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Evaluation of Oocyte Developmental Competence and Potential Strategies to Improve Oocyte Quality

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EVALUATION OF OOCYTE DEVELOPMENTAL COMPETENCE AND POTENTIAL STRATEGIES TO IMPROVE OOCYTE QUALITY

by

Min Yang

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ABSTRACT

Evaluation of Oocyte Developmental Competence and Potential Strategies to Improve Oocyte Quality

by

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Oocyte quality, or developmental competence, is acquired as the oocyte grows in the follicle and during the period of oocyte maturation. Oocyte quality affects early embryonic development, the establishment and maintenance of pregnancy and even fetal development. The developmental competence of the oocyte is regarded as a limiting factor that contributes to the failures in the applications of assisted reproductive technologies (ARTs) in both human medicine and animal production. The objectives of this research were to elucidate mechanisms involved in the oocyte developmental competence and find potential strategies to improve the efficiency of ARTs by increasing oocyte quality.

Two oocyte-selecting criteria (follicle size and Glucose-6-phosphate dehydrogenase (G6PDH) activity) were used to separate oocytes and consequently assess their ability to develop. Both *in vitro* development and *in vivo* development following somatic cell nuclear transfer (SCNT) were used to investigate oocyte developmental competence. Our data showed that oocytes derived from large follicles have the higher

potential to reach the metaphase II (MII) maturation phase. We also confirmed that G6PDH is negatively associated with oocyte developmental competence.

Using gene expression profile analysis, we have identified three genes (*MATER*, *IGF2R* and *GRB10*) that had higher level of mRNA in MII oocytes derived from large follicles compared to those in oocytes from small follicles. Furthermore, among the genes differentially expressed between oocytes with different G6PDH activity, the *CPEB1* gene was expressed at a higher level in oocytes with higher developmental competence. Based on this finding, we proposed a potential strategy for improving oocyte developmental competence by injecting exogenous *CPEB1* mRNA into *in vitro* matured MII oocytes. We observed the increased blastocyst rate in this treatment group, which reaffirmed the essential role of CPEB1 in early embryonic development.

Last but not least, we also compared the effect of three different *in vitro* maturation (IVM) media on goat oocytes. The results contribute to the understanding of how the nutrients in culture medium facilitate oocyte maturation.

The knowledge obtained from this research provides information that helps us to gain understanding on how oocytes acquire their developmental competence at the molecular level that should lead to improvements of ART efficiency.

(188 pages)

PUBLIC ABSTRACT

Evaluation of Oocyte Developmental Competence and Potential Strategies to Improve Oocyte Quality

Min Yang

Assisted reproductive technologies (ARTs) have now been extensively used to promote reproductive efficiency as a fertility treatment not only in human medicine but also animal reproduction. ARTs serve as an important tool to advance the fundamental knowledge of reproductive processes. The quality of female's eggs defines its ability to undergo maturation, fertilization, and development. This quality is determined by various factors and is crucial for the success of ARTs. Any alternations happening during the egg growth and maturation process can result in the decreased quality, which could have long-lasting effects on development. Improving the developmental efficiency of the egg is quite challenging due to the limited knowledge on the underlying mechanism of how the egg regulates biological processes during the growth and maturation phase. We compared good-quality and poor-quality eggs to detect the key players in determining the egg quality at the molecular level. Our finding also provides information that benefits the understanding of how the nutrients in culture medium facilitate oocyte maturation, which will eventually help optimize the condition for oocyte culture. Based on the results from these comparative studies, we proposed a potential strategy for improving egg quality. The knowledge obtained from our research offers promise for many applications in the treatment of infertility and improvement of ART efficiency.

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CHAPTER I

REVIEW OF LITERATURE

Artificial reproductive technologies (ARTs), including artificial insemination, embryo transfer, *in vitro* maturation, *in vitro* fertilization, intracytoplasmic sperm injection, etc., not only have a practical use in the medical field, but also have been successfully used in animal reproduction (Baldassarre and Karatzas 2004; Mapletoft and Hasler 2005). However, *in vitro* produced embryos have shown a reduced developmental competence compared with *in vivo*- derived embryos (Enright, Lonergan et al. 2000; Manjunatha, Gupta et al. 2008; Nagashima, Grupen et al. 1996; Papadopoulos, Rizos et al. 2002). Somatic cell nucleaer transfer (SCNT) is also a powerful tool to generate transgenic animals, however, its efficiency remains extremely low with typically only 1-5% of manipulated embryos developing to term. The quality of the oocyte is generally accepted as a key factor for the success rate of ARTs and profoundly affects early embryonic development, the maintenance of pregnancy, and even fetal development. Maternal factors that accumulated during oogenesis, such as subcellular organelles, macromolecules and stored mRNA transcripts are believed to contribute to oocyte developmental competence (Krisher 2004). Improvement of SCNT technique can be achieved by enhancing the competence of the donor cytoplast (Lee, Hyun et al. 2003).

Oocyte Development

Oocyte development competence is acquired during oogenesis with the interaction with somatic cells (Royère 2006). This process is regulated by a vast number of intra- and extra-ovarian factors and stores a number of cytoplasmic enzymes,

mRNAs, organelles, and metabolic substrates, which contains all the materials needed to initiate and maintain metabolism and development (Sanchez and Smitz 2012).

Oogenesis

Oogenesis is a complex differentiation process of oogonia developing into mature oocytes that are competent and capable of being fertilized. Oogenesis initiates following the migration of the primordial germ cells (PGCs) into the genital ridge where they start to proliferate and interact with surrounding somatic cells (Bukovsky, Caudle et al. 2005). This migration of PGCs gives rise to the development of ovarian follicles, the functional unit of the ovary. In mammals, oogenesis has three major phases:

1. Multiplication phase

In the first phase, PGCs become the oogonia in the forming gonad (ovary). The oogonia then undergo extensive proliferation by mitotic division (Senger, Oki et al. 2012).

2. Growth phase

When cell division ceases, the oogonia enlarge to form primary oocytes and enter into the growth phase. In this phase, primary oocytes become arrested at the diplotene stage of the prophase of the first meiotic division (Prophase I). Each oocyte at the end of prophase I have enveloped by a single layer of flat, follicular epithelial cells. Primary oocytes together with the surrounding support somatic cells formed the primordial follicles. The contribution of substance from oocytes is much greater than from the paternal gametes during the early embryo development. Therefore, the growth of gametes in oogenesis is crucial and takes a longer period of time than in spermatogenesis. The meiosis of primary oocytes begins before birth but arrests at prophase I until puberty

starts. During the growth phase, the size of the primary oocyte increases enormously along with folliculogenesis. A large number of proteins, organelles, mRNA transcripts and other substrates and nutrients that are required to achieve oocyte developmental competence become accumulated in the cytoplasm (Watson 2007). The later oocyte maturation and embryo development rely upon the storage of the nutrients and proper possessing, and activation of the maternal mRNA stored during this phase. In addition to the cytoplasmic change, the nucleus of primary oocytes also undergoes tremendous changes that occur in the growth phase (Zhang and Xia 2012). The nucleus is called germinal vesicle at this stage because it becomes large and watery due to the increased amount of nucleoplasm. The transcriptional activity in the growth phase of the oocytes is greatly increased as a result the oocytes remaining tetraploid for a long period of time, which also prepare the oocytes for the long transcriptional quiescent stage that occurs after meiosis.

3. Maturation phase

After animals enter puberty, primary oocytes undergo germinal vesicle break down (GVBD) and meiosis resumption occurs, which is triggered by Luteinizing hormone (LH) surge and leads to the maturation phase of oocytes. The underlying mechanism that drives the resumption of meiosis is still not fully understood. By the time of ovulation, oocytes are arrested at metaphase of the second meiotic division (Metaphase II) with the first polar body extruded. During this phase, oocytes do not only undergo nuclear maturation but also cytoplasmic maturation (Sirard and Coenen 2006). Nuclear maturation refers to the ability of the oocyte nucleus to resume meiosis. However, the cytoplasmic maturation is not as visible and clearly defined. As we know, transcription

event ceases after germinal vesicle undergoes the breakdown during meiosis. Therefore, the mRNA, proteins and other nutrients stored during the oocyte growth and maturation phase are crucial for early embryonic development before embryonic genome is activated (Watson 2007). Extensive post-translational, transcriptional and post-transcriptional regulations are observed in the oocyte cytoplasm at this stage (Kang and Han 2011). Cytoplasmic maturation includes all these events occurring in oocyte cytoplasm that support the oocytes to complete nuclear maturation, and later embryo development after fertilization.

Folliculogenesis

As primary oocytes are formed, a single-layer of epithelial follicle cells derived from the oocyte-surrounded support somatic cell at the genital ridge slightly encloses the primary oocytes. Those somatic cells (granulosa cells) and the enclosed primary oocytes constitute a primordial follicle (Johnson 2012). During oogenesis, germ cells and granulosa cells in the ovarian follicles grow and develop in a coordinated and dependent manner. The oocyte plays a crucial role in folliculogenesis including the follicle growth and granulosa cell differentiation. As the enclosed oocyte grows and matures, the primordial follicle later grows into a preantral follicle followed by an antral follicle in which an antrum is formed. In the antral follicle, granulosa cells that are in intimate contact with the oocyte differentiate into cumulus cells. The follicle finally grows into a preovulatory follicle and ruptures to release the mature cumulus-oocyte complex (COCs) during ovulation.

