIP6, PTGS2 and gremlin 1 (GREM1)]. Other candidates identified are inhibin βA (INHBA), epidermal growth factor receptor (EGFR), betacellulin (BTC) and CD44 molecule (CD44) (Assidi et al. 2008). Another recent study using prepubertal calf oocytes as a model of poor oocyte competence; the transcript abundance of genes encoding the cathepsin family of cysteine proteinases (CTSB, CTSS and CTSZ) is negatively associated with bovine oocyte competence (Bettegowda et al. 2008).

Overall, the reported differences in developmental competence of bovine oocytes derived from the two models are also accompanied by differences in the relative abundance of transcripts related to the various molecular events and processes regulating oocyte quality. In addition, we have detected differences in the mRNA transcript abundance of some selected candidate genes between cumuli cells derived from oocytes with different developmental potential. Finally, this study provides a genome-wide expression profiling of genes that could be associated with functional relevance for the establishment of developmental competence in oocytes. However, further functional investigations based on these data could help to define the exact key regulatory genes controlling oocyte quality, which could be considered as good biomarkers for oocytes with high or low developmental competence.

6 Summary

The present study was performed, (1) to compare the transcript abundance of bovine oocytes with different developmental potentials of two distinct models (follicular development phases and Brilliant Cresyl Blue staining) using cDNA microarray, (2) to identify differences in transcript abundance of some transcripts of interest in cumulus cells using quantitative real-time PCR and (3) further characterization of selected candidate (MSX1) gene at mRNA and protein level using in situ hybridization and immunohistochemistery.

In this study, we have analyzed gene expression profile of immature oocytes derived from two different well-established models for oocyte selection in two different experiments. The first model is based on retrieval of the oocyte at different stages of ovarian follicular turnover which have previously shown that development of early embryos to the blastocyst stage was greater when oocytes are obtained during follicular growth/stagnation phase (G/S) than in the dominance/regression phase (D/R). In the second model, oocytes were screened based on BCB staining which have also shown to be different in their developmental potential. BCB^+ oocytes (colored cytoplasm, low G6PDH activity) are more competent than BCB^- (colorless cytoplasm, high G6PDH activity) in terms of blastocyst rate and quality.

Bovine cDNA microarray with ~ 2000 clones was used to compare the gene expression profiles of these two groups of oocytes of each model. Three independent biological replicates were used for mRNA isolation and subsequent RNA amplification. Approximately 3µg of amplified RNA from each sample was used as a template in reverse transcription, indirect aminoallyl labelling and subsequent Cy3 or Cy5 dye incorporation. Images were scanned and analyzed using Axon GenePix 4000B scanner and GenePix® Pro 4.0 software. Data were normalized using GPROCESSOR program and finally analyzed using Significant Analysis of Microarray (SAM) procedure. Real-time PCR procedure was used to validate the expression of candidate genes using independent RNA samples.

A total of 51 and 185 genes has been identified to be differentially expressed between the oocytes derived from growth compared to dominance follicular phase and BCB^+ compared to BCB^- ones, respectively. We have observed that certain functional annotations were more represented in either competent or incompetent oocytes. Oocytes

derived from growth phase were enriched with transcripts involved in protein biosynthesis or as translation elongation, cytoskeleton or chromosome organization, calcium ion binding, signal transduction and thiol-disulfide exchange intermediate. Oocytes derived from dominance phase were enriched with genes involved in cell cycle, transcription factors, aldehyde reductase activity, nucleotide binding). In the second model, genes related to protein binding, enzymatic activity, structural constituent of ribosome, nucleic acid binding, ubiquitin-protein ligase activity, calmodulin binding, translation elongation factor activity were more represented in BCB^+ oocytes. On the other hand, BCB^- oocytes were enriched with transcripts involved in oxidoreductase activity, enzymatic activity and hydrogen ion transporting ATPase activity.

We found that distinct biological processes were similar in their expression profile in competent compared to incompetent oocytes of both models. For example, genes involved in protein biosynthesis as structural constituent of ribosome (RPL24, ARL6IP, RPS14 and RPS15), translation elongation factor activity (EEF1A1), chromosome organization and biogenesis (H2AFZ) and signal transduction (GNB2L1) were commonly up-regulated in competent oocytes. On the other hand, incompetent oocytes from both models were enriched with transcripts regulating transcription (PTTG1) and growth factor activity (BMP 15).

Quantitative real-time PCR using independent samples has confirmed the relative abundance of 8 out of 10 and 9 out of 10 genes to be in accordance with microarray analysis for follicular phase and BCB staining models, respectively.