Molecular regulation during oogenesis and folliculogenesis

The synthesis and accumulation of proteins, mRNAs, and other nutrients during oogenesis are crucial for the acquisition of oocyte competence for growth, maturation, and the ability to develop into a viable embryo after fertilization. Oocyte and granulosa cells communication is essential for this process. The developmental competence of the oocyte is dependent on the differentiation of cumulus cells, which provides the oocyte with nutrients and signaling factors that promote maturation and development (Brower and Schultz 1982). Furthermore, since oocyte cytoplasm contains plenty of maternal transcripts and protein and lacks transcription activity for a long period of time, the proper post-transcriptional and post-translational regulations are vital for the oocyte maturation, embryo development and the maternal to zygotic transition after fertilization. The oocyte-derived growth factors (such as Growth Differentiation Factor 9 (GDF9) and Bone Morphogenetic Protein 15 (BMP15) stimulate the proliferation of granulosa cells and follicle growth during this early stage, which is followed by follicle stimulating hormone(FSH)-independent stage of follicle growth (Vitt, Hayashi et al. 2000). The theca cells play an important role in follicle growth by secreting several regulating cytokines including tumor necrosis factor-α (TNF) and interleukins 1α and 1β (IL-1) (Nelson-Degrave, Wickenheisser et al. 2005). FSH becomes critical when follicle reaches antral stage. FSH facilitates the follicle growth by binding to the FSH-receptors in the follicular somatic cells, which stimulates a number of downstream signaling pathways, such as cAMP-dependent pathway, that eventually lead to the growth of oocyte and follicle (Gutierrez, Campbell et al. 1997).

Maintenance of oocyte meiotic arrest before maturation is dependent on follicular somatic cells. The mechanism of meiotic arrest is not fully understood. One possible

mechanism is that arrest is maintained by a high concentration of the second messenger cyclic AMP (cAMP, Hinckley, Vaccari et al. 2005; Knecht, Amsterdam et al. 1981). The cAMP is mostly derived from the surrounding somatic cells via gap junction, but also can be produced within the oocytes themselves. High level of cAMP within the oocyte activates protein kinases A (PKA), which then phosphorylates CDC2. This phosphorylation keeps the maturation promoting factors (MPF), which is a CDC-cyclin complex (CDC2 and CyclinB), inactive (Polanski, Homer et al. 2012). Cyclic GMP is also an important signaling factor for maintenance of meiotic arrest by inhibiting the activity of oocyte-specific Phosphodiesterase 3A (PDE3A), which is an enzyme that initiates multiple pathways leading to meiosis (Norris, Ratzan et al. 2009). Under the stimulation of FSH, regulatory signals such as peptide precursor type3 (NPPC) are expressed by somatic granulosa cells and increase the level of cGMP in cumulus cells, which leads to the increase of cGMP in oocytes through gap junction (Zhang, Su et al. 2010). These events together block meiosis progression. The preovulatory LH surge initiates a series of events in ovarian follicles, including meiotic resumption, luteinization, expansion and maturation of cumulus cells, and follicle rupture. LH binds to its receptor on the membrane of somatic cells in the follicle and triggers more than one signaling pathways. LH suppresses the expression of connexin43 (CX43, the most important gap junction protein) by reducing its translation rate, which is mediated by both PKA and stimulate mitogen-activated protein kinase (MAPK, Kalma, Granot et al. 2004). The suppression of CX43 might cause the closure of gap junctions between follicular somatic cells and the oocyte, thus preventing the passage of cAMP, cGMP or other meiosis-inhibitory molecules (Norris, Freudzon et al. 2008). Without a high level of

cAMP, PKA is inactive. Active PKA that phosphorylates the CDC2 keeps MFP inactive. Therefore, at the time of the LH surge, MFP becomes active and then regulates a number of downstream pathways to eventually lead to maturation of the oocyte and extrusion of the 1st polar body. Also, decreased cGMP allows PDE3A to be active. Active PDE3A hydrolyzes cAMP to further decrease the PKA activity (Richard, Tsafriri et al. 2001). Gap junction closure itself can initiate the prophase-to-metaphase transition. LH could also reinitiate meiosis by other redundant mechanisms. However, how this gap junction closure-independent pathway regulates meiotic resumption remains unclear. In the absence of transcription, completion of oocyte growth, maturation, and early embryo development relies ominously on maternally synthesized and stored RNAs and proteins. These processes are mainly controlled by the post-transcriptional regulation of maternal stored mRNA and post-translational modification of proteins. Temporal and spatial regulation of translation in the mammalian oocyte cytoplasm is essential for these processes, which is often regulated via increases in poly(A) length (Dickson, Thompson et al. 2001). Cytoplasmic polyadenylation and deadenylation are generally associated with translational activation and translational repression, respectively (Gale, Tan et al. 2000; Gray and Wickens 1998). Stability of the large amounts of mRNAs accumulated during the growth phase is vital to sustain the following transcriptionally quiescent phase and is regulated by a number of RNA-binding proteins such as DAZL and MYS2 (Clift and Schuh 2013). Many mRNAs can also be held in a translationally -repressed state by CPE-binding protein (CPEB) to their 3′ untranslated regions.

CPEB is required for poly-adenylation of most mRNAs during oocyte maturation (Stebbins-Boaz, Hake et al. 1996). The mechanism of how CPEB regulates mRNA

translation and stability is still not fully understood. However, it is generally believed that CPEB can either activate or depress a gene by binding with different regulatory proteins (Minshall, Reiter et al. 2007; Racki and Richter 2006).

In all, oocyte development is an extremely complex process regulated by a large number of maternal factors.

Somatic Cell Nuclear Transfer

Assistant reproductive technologies are used very broadly in livestock to improve desemination of desired production traits, advance our knowledge of reproductive processes, preserve valuable genetics and generate transgenic animals. These technologies include artificial insemination (AI), *in vitro* maturation (IVM), *in vitro* fertilization (IVF), *in vitro* embryo production (IVEP), multiple ovulation embryo transfer (MOET), intracytoplasmic sperm injection (ICSI), somatic cell nuclear transfer (SCNT), etc.

Somatic cell nuclear transfer is also known as cloning. This technology produces animals genetically identical to the donor somatic cells used as nuclear donor for the SCNT procedure. Since the first nuclear transfer mammalian animal has been created from a cultured differentiated sheep cell in 1996 (Campbell, McWhir et al. 1996), nuclear transfer technique has been successfully applied in numerous species including cattle (Cibelli, Stice et al. 1998), mice (Wakayama, Perry et al. 1998), goats (Baguisi, Behboodi et al. 1999), pigs (Polejaeva et al. 2000), cats (Shin, Kraemer et al. 2002) using a variety of somatic cell types as donors. Briefly, in the SCNT process, diploid donor nuclei (G1/G0) are transferred into enucleated MII oocytes, followed by fusion and either electrical or chemical artificial activation *in vitro* (Wilmut, Beaujean et al. 2002). Then

the reconstructed embryos are cultured and transferred into estrus syncgronized female animal recipients for further development.

SCNT is the only cell mediated platform for production of transgenic livestock as true chimera contributing pluripotent embryonic stem cells (ESCs) are only reported for mice and rats (Polejaeva and Mitalipov 2013). Somatic cells (typically fetal fibroblasts) can be genetically modified *in vitro*, screened to confirm the presence of the transgene or other genetic modification of interest and then used for SCNT. Transgenic livestock (pigs, cattle, goats and sheep) were generated using SCNT for variety of applications including large animal models of human disease (Polejaeva, Ranjan et al. 2016), pharmaceutical protein production and improving agricultural traits (Ogura, Inoue et al. 2013; Vajta 2007).

The U.N. predicts world population will exceed 9.5 billion by mid-century and much of this population growth will occur in developing countries. This will require innovative strategies and new technologies to double food production by 2050 in order to satisfy global food needs. Both genetic- and management-based increases in sustainable productivity are required to prevent the possible future food shortage (Fahrenkrug, Blake et al. 2010). Recent development of genome editing tools allow for introduction of specific traits from one breed to another as well as potentially between different species. Livestock genetic engineering also plays a critical role in human medicine by serving as the material source for tissue and organ transplantation using genetically modified pigs (xenotransplantation) as well as models of human diseases (Kues and Niemann 2004; Snaith and Törnell 2002). Compared to small model animals like mice and rats, some large livestock animals are more similar to human in size, physiology, organ

development, and disease progression (Kuzmuk and Schook 2011; Lunney 2007). With the help of next generation genome editing tools such as TALENs and CRISPR/Cas9, production of transgenic large animals has become much more effective in the past decade. In SCNT, transfected donor cells with the gene of interest or precisely modified genome can be screened and confirmed before nuclear transfer; thus 100% of the resulting offspring will be transgenic. Therefore, producing genetically modified large animals using SCNT technology is more efficient than using pronuclear DNA/RNA-microinjection, which has generally been used for the production of transgenic mice and other small model animals. Somatic cell nuclear transfer combined with TALENs or CRISPR/Cas9 has been successfully used in multiple species of farm animals including pigs (Xin, Yang et al. 2013; Zhou, Xin et al. 2015), cattle (Jeong, Kim et al. 2016), sheep (Crispo, Mulet et al. 2015) and goats (Ni, Qiao et al. 2014). Increasing attention has been paid to transgenic goats due to their great potential in agriculture and as a bioreactor for the production of recombinant proteins. Transgenic goats can be efficiently used for producing valuable therapeutic protein due to their high milk production, short generation interval and low incidence of disease (Huang, Huang et al. 2007; Liu, Luo et al. 2013). The very first pharmaceutical product derived from transgenic animals was produced in transgenic goats modified to produce therapeutic proteins in their milk. The product, ATryn (an antithrombrin) received regulatory approval in the EU in 2006 and in the U.S. in 2008. Our previous study showed that the CRISPR/Cas9 system combined with SCNT technology is a highly efficient strategy for goat genome editing (Ni, Qiao et al. 2014). In all, although cloning efficiency is low, it is still a promising technology especially for genetic engineering with potential applications in both agricultural and biomedical fields.

SCNT reprograms somatic cells to a pluripotent state and thus has tremendous potentials in regenerative medicine. The reprogramming process of a cloned couplet represents the reversal of a somatic cell from a differentiated state to a pluripotent state, giving cell an ability to differentiate into multiple cell types. Therefore, patient-specific pluripotent stem cells could be generated by SCNT upon the completion of nuclear reprogramming. Theoretically, embryonic stem cells (ESCs) can be derived from the cloned embryo. Since these stem cells are genetically identical to the patient, immune rejection would be avoided when transplanting the differentiated therapeutic cells back to the same donor. This perspective of SCNT concept is known as ‘therapeutic cloning,' which can be served as a method to derive human ESC lines for research use and ultimately for therapy. Mouse and monkey ESCs have been successfully generated using SCNT (Byrne, Pedersen et al. 2014; Munsie, Michalska et al. 2000). However, generation of patient-specific ESCs from human SCNT has been technically and ethically challenging. The number of cloned embryos that could develop to blastocyst in human SCNT is extremely low and only a few groups were able to create ESCs from SCNT so far (Chung, Eum et al. 2014; Lee, Chung et al. 2016; Tachibana, Amato et al. 2013).

Over the past decades, induced pluripotent stem cells (iPSCs), which closely resemble ESC biologic and phenotypic characteristics, have been generated in both mice and humans through reprogramming of somatic cells via the introduction of specific pluripotent factors (Oct4, Sox2, Klf4 and c-Myc; Takahashi and Yamanaka 2006; Yu, Vodyanik et al. 2007). This demonstrated that generation of iPSCs might be a more practical approach than the generation of ESCs from SCNT. However, concerns about the safety of iPSCs have been raised due to the compromised genomic integrities (Gore, Li et

al. 2011; Hussein, Batada et al. 2011; Mayshar, Ben-David et al. 2010) and the immunogenicity of these cells (Zhao, Zhang et al. 2011). Residual epigenetic signature characteristics from donor cells, which could restrict their differentiation, were found in mouse iPSCs. In contrast, mouse ESCs derived from SCNT showed no such epigenetic memories. Therefore, it is important to study the induced reprogramming by oocytes using SCNT to fully understand the somatic reprogramming mechanism, which would also help unravel the important proteins and factors that facilitate reprogramming in the oocytes and further improve the efficiency and safety of iPSC generation. Contrariwise, new factors revealed in the oocytes that are critical in the reprogramming process may be validated in the iPSC system for further understanding of the mechanism.

Overall, SCNT has enormous potential application as an essential tool for the generation of genetically modified animals and a promising technique for human therapeutics. However, SCNT has a very poor efficiency due to the extraordinary demands placed on the cytoplasmic donor in reprogramming a somatic nucleus. Only 1–5% of cloned embryos transferred to recipient animal are able to develop to term into offspring. SCNT is a complex procedure. Thus, multiple factors could affect its efficiency, including oocyte quality, donor cell types, timing and method of oocyte activation, embryo culture, etc. Failure to reprogram the donor genome is the main reason for the low efficiency of cloning (Rodriguez-Osorio, Urrego et al. 2012). The cytoplasmic donor plays the major role in reprogramming of epigenetic status of the incoming somatic cell nucleus. Oocytes provide mRNA, nutrition and substances that are required during early embryonic development (Yu, Mai et al. 2008). Thus, oocyte quality is generally regarded as the most important factor in SCNT. Having a better

understanding of oocyte developmental and reprogramming mechanism as well as optimizing each step of SCNT is necessary to improve cloning efficiency and further our knowledge of regenerative biology.

Developmental Competence of Oocytes

Many factors affect embryonic development and contribute to the low efficiency of SCNT. The quality, or developmental competence, of the oocytes is the ability of an oocyte to undergo maturation, fertilization and development to blastocyst stages or live offspring and has been generally accepted as a key factor that determines the success of an embryo developing (Duranthon and Renard 2001). 1.6 million couples in America fail to conceive due to the poor oocyte quality (Krisher 2004). Oocyte developmental competence is established as oocytes grow and mature. It is believed that *in vitro* maturation, or other assisted reproductive technologies disturb this process and reduce the competence of oocytes. Previous studies in multiple species demonstrated that, even though the offspring can be produced using *in vitro* matured oocytes, the overall developmental efficiency of these oocytes are lower than the *in vivo* matured oocytes (Curnow, Ryan et al. 2010; Gendelman and Roth 2012; Johnston, Parrish et al. 1994; Thompson 1997). This is probably due to the fact that the nuclear maturation of oocytes alone is not sufficient to complete developmental competence, a variety of factors in the oocyte cytoplasm acquired during oocyte growth and maturation are required in this process as well. Completion of the events relevant to developmental competence in oocyte cytoplasm is independent of nuclear maturation and is generally regarded as cytoplasmic maturation. Oocytes that fail the completion of cytoplasmic maturation are considered of bad quality and not able to achieve normal developmental processes

(Krisher 2004). Compared to nuclear maturation, which is the resumption of meiosis, cytoplasmic maturation is more complex to define. The cellular mechanisms how an oocyte acquires developmental competence remain unclear.

Although oocyte quality is greatly determined by oocyte cytoplasmic maturation, there are no methods capable of measuring completion of this process. As mentioned earlier, while the oocyte is acquiring competence, there are a number of cellular processes occurring in the oocyte cytoplasm including mRNA transcription and storage, post-transcriptional regulation, protein translation, and post-translational modification (Kang and Han 2011). The transcripts or proteins stored during oocyte growth and maturation are critical for embryo development before and even after zygotic genome activation. Therefore, oocyte quality is affected by the transcripts and proteins stored in the cytoplasm and therefore, identification of differentially expressed genes could help to better understand a mechanism of oocyte developmental competence acquisition. Communication of the oocyte with surrounding cumulus cells is crucial for oocyte acquisition of developmental competence. Thus, examining the transcription in the cumulus cells is also important in the investigation of oocyte developmental competence.

Correct regulation of both energy and nutrition metabolism is critical for oocyte developmental competence. Previous studies demonstrated that the ability of embryos and oocytes to regulate their metabolism reduced after *in vitro* culture (Lane 2001; Thompson 1997). Energy metabolism is essential for oocyte maturation due to the fact that all dynamic processes during this phase require energy which is produced from various substrates including carbohydrates, amino acids and lipids (Collado-Fernandez, Picton et al. 2013; Songsasen 2012). During oocyte maturation, oxidative metabolism

provides the major energy source to fulfill these essential cellular processes (Biggers, Whittingham et al. 1967; Rieger and Loskutoff 1994). Oxidative metabolism is associated with glucose metabolism, which is essential in the regulation of meiosis in oocytes. It is believed that glycolytic activity is positively correlated with oocyte developmental competence (Krisher and Bavister 1999; Xie, Wang et al. 2016). *In vivo* maturated oocytes that are confirmed to have higher developmental competence have more active glucose metabolism (Krisher 2004). Glucose metabolism is also believed to be important in the production of NADPH used in cellular processes and this involvement in the pentose phosphate pathway confirms that glucose metabolism has a fundamental role in regulating the nuclear maturation and maintaining the oocyte developmental competence (Xie, Wang et al. 2016). In oocytes, energy production and the level of reactive oxygen species (ROS) are closely connected (Dumollard, Duchen et al. 2007). Abnormalities in glucose metabolism is associated with oxidative damage, oxygen radical formation, and decrease of intracellular glutathione (GSH), all of which could lead to a reduction in developmental competence (Yoshida, Ishigaki et al. 1993). Metabolic influence is critical on oocyte quality and could be controlled by multiple factors including nutrition in the environment, which suggests that the optimal *in vitro* culture condition that meets metabolic requirement is crucial for oocytes to acquire developmental competence (Absalón-Medina, Butler et al. 2014).

Recent studies of oocyte developmental competence and its maintenance during early embryonic stages have been focused more on mitochondrial function. Mitochondria play a key role in cytoplasmic activity. Mitochondria contain their own genetic material (mtDNA) that are maternally inherited. Mitochondrial DNA copy number expands in

oocyte cytoplasm during oogenesis and its replication is arrested during early embryogenesis until the pre-implantation stage, thus the mitochondrial content of oocyte is sufficient to support the embryo development. High mitochondria content increases the developmental competence of oocytes regarding fertilization outcome (Santos, El Shourbagy et al. 2006). Previous studies have also demonstrated that maternal age and oocyte mtDNA copy number are negatively correlated and oocytes derived from patients with ovarian inefficiency have less mtDNA (De Boer, Jansen et al. 1999; May-Panloup, Chretien et al. 2005). Mitochondria are active in oxidative phosphorylation and provide the major source of ATP in mammalian oocytes and embryos (Dumollard, Duchen et al. 2007). Mitochondrial dysfunction increases ROS, interrupts spindle formation in meiosis, and leads to oocyte and embryonic death (Navarro, Liu et al. 2004). Therefore, determining the mitochondrial function during development holds great promise for treatment for infertility caused by reduced oocyte competence.