Collectively, results presented here clearly demonstrate that the differences in developmental competence of bovine oocytes selected based in distinct models are also associated with differences in relative abundance of developmentally relevant genes in both the oocytes and their surrounding cumulus cells. Consequently, we have identified candidate genes related to oocyte developmental competence, which need further investigation to get insight into their exact role in controlling the developmental potential of oocytes and early embryo development.

7 Zusammenfassung

Die vorliegende Untersuchung wurde durchgeführt, (1) um die gen expressionsprofile bei Rinderoozyten mit unterschiedlichem Entwicklungspotential anhand von zwei eindeutigen Modellen (follikulare Entwicklungsphasen und "Brilliant Cresyl Blue" Färbung) unter Verwendung eines cDNA Microarrays zu vergleichen, (2) um Unterschiede bezüglich der Transkripthäufigkeit von einigen Genen in den Kumuluszellen unter Verwendung quantitativer Realtime PCR zu identifizieren und (3) um eine weitere Charakterisierung eines Kandatengens (MSX1) auf mRNA und Proteinebene, unter Verwendung von In-situ Hybridisierung und Immunohistochemie, vorzunehmen.

In dieser Studie haben wir Genexpressionsprofile von unreifen Oozyten analysiert, die mit Hilfe von zwei verschiedenen Methoden aus zwei verschiedenen Experimenten gewonnen wurden. Das erste Modell basiert dabei auf der Gewinnung von Oozyten aus verschiedenen Stadien der follikulogenese im Eierstock. Wie gezeigt wurde, kann die Entwicklung eines frühen Embryos zum Blastozysten Stadium besser stattfinden, wenn die Oozyten während der follikularen Wachstum-/Stagnationsphase (G/S) anstatt in der Dominanz-/Rückbildungphase (D/R) gewonnen werden. Im zweiten Modell wurden Oozyten anhand der BCB Färbung sortiert, welche für verschiedenes Entwicklungspotential der Eizellen aussagekräftig ist. BCB^+ Oozyten (farbiges Zytoplasma, niedrige G6PDH Tätigkeit) sind kompetenter als BCB^- Oozyten (farbloses Zytoplasma, hohe G6PDH Tätigkeit), wenn man dies in Form der Blasozystenrate (Anzahl von Oozyten, die das Blastozystenstadium erreichen) bzw. der Blastozysten qualität ausdrückt.

Ein Rinder cDNA Microarray mit ~ 2000 Klonen wurde verwendet, um die Genexpressionprofile dieser zwei Gruppen von Oozyten zu vergleichen. Drei unabhängige biologische Replikate wurden für mRNA- Isolierung und folgende RNA-Amplifikation verwendet. Etwa 3µg der amplifizierten RNA von jeder Probe wurde als Muster für die reverse Transkription, die indirekte Aminoallyl Kennzeichnung und die folgende Cy3 oder Cy5 Färbung verwendet. Bilder wurden unter Verwendung des Neurit GenePix 4000B Scanners und der GenePix® Prosoftware 4.0 gescannt und analysiert. Die Daten wurden unter Verwendung des GPROCESSOR Programms normalisiert und schließlich mit Hilfe der Significant Analysis of Microarray (SAM)

Methode ausgewertet. Das Realtime-PCR-Verfahren wurde verwendet, um die Expression der Kandidatengene mit Hilfe von unabhängigen RNA-Proben zu validieren.

Insgesamt konnten 51 bzw. 185 unterschiedlich exprimierte Gene identifiziert werden, zum einen aus der Gruppe mit unterschiedlichen follikularen Phasen und zum anderen aus der Gruppe mit unterschiedlichen Färbungen. Wir konnten beobachten, dass bestimmte funktionelle Gruppen entweder häufiger in den kompetenten oder in den inkompetenten Oozyten präsent waren. Bei Oozyten, die in der Wachstumphase gewonnen wurden, zeigten Gene die in der Proteinbiosynthese, der Translationselongation, der Cytoskelett- oder Chromosomenorganisation, der Kalziumionenbindung, der Signaltransduktion sowie dem Thioldisulfid Austausch involviert waren, eine höhere Expression. Bei Oozyten, die in der Dominanzphase gewonnen wurden, zeigten Gene die in den Zellzyklus, in Transkriptionsfaktoren, in Aldehydreduktase Tätigkeit, sowie in der Nukleotidbindung involviert waren, eine höhere Expression. Im zweiten Modell waren Gene, die mit Proteinbindung, enzymatischen Aktivitäten, strukturellen Bestandteilen des Ribosoms, Nukleinsäurebindung, Ubiquitinprotein Ligaseaktivität, Calmodulinbindung sowie Translationselongations-Faktoren in Zusammenhang stehen und dabei eine BCB^+ Färbung zeigten, stärker exprimiert. Bei BCB^- gefärbten Oozyten exprimierten Gene stärker, die in Oxydoreduktaseaktivität, in enzymatische Aktivität und in Wasserstoffttransport ATPaseaktivität involviert waren.