Overall, the cellular mechanism of oocytes acquiring developmental competence is poorly understood. Comparative approaches have generally been used to study how certain possible cellular interactions are related to oocyte developmental potentials. By comparing oocytes with high and low developmental competence, we can not only detect the key players in determining the oocyte quality but also identify the potential biochemical markers that are associated with oocyte developmental competence. Oocyte population of different developmental potential could be decided by traditional assessing parameters, for example *in vivo*-matured and *in vitro*-matured or large- and small follicle-derived. Understanding the mechanisms involved in oocyte developmental competence holds the promise to solve human female infertility and increase the efficiency of ARTs.

Oocyte Quality Assessment

The quality of oocytes collected for assisted reproductive technologies varies in the aspects of viability, integrity and maturation and later embryo development. Oocyte developmental competence is essential for the success of ARTs. There is however a lack of efficient and rapid method to evaluate it. Evaluation of oocyte quality based on morphology remains the major approach in predicting the oocyte developmental potential in the ARTs including IVF, ICSI, and SCNT. Certain morphological characteristics indicate whether or not the oocyte has a high likelihood to develop normally. However, morphological selection is subjective and the criteria of grading oocyte quality are not well defined. There is a general need for non-invasive and high throughput oocyte quality parameters that allow the early selection of competent oocytes in a quick and simple manner. Different non-invasive selecting approaches have been used to determine oocytes with high developmental potential, which includes separating oocytes by oocyte diameter, follicle size, brilliant cresyl blue (BCB) test or even biomarkers of cumulus cells. Other intrinsic parameters, such as mitochondrial status, GSH and ROS activity, have also been used as an indicator of oocyte developmental competence. The approaches to detect intrinsic parameters are usually invasive. However, these cellular and molecular predictors are demonstrated to reflect the oocyte developmental competence more objectively and precisely and can be used for optimizing non-invasive selecting approach and culture condition.

There are no comprehensive approaches available that can precisely measure the quality of oocytes. Further understanding of the differences of molecular and cellular

levels within individual oocytes could provide us a more accurate way to predict oocyte potential in development.

Morphological criteria for oocyte quality

Morphological assessment is the most basic method to evaluate oocyte viability and competence. In most mammals, oocytes in antral follicles are surrounded by compact layers of cumulus cells, which form the cumulus-oocyte complexes (COCs). Therefore, the evaluation of oocyte morphology before IVM is difficult due to the presence of cumulus cells. The quality of oocytes is usually evaluated by examining the COC structure. The establishment of oocyte developmental competence is believed to depend on the bi-directional communication between the oocyte and cumulus cells. Oocytes that are surrounded by more cumulus cells are more viable and less apoptotic. The cumulus cell density is positively correlated with oocyte maturation and fertilization capability (Hashimoto, Saeki et al. 1998; Lourenço, Sousa et al. 2014). The COCs with complete compact multilayered cumulus cells are generally considered to have better quality than the COCs with none or only a layer of cumulus cells. Therefore, only the oocytes with 3-4 layers of compact cumulus cells are typically selected for *in vitro* maturation. Previous studies in several species created an oocyte grading system based on the cumulus cell density (Dutta, Li et al. 2016; Khurana and Niemann 2000).

After *in vitro* maturation, cumulus cells are removed from the oocyte. Therefore, more accurate assessments of oocyte morphological features can be conducted, which include the assessment of oocyte size, cytoplasm, perivitelline space and zona pellucida. Oocyte cytoplasm is evaluated according to the coloration and distribution of organelles. A previous study in bovine demonstrated that oocytes with dark cytoplasm have greater

development potential after *in vitro* fertilization than light-colored cytoplasm, which is possibly due to the fact that dark color of cytoplasm indicates an accumulation of lipids and light color is caused by the low density of organelles (Nagano, Katagiri et al. 2006). In addition, oocytes with large granules in the periviteline space showed poorer developmental competence after fertilization compared to those without granules. Zona pellucida thickness is considered to affect sperm penetration in IVF. However, another human study showed the developmental competence was not compromised using the oocytes with undesired morphological characteristics. In this study, the fertilization rate, embryo quality, and implantation rate were not affected by oocyte morphology after ICSI (Balaban, Urman et al. 1998).

Oocyte selection based on morphological features is tricky and insufficient. However, due to its simplicity, the morphological assessment remains the primary approach to increase ART efficiency.

Follicle size

Oocyte selection based on the follicle size is a non-invasive approach that assesses oocyte quality using an extrinsic factor. Compared to a morphological assessment, the measurement of follicle size is more objective. This approach is based on the assumption that the size of the follicles from which the oocytes are derived is positively correlated with the oocyte maturational and developmental competence.

Follicle size measurement has been commonly used in bovine oocyte collection, where oocytes are only aspirated from follicles with decent size (standards vary from lab to lab). In a bovine study, the blastocyst rate obtained from oocytes derived from follicles over 6mm was significantly higher compared to those from 2-6mm follicles (65.9% vs.

34.3%, Lonergan, Monaghan et al. 1994). Another bovine study showed that oocytes from follicles smaller than 2 mm have the ability of fertilization, however, are not capable of cleaving beyond the 8-cell stage (Pavlok, Lucas-Hahn et al. 1992). In goat, oocyte from small (2-3mm) and medium (3.1-5mm) follicles yielded a significantly lower blastocyst rate than oocyte from large (>5mm) follicles (Crozet, Ahmed-Ali et al. 1995). Similar data were observed in sheep (Cognie, Benoit et al. 1998), and buffalo (Raghu, Nandi et al. 2002). Additionally in goats, oocytes from large follicles have greater maturation rate after IVM and blastocyst development rate after IVF than oocytes from small follicles. Therefore, it is believed that developmental competence of oocytes is acquired progressively during follicle growth and that only the oocytes isolated from large antral follicles have the ability to progress to the blastocyst stage following *in vitro* culture. Follicle size characterizes the developmental stage of the follicle and could be a straightforward indicator of the advancement of developmental competence of the oocyte within that follicle.

Brilliant cresyl blue staining

Glucose-6-phosphate dehydrogenase (G6PDH) is an important protein enzyme synthesized in oocytes during the growth phase of oogenesis. This enzyme plays a role of the rate controlling the pentose phosphate pathway (PPP) by catalyzing the chemical reaction

D-glucose 6-phosphate + NADP+ ⇌ 6-phospho-D-glucono-1, 5-lactone + NADPH + H+.

A close relationship between PPP activity and oocyte meiotic and cytoplasmic maturation was confirmed in bovine COCs (Gutnisky, Dalvit et al. 2014). The PPP has

two major metabolic roles: (1) to produce NADPH, which is a cofactor in anabolic reaction; and (2) to yield ribose 5-phosphate, which is the nucleotide precursor. Thus, G6PDH is crucial in the various oocyte cellular processes such as synthesis of fatty acid, steroids, and nucleotides. Furthermore, NADPH reduces oxidized glutathione. Thus, G6PHD also plays a key role in protection against reactive oxygen species (ROS) and reduction of glutathione (GSH) content (Berg, Tymoczko et al. 2002).

The activity of G6PDH is intense in growing oocytes, but decreases when oocytes are fully grown (Ericsson, Boice et al. 1993). Fully-grown oocytes are generally more competent than the growing oocytes due to the fact that more maternal factors and nutrients essential for oocyte maturation and early embryonic development are stored in fully-grown oocytes. Previous studies in a variety of species have shown that G6PDH-activity is negatively associated with developmental competence of oocytes (Abazari-Kia, Mohammadi-Sangcheshmeh et al. 2014; Alm, Torner et al. 2005; Catalá, Izquierdo et al. 2011; Manjunatha, Gupta et al. 2007; Rodrıguez-González, Lopez-Bejar et al. 2002). Therefore, G6PDH activity in oocytes has been regarded as the indicator of developmental competence.

The brilliant cresyl blue (BCB) stain is an electron acceptor and has been used to measure the level of G6PDH activity in the oocytes (Tian, Braunstein et al. 1998). Because G6PDH converts BCB dye to colorless, BCB staining has been used as a marker of G6PDH activity. BCB staining has been used to differentiate between fully-grown oocytes and growing oocytes. Due to the high level of active G6PDH in oocytes undergoing growth, the BCB dye is reduced to colorless (BCB-). Fully-grown oocytes remain blue (BCB+) due to the decreased amount of cytoplasmic G6PDH-activity. BCB

staining has been used for oocyte selection in large animals and previous studies showed that BCB+ oocytes were larger and more competent in maturation and development than BCB− oocytes. However, not all BCB+ oocytes are able to successfully achieve maturation and development. The groups divided by BCB staining may not constitute a homogeneous population. BCB staining is not sufficient enough to identify oocytes that are competent for embryo development. However, BCB staining remains a practical oocyte selection approach because it is a non-invasive, non-perturbing and rather objective. Studies on the factors that determine the developmental success of BCB- oocytes will help us identify more specific criteria for oocyte selection (Wu, Liu et al. 2007).

Optimizing Oocyte and Embryo Culture System

Higher incidence of developmental abnormalities was observed during the procedure of ARTs for production of embryos compared to natural pregnancy. Although the precise cause of such developmental abnormalities remains unclear, the culture environment of oocytes and pre-implantation embryos is demonstrated to play a critical role in the development process (Behboodi, Anderson et al. 1995; Farin and Farin 1995; McEvoy 2003; Wilson, Williams et al. 1995). Previous studies in various species have shown that one of the major factors affecting oocyte and embryo developmental competence is the origin of the oocyte by comparing the oocyte maturation and embryo development *in vivo* and *in vitro* (Izquierdo, Villamediana et al. 2002; Motlik, Crozet et al. 1984; O'Brien, Catt et al. 1997; Rizos, Ward et al. 2002). These results reaffirmed the hypothesis that oocyte maturation and embryo culture environment is critical in determining the oocyte developmental competence for the *in vitro* production of

embryos. Oocyte growth, maturation, and acquisition of developmental competences relies on the microenvironment of ovarian follicles and the maternal signaling pathway between oocytes and surrounded somatic cells. The mechanism underneath is complex and still poor understood, which made it very difficult to identify the crucial factors that are required for oocytes to achieve the competence for further development (Zuccotti, Merico et al. 2011). Mimicking the *in vivo* condition of oocyte and embryo development is the main strategy during *in vitro* culture to improve the efficiency of ARTs. *In vitro* culture conditions have constantly been enhanced by optimizing the media formulations for both IVM and *in vitro* embryo culture (*in vitro* culture, IVC).

Oocyte maturation environment is essential for the production of embryos with good quality and high yield and is one of the key factors in determining the oocyte developmental competence. The follicular fluid that COCs are enveloped with during antral follicle stage contains a variety of maternal factors and nutrients including proteins, cytokine/growth factors, metabolites, hormones, steroid, mRNA transcripts (Wrenzycki and Stinshoff 2013). The composition of follicular fluid varies through each follicle and the determining components of oocyte competence in it remain unknown. Therefore, understanding of the intra-follicular condition is important for improving oocyte selection and *in vitro* maturation. Some of the follicular fluid components are believed to be more important than others and generally used in the IVM media.

As the main energy source, adequate supply of glucose is required for oocyte metabolism. The oocyte glucose utilization capability correlates positively with subsequent embryo development. Glucose is often added to maturation medium, which is demonstrated to facilitate the meiotic resumption, embryo cleavage and *in vitro*

development (Krisher and Bavister 1998; Rose-Hellekant, Libersky-Williamson et al. 1998). However, glucose has an adverse effect on oocyte developmental competence if the concentration is too high, which is possibly due to the decreased GSH levels and increased generation of intracellular ROS (Hashimoto, Minami et al. 2000).

Gonadotropin additives FSH and LH are commonly supplemented to maturation medium to induce nuclear and cytoplasmic maturation, and cumulus cell expansion. FSH stimulates the follicle development and induces steroidogenesis (progesterone and estradiol). FSH retards nuclear maturation, perhaps thereby enhancing subsequent developmental competence by allowing more time for cytoplasmic maturation to occur before fertilization. LH surge is believed to induce the production of progesterone, prostaglandin and epidermal growth factor (EGF), which subsequently mediates the cumulus cell expansion and meiosis resumption. Because FSH and LH receptor (FSHR and LHR) proteins and mRNA are expressed by the cumulus cell, it is important to culture COCs for the gonadotrophins to act *in vitro.*

Steroid hormones also play a crucial role in the maturation of mammalian oocytes *in vivo* and *in vitro*. In a previously published study, the best development of embryos was obtained from the oocytes matured in the medium supplemented with gonadotropins (FSH and LH) and either estradiol or progesterone, while the control oocytes matured without steroid hormones gave the lowest development rate (Zheng, Si et al. 2003). Furthermore, COCs secrete testosterone during *in vitro* maturation (Schoenfelder, Schams et al. 2003). In a bovine study, the addition of exogenous testosterone to oocyte maturation medium also improved their developmental competence followed by IVF (Dieleman, Kruip et al. 1983). Contradictory results were observed in a mouse study that

the exposure of murine oocytes to testosterone during maturation significantly reduces the maturation rate and embryonic developmental competence (Anderiesz and Trounson 1995).

A number of studies were performed to improve *in vitro* culture media by supplementing them with components that might be beneficial for the acquirement of oocyte and embryo developmental competence. These components include energy substrates, amino acids, growth factors, albumin and serum (Çevik, Taş et al. 2009; Choi, Lee et al. 2002; Lonergan, Carolan et al. 1996; Sirisathien, Hernandez-Fonseca et al. 2003).

Growth factors such as EGF and IGF-1 have been used in *in vitro* maturation media and improved maturation rate and blastocyst formation rate following IVF and SCNT (Moreira, Paula-Lopes et al. 2002; Wadhwa, Kunj et al. 2009; Wang, Xiong et al. 2012; Wasielak and Bogacki 2007). EGF is believed to improve the developmental potential of oocytes. EGF is also demonstrated to act on both oocytes and the surrounding cumulus cells since EGF receptors were found in both cell types. IGF-I induces the mitosis of the surrounding somatic cells, which contributes to cumulus cell expansion during oocyte maturation. IGF-I also increases the mitosis in the embryonic development. Thus, growth factor IGF-I is often used in *in vitro* culture medium for oocyte maturation and embryo development. Cysteamine is another chemical compound often supplemented to IVM and IVC media. Cysteamine is a degradation product of amino acid cysteine and a critical factor in supplying cysteine for GSH synthesis (De Matos, Herrera et al. 2002). The intracellular GSH content in oocytes is crucial for oocyte competence due to the crucial role of GSH in preventing cellular damage from ROS. The

synthesis of GSH depends on the availability of cysteine. However, cysteine is not stable outside the cell. Thus precursor amino acid cysteamine available in the culture medium could contribute to the cysteine supply and control the GSH synthesis. Previous studies in various species confirmed cysteamine is beneficial for oocyte maturation and embryo development.

Tissue Culture Medium 199 (TCM-199) and synthetic oviduct fluid (SOF) medium are the basic culture medium for IVM and IVC, respectively. Numerous studies had been conducted to optimize the culture environment by adjusting the supplementary components of these culture media. It has been demonstrated that the difference in the gene expression profiles of individual oocytes or embryos reflect their developmental competence and potential to produce viable offspring. Gene expression analysis can also reflect the stored mRNA transcripts level in oocytes during the transcriptional quiescent stage. Thus understanding the oocyte or embryo gene expression patterns related to the environmental change might help us understand the underneath mechanism of oocyte growth, maturation and embryonic development, which will eventually unravel the cellular processes that contribute to the acquisition of oocyte developmental competence. In addition, studies of epigenetic profiles of embryos with different origins could also help us reveal the critical factors contributing to developmental competence.

In all, oocyte environment in terms of *in vitro* culture condition affect the embryo development and subsequent blastocyst fate. Optimization of culture condition for IVM and IVP could help us achieve better efficiency of assisted reproductive technologies.

Oocyte Activation

Oocyte activation is the cellular transition from a highly differentiated state of

oocyte into a totipotent state of embryos. Oocytes undergo this transition to finally achieve the capability to further develop into pluripotent embryos. This transition is usually initiated by fertilization when a sperm enters the oocyte (Jaffe 1983). In fertilization, calcium signaling triggers the initiation of activation. The oocyte activation can also be triggered artificially by chemical or physical stimulation. Different protocols have been invented for artificially activating oocyte including chemical, electrical and mechanical methods (Eftekhar, Janati et al. 2013). Chemical activation is the most commonly used approach among them. Artificial oocyte activation has frequently been used in assisted reproductive technologies such as SCNT and ICSI, with the aim of mimicking physiological activation mechanism taking place during fertilization.