Wir fanden heraus, dass beim Vergleich von kompetenten mit inkompetenten Oozyten beider Modelle, spezifische biologische Prozesse sich in ihrem Expressionsprofil ähneln. Beispielsweise wurden die Gene, die in Proteinbiosynthese als struktureller Bestandteil des Ribosoms (RPL24, ARL6IP, RPS14 und RPS15), der Translationselongationsfaktoraktivität (EEF1A1), der Chromosomenorganisation und der Biogenese (H2AFZ) sowie der Signal Transduktion (GNB2L1) involviert sind, grundsätzlich in den kompetenten Oozyten hochreguliert. Andererseits waren inkompetente Oozyten aus beiden Modellen mit Genprodukten angereichert, die für Transkriptionsregulation (PTTG1) und Wachstumfaktoraktivität (BMP 15) verantwortlich sind. Quantitative Real-time PCR, unter Verwendung von unabhängigen Proben, konnte die relative Abundanz bei acht von zehn Genen im ersten Modell sowie bei neun von zehn Genen im zweiten Modell bestätigen.

Insgesamt zeigen die hier dargestellten Ergebnisse, dass Unterschiede in der Entwicklungskompetenz von Rinderoozyten stark mit der Expression von entwicklungsrelevanten Genen assoziert sind, die sowohl in den Oozyten als auch in den umgebenden Kumuluszellen transkribieren. Infolgedessen haben wir Kandidatengene identifiziert, die einer weiteren Untersuchung bedürfen, um Mechanismen der Genregulation von Oozyten in der frühen Embryoentwicklung besser zu verstehen.

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Curriculum vitae

1. Personal information

Full name	: Nasser Ghanem Osman Mohamed
Date of birth	: 24/11/1973
Place of birth	: Cairo, Egypt
Nationality	: Egyptian
Marital status	: Married

2. Graduation

1992 – 1996: Bachelor of Agriculture. Department of Animal Production, Faculty of Agriculture, Cairo University, Egypt.

1997 – 2003: Master of Science with a major in animal breeding (Female reproduction). Department of Animal Production, Faculty of Agriculture, Cairo University, Egypt.

2005 – 2009: Ph.D student. Institute of Animal Science–Animal Breeding and Husbandry Group, University of Bonn, Germany.

3. Training courses

1) Female reproductive ultrasonic (cattle, buffaloes, sheep) Animal Reproduction Research Institute,Agriculture Research Center, Giza, Egypt (from August 1998 to January 1999)
2) In Situ Hybridization: INRA, Nouzilly, Tours, France (June and July, 2000)
3) Embryo transfer in cattle, University of Azores, Faculty of Agrarian Sciences, Terceira, Portugal (February and March, 2003).
4) Gene expression of preimplantation bovine embryos, Institute of Animal Science–Animal Breeding and Husbandry Group, University of Bonn, Germany (from July to October, 2004).

5) Gene expression analysis workshop (Holistische Genexpressionsanalysen-Kurs), Research Institute for the biology of farm animals, Molecular Biology Research Division, Dummerstorf (from 29th September till 2nd October 2008)

4. Publications

4. 1 Full length Articles

Barkawi AH, Hafez YM, Ibrahim SA, El-Asheeri AK, **Ghanem N** (2002): Progesterone profile in relation to corpus luteum development throughout the normal estrous cycle of Egyptian buffaloes. Egyptian Journal of Animal Production 39, 87-94

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Barkawi AH, Ibrahim SA, Hafez YM, Ashour G, El-Asheeri AK, **Ghanem N** (2002): Pattern of Follicular Waves and Corpus Luteum Development throughout the Abnormal Estrous Cycle of Egyptian Buffaloes. Association of Animal Production (EAAP), 53rdAnnual Meeting, Egypt, Cairo, 1-4 September, (Theatre)

Ghanem N, Hoelker M, Jennen D, Tholen E, Sirard MA, Schel-lander K, Tesfaye D (2006): Differential regulation of genes in bovine oocytes collected at growth and dominance phases of follicular development. 22nd Scientific Meeting of

the European Embryo Transfer Association / Association Européenne de Transfert Embryon-naire (A.E.T.E.), 8./9.9.2006, Zug, Switzerland (Abstr)

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