During natural fertilization, oocyte activation consists of a series of events. The rise of intracellular calcium occurring when a sperm enters an oocyte. This event appears to be the universal signal that initiates oocyte activation. The mechanism of how the entry of sperm triggers the Ca^{2+} release stays controversial still. The most common hypothesis is that sperm factors are released into the oocyte upon fusion and subsequently leads to the signaling cascade to occur. Oocyte activation by fertilization is characterized by two basic molecular events: the initial elevation of Ca^{2+} level and a transient Ca^{2+} elevation, which is referred to as calcium oscillation (Krauchunas and Wolfner 2013). In mammals, fertilization instantly results in a rise in cytosolic Ca^{2+} in the oocyte, which is mediated by sperm-specific phospholipase C zeta (PLCζ). After the penetration of the oocyte plasma membrane, the interaction and fusion of the sperm with the membrane cause it to introduce the already-active PLCζ into the oocyte membrane. PLCζ hydrolyzes 4,5-bisphosphate (PIP2) into inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) in

oocytes (Nomikos, Swann et al. 2012; Xu, Kopf et al. 1994). IP3 then binds IP3 receptors on the endoplasmic reticulum (ER) in the cytoplasm, which elicits Ca^{2+} release from internal stores of the endoplasmic reticulum and then results in an overall rise of cytosolic Ca^{2+} in oocytes. DAG may also contribute to Ca^{2+} release from ER by stimulating a pathway leading to the production of cyclic ADP ribose (cADPR), which releases the Ca^{2+} from ryanodine receptors on the ER. During fertilization, calcium level in the oocyte continues to rise and fall in an oscillatory pattern and, in mammals, the Ca^{2+} oscillation lasts for several hours (Stricker 1999). The downstream molecules that are regulated by calcium oscillation during oocyte activation are still not fully elucidated. Some calcium-dependent effector molecules have been demonstrated to play an important role in oocyte activation. Phospholipid-dependent protein kinase C (PKC) is activated after fertilization (Viveiros, Hirao et al. 2001). PKC activity is believed to promote cortical granule exocytosis, second polar body extrusion and remodeling of the oocytes internal cytoskeleton. The release of Ca^{2+} also results in the increase of calmodulin-dependent protein kinase II (CAMKII), which inhibits cytostatic factor (CSF) by inhibiting the anaphase promoting complex (APC) within the oocyte and then allows the oocyte to resume meiosis II (Hansen, Tung et al. 2006). Previous studies demonstrated the increase in intracellular Ca^{2+} level is required for the oocyte to fully complete meiosis. On the contrary, some studies have shown that the lack of subsequent calcium oscillations seems not to affect the completion of oocyte activation (Rogers, Halet et al. 2006; Suzuki, Yoshida et al. 2010). Overall, the release of Ca^{2+} initiated by fertilization leads to the activation of subsequent molecules that cause the oocyte to undergo a number of cellular and molecular changes. This eventually allows the oocyte to achieve the pluripotent state.

In nature, parthenogenesis is a reproduction strategy found in some vertebrate species, in which embryos can be activated from oocytes with no sperm involved, and offspring with no paternal inheritance can be generated. In this case, fertilization is not required; instead, changes in the ionic environment, pH, or mechanical stimulation trigger activation (Horner and Wolfner 2008). A common mechanism of this process is the mechanical stress on oocyte during ovulation. It is believed that the mechanical stimulation causes an increase of Ca^{2+} content in oocytes. One possible activation mechanism is the mechanical stimulus that can directly allow the Ca^{2+} influx by activating a stretch-activated calcium channel (Reifarth, Clauss et al. 1999). Another possible mechanism is that the activation of mechanosensitive PLCζ generates IP2 to release Ca^{2+} (Boitano, Sanderson et al. 1994; Brophy, Mills et al. 1993). In addition, exposure to new ionic environment triggers oocyte activation in some marine animals like shrimp (Lindsay, Hertzler et al. 1992). For oocyte activation in both fertilization and parthenogenesis, the Ca^{2+} signaling is the critical initial step of the oocyte activation. Further understanding of how an oocyte becomes developmentally competent through oocyte activation is important in the solution of diagnosis and treatment of female infertility and improving the efficiency of ARTs.

In mammals, parthenogenesis does not occur naturally, but mammalian oocyte can be successfully activated using a variety of stimulation *in vitro* to mimic the oocyte activation taking place during fertilization. Parthenogenetic activation of oocytes has been successfully conducted in numerous species (e.g., mice (Barton, Adams et al. 1985), sheep (Loi, Ledda et al. 1998), cattle (Fukui, Sawai et al. 1992), pigs (Kure-Bayashi, Miyake et al. 2000), rabbits (Ozil 1990)). The extent of how well the parthenogenetic

embryos can develop varies among species. Mouse and pig parthenotes are able to develop beyond the post-implantation stage when transferred back to recipient female (Kaufman, BARTON et al. 1977; Surani, Barton et al. 1984, Zhu, King et al. 2003). Primate parthenotes have only been shown to develop up to implantation stage (Marshall, Wilton et al. 1998).

Parthenogenetic activation of oocytes has been commonly used for studying the mechanism of human embryonic development since it is considered less ethically controversial. Parthenotes are very much comparable to embryos during the *in vitro* development to blastocysts. Thus, parthenogenetic activation is also a useful tool to investigate the effect of different treatment options, culture conditions and exposure of chemicals to oocytes or embryos on embryonic development.

Artificial oocyte activation is commonly used in basic research studying reproductive technologies. A number of assisted reproductive technologies bypass some crucial stage in normal fertilization. Therefore, artificial activation of the oocyte is required for the success of these ARTs such as ICSI and SCNT. Multiple strategies have been used to activate oocyte artificially. Previous studies showed that mechanical stimulation alone is capable to activate oocyte in ICSI but not efficiently (Shirazi, Ostad-Hosseini et al. 2009). The most commonly used protocol to induce oocyte activation consists of an electrical or chemical stimulus such as calcium ionophore treatment to mimic the intracellular calcium wave. Treatment with calcium ionophores such as ionomycin can increase the Ca^{2+} permeability of the cell membrane, which allows extracellular Ca^{2+} to flow into the cell, and therefore activate oocytes (Swann and Ozil 1994). Electrical stimulation is often used in PA and cloning. The electric field induces

the movement of charged proteins in the cell membrane, which forms the pores in the membrane, and therefore allows the extracellular Ca^{2+} in the culture medium flow into the oocyte through these pores (Yanagida, Katayose et al. 1997). This induced Ca^{2+} influx subsequently activates the oocyte.

Summary

Due to the unique and fundamental role of the oocyte in embryonic development and genomic reprogramming, a number of research efforts have been focused on studies of its developmental mechanism. Decreased developmental competence of the oocyte causes many fertility issues in the process of fertilization, implantation, and gestation. Further understanding of how the oocyte acquires developmental competence through the process of growth, maturation, and activation is important for improving the oocytes quality. Oocyte quality has also been regarded as one of the determining factors of the ART success. Due to the great potentials that ARTs holds in the clinical, biomedical and agricultural field, improvement in its efficiency is urgently needed and could be achieved by increasing developmental competence of oocytes. The overall oocyte developmental efficiency could be improved by efficiently selecting the good-quality oocytes from the pool. Efficient and non-invasive methods are desired for oocytes selection. Environment surrounding the oocyte also has a role in determining the oocyte's ability to achieve developmental competence. Characterizing the mechanism of oocyte maturation and development process on the cellular and molecular levels could lead to identification of potential biomarkers of oocyte quality that would assist with creating more enhanced oocyte/embryo culture conditions.

Research Goals and Impacts

The focus of this work consists of two primary objectives; first to characterize the cellular and molecular differences between oocytes with different developmental competence, and second to identify an efficient method to improve oocyte's ability to develop. The overall hypothesis is that characteristic differences in good-quality and bad-quality oocytes could be identified by comparing these two oocyte populations, and will provide us intrinsic information of oocyte development mechanism and help increase the oocyte and embryo development success.

In the first objective, two independent studies had been done; the first study (chapter II) was designed to determine whether the follicle size (from which the oocytes were derived from) affects the developmental competence following SCNT and identify the molecular differences between oocytes from follicle with different sizes by analyzing the gene expression profiles. The second study (chapter III) was aimed to determine the molecular and sub-cellular characteristics of oocytes with different developmental competence based on the G6PDH-activity. We expected to identify the key factors in determining oocyte quality and explain what caused the decrease in oocyte developmental ability. The third study (chapter IV) was designed to compare the behavior of oocytes submitted to different *in vitro* maturation treatments. This study is expected to reveal the effect of each culture condition on oocyte acquiring developmental competence and provides information on the molecular level, which could help understand how the nutrients in culture medium facilitate oocyte maturation and eventually benefit optimizing the culture environment for oocytes. The last study (chapter V) aims to improve oocyte developmental competence by directly injecting the oocyte

cytoplasm with a developmental important gene (CPEB), which has been confirmed up-regulated in good quality oocyte based on our previous study.

This work not only advanced our understanding in how oocytes acquire developmental competence and identified potential key factors and biomarkers for oocyte quality but also revealed specific promising methods of improving oocyte quality. This knowledge offers promise for many applications in the treatment of infertility and improvement of ART efficiency.

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CHAPTER II

OOCYTES FROM SMALL AND LARGE FOLLICLES EXHIBITS SIMILAR DEVELOPMENT COMPETENCE FOLLOWING GOAT CLONING DESPITE THEIR DIFFERENCE IN MEIOTIC AND CYTOPLAMIC MATURATION[1]

Abstract

Reduced developmental competence following IVF has been reported using oocytes derived from small follicles in several species including cattle, sheep and goats. No information is currently available about the effect of follicle size of the cytoplast donor on *in vivo* development following somatic cell nuclear transfer (SCNT) in goats. Oocytes collected from large (≥3mm) and small follicles (<3mm) were examined for maturation and *in vivo* developmental competence after SCNT. Significantly greater maturation rate was observed in oocytes derived from large follicles compared to that of small follicles (51.6% and 33.7%, $P < 0.05$). Greater percent of large follicle oocytes exhibited a low glucose-6-phosphate dehydrogenase (G6PDH) activity at germinal vesicle stage compared to small follicle oocytes (54.9% and 38.7%, $P < 0.05$). Relative mRNA expression analysis of 48 genes associated with embryonic and fetal development revealed that three genes (MATER, IGF2R and GRB10) had higher level of expression in metaphase II (MII) oocytes from large follicles compared to oocytes from small follicles. Nevertheless, no difference was observed in pregnancy rates (33.3% vs. 47.1%) and birth rates (22.2% vs. 16.7%) after SCNT between the large and small follicle groups). These

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results indicate that MII cytoplasts from small and large follicles have similar developmental competence when used in goat SCNT.

Introduction

Somatic cell nuclear transfer (SCNT) has nearly 20 years of history and has been successfully used for cloning of various livestock species (Campbell, McWhir *et al.* 1996; Keefer, Keyston *et al.* 2002; Polejaeva, Chen *et al.* 2000; Tian, Xu *et al.* 2000). One of the greatest benefits of this technology is its ability to provide a cell-mediated platform for livestock genetic engineering. Somatic cells can be genetically modified *in vitro* and screened to identify the cells containing the gene of interest, which then are used to generate transgenic animals via nuclear transfer. Despite the nearly two-decade history, SCNT efficiency remains low with typically only 1-4% of manipulated embryos developing to term.

Due to the low efficiency of SCNT, a large number of cytoplast donors are required for the procedure. A local abattoir is the best option to obtain goat ovaries for oocyte collection on a regular basis. However, goat ovaries from abattoirs are often derived from prepubertal goats. In prepubertal goats, most of the oocytes come from the follicles with a diameter smaller than 3 mm (Martino, Mogas *et al.* 1994), making it difficult to release the cumulus-oocyte complexes (COCs) by traditional aspiration. For this reason, oocytes are routinely obtained by slicing the surface of the ovary, resulting in oocytes with a heterogeneous diameter, different COC morphology and varying stages of development. Low SCNT efficiency is believed to be attributed to the quality and competence of the cytoplast donor, which is generally accepted as a key determining factor for the success of embryonic development (Krisher 2004). The size of the follicles

from which the oocytes are collected is one of the factors that influence the maturational and developmental competence using assisted reproductive technologies (ARTs) (Bagg MA 2004; Otoi, Yamamoto *et al.* 1997). The existing data indicates that significantly fewer oocytes derived from follicles with a diameter of less than 3mm reached metaphase II after *in vitro* maturation (IVM) either in adult or prepubertal goats (Crozet, Ahmed-Ali *et al.* 1995; Romaguera, Casanovas *et al.* 2010). Furthermore, in cattle (Lequarre, Vigneron *et al.* 2005), sheep (Cognie, Benoit *et al.* 1998), and buffalo (Raghu, Nandi *et al.* 2002) , oocytes from large follicles have greater blastocyst development rates following *in vitro* fertilization (IVF) than oocytes from small follicles. However, to our knowledge, no reports have been published on the effect of the follicle size of the cytoplast donor on the efficiency of goat cloning.

The aims of the present study were to investigate meiotic maturation of goat oocytes obtained from large and small follicles, and compare the ability of cytoplast donors derived from small and large follicles to support embryonic and fetal development to term following goat SCNT. Additionally, the enzymatic activity of glucose-6-phosphate dehydrogenase (G6PDH) was assessed in these two oocyte groups. G6PDH is an enzyme synthesized in oocytes during oogenesis and folliculogenesis. The activity of G6PDH in oocytes has been previously correlated with several indicators of cytoplasmic maturation including cortical granule organization (Silva, Rodriguez *et al.* 2013), activity of the mitochondria, cytoplasmic lipid content and intracellular glutathione level (Catala, Izquierdo *et al.* 2011; Egerszegi, Alm *et al.* 2010; Wu, Liu *et al.* 2007), and therefore could be used as a marker of cytoplasmic maturation and developmental competence. Furthermore, we assessed gene expression levels of 48 genes in metaphase II (MII)

oocytes derived from small and large follicle groups. The selected genes were previously implicated in embryonic and fetal development (Fair, Murphy *et al.* 2004; Leoni, Bebbere *et al.* 2007; Mourot, Dufort *et al.* 2006; Sirard 2001).

Materials and Methods

All animal procedures were approved by and conducted following the guidelines of the Utah State University Animal Care and Use Committee. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

Oocyte collection

Ovaries from prepubertal domestic goats (*Capra aegagrus hircus*) were obtained from a local abattoir (Springville, Utah) and transported to the laboratory within 4 hours after collection. The ovaries were transported at 20-27°C in saline containing 100 U/mL penicillin/streptomycin. Upon arrival to the laboratory the ovaries were washed three times in saline and then three times in Dulbecco's phosphate-buffered saline (DPBS, Hyclone, Logan, UT) containing 100 U/mL 1% penicillin/streptomycin. Cumulus-oocyte complexes were recovered from ovaries in Modified TL-Hepes medium (Lonza, Walkersville, MD) supplemented with 1% fetal bovine serum (FBS; Hyclone, Logan, UT), 100 U/mL penicillin/streptomycin and 30 μg/mL heparin using a modified slicing technique in which only the surface of the ovaries was dissected (Pawshe, Totey *et al.* 1994). First, only large follicles (≥3mm) were dissected and rinsed with Modified TL-Hepes medium. Ovaries were then moved to another dish to dissect the remaining follicles. Oocytes with three or more layers of compact cumulus cells and homogeneous cytoplasm were used. The groups of oocytes derived from large and small follicles

(<3mm) were treated separately throughout the process.

Brilliant Cresyl Blue (BCB) test

BCB staining was conducted to measure the G6PDH activity as previously described (Rodrıguez-González 2002). Immediately after collection, COCs were washed three times in modified DPBS (mDPBS, PBS supplemented with 1 g/L glucose, 36 mg/L sodium pyruvate, 0.5 g/L BSA and 0.05 g/L gentamicin). The oocytes were treated with 26 μM BCB diluted in mDPBS at 38.5°C in 5% CO_2 in air for 30 min and then washed two times in mDPBS. The blue color of BCB dye is reduced to colorless in the oocytes with high G6PDH levels. However, the blue color will remain in the cytoplasm of oocytes containing low levels of G6PDH (Ericsson 1993). We classified the oocytes into two groups according to their cytoplasm coloration. Oocytes with or without blue cytoplasmic coloration were designated as BCB+ (low G6PHD activity) and BCB− (high G6PDH activity).

In vitro maturation

The COCs were washed three times in maturation medium (TCM-199 (Gibco, Grand Island, NY), containing 10% FBS, 10 μg/mL LH, 5 μg/mL FSH, 1 μg/mL estradiol-17β and 0.05 g/L gentamycin) and then incubated in maturation medium for 22 h at 38.5°C in 5% CO_2 in air. The COCs were cultured in groups of 50 in 4-well plates containing 500 μL of maturation medium. After 22 h of culture, cumulus cells were removed from oocytes by vortexing the COCs for 1-2 min in TL Hepes containing 1 mg/mL hyaluronidase. Maturation status was assessed by the presence of a first polar body. Oocytes at this stage are termed MII oocytes.

Somatic cell nuclear transfer

Skin ear biopsies were collected from a neonatal domestic goat for fibroblasts isolation. The cells were cultured in DMEM/high-glucose medium (Hyclone, Logan, UT) supplemented with 15% FBS and 100 U/mL penicillin/streptomycin. The fibroblasts were grown to 80-90% confluence and used as nuclear donor cells for SCNT after 24-48 h of serum starvation (0.5% FBS). The first polar body and metaphase plate were removed from an oocyte and a single donor cell was subsequently transferred into the perivitelline space of the enucleated oocyte. Fusion of somatic cells with oocyte cytoplasm was performed in the 0.28 M sorbitol fusion medium containing 0.1 mM Calcium, 0.5 mM magnesium, 0.5 mM Hepes and 1 mg/mL BSA) by a single DC electric pulse of 1.75 kV/cm for 15 microseconds. Fusion of the donor cell with the oocyte cytoplasm was evaluated by microscopy 30 min after the pulse. Following fusion, embryos were held in G1 medium (VitroLife, Engelwood, CO) supplemented with 3% FBS for 2.0 hours prior to activation. Fused embryos were activated between 27 and 29 h after the onset of maturation by exposure to 5 μM ionomycin for 5 min followed by a four-hour incubation in 2 mM DMAP and 10 μg/mL cycloheximide. Following activation, embryos were cultured under oil in 50 μL droplets of G1 medium for 8-12 h prior to the transfer into the synchronized recipient females.

Recipient synchronization and embryo transfer

Recipient synchronization and embryo transfers were conducted as previously described (Rutigliano, Wilhelm *et al.* 2015). Briefly, SYNCRITE Vaginal Sponges (Animal Health Supplies, Ulladulla, Australia) that contain 40 mg of flurogesterone acetate were inserted vaginally with an appropriate disinfectant and lubricant and then

removed after 10 days. Estrus occurred at 36-48 hours after sponge removal and ovulation usually occured at 12-24 hours after the occurrence of estrus. Twenty-one domestic goats (*Capra aegagrus hircus*) were used as recipients for embryo transfers. All recipient goats were housed in an open-sided barn with free access to food and water. SCNT pregnancies were established by surgically transferring 16±5 embryos into the oviduct of synchronized recipients that showed estrus within 12 h of SCNT. Pregnancy confirmation was done 35±3, 60±3 and 90±3 days after embryo transfer by transabdominal ultrasonography. All animals containing a viable conceptus as determined by the presence of a heartbeat were considered pregnant. After birth, the offspring were allowed to remain with the dams and nurse freely until weaning between 2.5 and 3 months of age.

Single-cell real-time PCR

Real-time PCR was conducted on individual oocytes. Denuded MII oocytes were washed three times and stored individually in 1 μL DNA suspension buffer at −80°C before gene expression analysis. Single-cell gene-expression experiments were performed using Fluidigm's 48.48 quantitative PCR (qPCR) Dynamic Array Integrated Fluidic Circuits (IFC; Fluidigm, San Francisco, CA). Fluidigm BioMark system was used following the manufacturer's recommended protocol. As required by the Fluidigm single cell gene expression protocol, a reverse transcription and specific target amplification (RT-STA) step was performed to each single-cell sample to convert the RNA to cDNA and to enrich target-specific cDNA prior to qPCR. In the preparation for RT-STA, a 200 nM pooled STA primer mix was made by diluting all the 48 pairs of primers in DNA suspension buffer. Each individual oocyte (stored in DNA suspension buffer) was added

with 9 μL of RT-STA solution composed of 5 μL of CellsDirect PCR mix (Invitrogen, Carlsbad, CA), 0.2 μL SuperScript-III RT/Platinum Taq (Invitrogen, Carlsbad, CA), 2.5 μL of 200 nM pooled primer mix and 1.3 μL DNA suspension buffer. In the RT-STA mixture solution, reverse transcription reaction was done at 50°C for 15 min, 95°C for 2 min and cDNA was subsequently pre-amplified for 20 cycles under the following conditions: 95°C for 15 s, 60°C for 4 min. After the RT-STA reactions, 4 μL ExoSAP-IT (ExoI; New England Biolabs, Beverly, MA) was added to every RT-STA reaction in order to remove any unincorporated primers and then placed in the thermal cycler at 37°C for digestion, followed by 15 min inactivation at 80°C. Each reaction was then diluted 5-fold with DNA suspension buffer. The Fluidigm 48.48 chip was primed before use according to manufacturer's instructions (Spurgeon, Jones *et al.* 2008). A 2 μL-aliquot of diluted sample was then mixed with 2.5 μL of the 2X TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA), 0.25 μL of 20X DNA Binding Dye Sample Loading Reagent (Fluidigm, San Francisco, CA) and 0.25 μL EvaGreen DNA Binding Dye (Biotium, Hayward, CA). The 5 μL sample mix solution was then inserted into one of the sample inlets of the primed chip. An assay (primer) mix solution was made for every primer set by using 2.5 μL of the 2X Assay Loading Reagent (Fluidigm), 1.25 μL DNA suspension buffer, and 1.25 μL of each 20 μM forward and reverse primer mix and then individually inserted into one of the "assay" inlets of the primed chip. The chip was then returned to the IFC Controller for chip loading (Load Mix (113x) script). When the Load Mix script had finished, the chip was then placed in the BioMark real-time PCR machine for quantitative PCR following manufacturer's instructions. Cycling parameters included a 5 min initial enzyme activation at 95°C, 35 denaturation/extension cycles

(95°C for 15 s followed by 60°C for 60 s), and a 3 min final extension cycle at 60°C. The melting curve (Tm) was examined for all assays to verify the quality of each amplicon. The real-time PCR data were analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The gene expression data presented in this study reflect the fold change of gene expression of two oocyte groups in target gene expression normalized to the internal control gene (GAPDH).

Experimental design

Experiment 1. Activity of G6PDH in immature goat oocytes derived from large and small follicles.

The effect of the follicle size on the activity of G6PDH in immature oocytes was analyzed. Oocytes derived from large and small follicles were divided into BCB+ and BCB− groups. The BCB+ oocytes percentages were recorded for each oocyte group.

Experiment 2. Effect of follicular size on oocyte maturation and fusion rates and developmental competence in cloned embryos *in vivo.*

Each round of oocyte collection (different dates) was considered an experimental replicate. Maturation rates of oocytes collected from large and small follicles were assessed after IVM. The MII oocytes were then collected and used as cytoplast donors for SCNT. Fusion rates were observed after the fusion of somatic cells with oocyte cytoplasts. In our laboratory, blastocyst development rate *in vitro* following SCNT typically ranges between 10% and 15% (data not presented). Thus, 16±5 reconstructed embryos were transferred to each synchronized recipient. Pregnancy rates and full-term development rates were determined to assess the *in vivo* development of SCNT embryos.

Experiment 3. Effects of follicle size on maternal mRNAs of MII oocytes.

The association between follicle size and expression of developmentally important genes (Table 1) was examined in MII oocytes using real-time PCR assays. The selected genes fell into one of nine functional categories: housekeeping genes, transcription factors, metabolism, growth factor, pluripotency, apoptosis, oogenesis and imprinting (Fig.1). The relative gene expression level of the selected genes was compared between the oocytes originated from large and small follicles. Oocytes from large-follicle and small- follicle groups both consisted of 125 oocytes collected from five separate rounds of IVM. Real-time PCR was conducted on individual oocytes.

Statistical analysis

Each round of oocyte collection or SCNT was considered a replication. The percentage of BCB+ vs. BCB− oocytes between large and small follicles was determined by ANOVA in SAS 9.4 (SAS Institute Inc.). For maturation and fusion rate, data were converted to percentages and tested for homogeneity of variance plotting the residuals. Data were then analyzed by ANOVA with follicle size as the main effect in SAS 9.4 (SAS Institute Inc.). All the experiments were repeated at least three times using different pools of oocytes collected from different dates. Pregnancy rate and full-term development rates including birth rate (live births/ recipients) and cloning efficiency (live births/ embryos) were analyzed by Chi-square with a two-tailed *P* value. The effect of follicle size on the gene expression in oocytes was assessed using Student's t-test. All the results are presented as the mean ± SEM. Effects were considered to be significant when the *P* value was less than 0.05. For gene expression data, the difference between the groups was considered to be significantly different when the following requirements were met: at

least 1.5-fold change in gene expression was observed and *P* value was less than 0.05 (FDR- adjusted).

Results

Experiment 1. Activity of G6PDH in immature goat oocytes derived from large and small follicles.

In total, 336 COCs from large follicles and 1154 COCs from small follicles were collected from 116 ovaries on nine different collection dates averaging 13.06 ± 0.86 oocytes per ovary (10.24 ± 0.85 were derived from small follicles and 2.82 ± 0.12 from large follicles). The percentage of BCB+ oocytes was greater for oocytes obtained from large follicles compared to those from small follicles ($P < 0.05$; Table 2). Therefore, oocytes from small follicles have higher activity of G6PDH compared to the oocytes from large follicles.

Experiment 2. Effect of follicular size on oocyte maturation and fusion rates and developmental competence in cloned embryos *in vivo.*

A total of 121 ovaries were obtained on seven different days for oocyte collections for this experiment. On average, 11.45 ± 0.79 oocytes per ovary were collected (8.45 ± 0.86 from small follicles and 3.00 ± 0.33 from large). Oocytes from large follicles had significantly greater maturation rates than oocytes from small follicles (Table 3; $P < 0.05$). No significant difference in fusion rates was observed between these two groups (Table 3). SCNT pregnancies were produced using oocytes from both large and small follicle groups. In total, 124 and 193 SCNT embryos derived from oocytes of large follicles and small follicles were transferred into 9 and 12 recipients, respectively.

No significant difference was observed in pregnancy rates at day 35 ± 3, 60 ± 3 and 90 ± 3 of gestation between the two groups of oocytes (Table 4). During gestation, 3 out of 5 and 1 out of 3 pregnancies were lost in the small follicle group and large follicle group, respectively. The birth rate and cloning efficiency were not statistically significant between the large follicle and small follicle groups (Table 4).

Experiment 3. Effects of follicle size on maternal mRNAs of MII oocytes.

The relative gene expression results are presented in Figure 1. Forty-four of the analyzed genes showed no statistical difference in relative expression level between oocytes from large and small follicles. One gene in the oogenesis category (*MATER*) and two imprinted genes (*IGF2R* and *GRB10)* were differentially expressed between MII oocytes derived from large and small follicles. The mRNA levels of *MATER, IGF2R* and *GRB10* were more abundant in the oocytes from large follicles compared with those from small follicles with a fold change of 2.87, 1.62 and 2.58, respectively ($P < 0.05$).

Discussion

This is the first study to determine the effect of follicle size of the cytoplast donor on the *in vivo* developmental competence following SCNT in goats. This study also aimed to analyze changes in the relative expression of genes that contribute to development in oocytes derived from two populations of follicles, small (<3mm) and large (≥3mm). In this study, our data are consistent with previous observations that oocytes derived from small follicles yield a significantly lower maturation rate than oocytes derived from large follicles (Crozet, Ahmed-Ali *et al.* 1995; Martino, Mogas *et al.* 1994; Romaguera, Casanovas *et al.* 2010). This result reinforced the fact that the

oocytes derived from large and small follicles compose two distinct populations. We also carried out the BCB staining on these two groups to measure the G6PDH activity of oocytes. G6PDH is an enzyme that is active in the growing oocytes and its activity is decreased in fully-grown oocytes. Studies in pigs (Ericsson 1993), goats (Rodrıguez-González 2002; Rodriguez-Gonzalez, Lopez-Bejar *et al.* 2003; Urdaneta, Jimenez-Macedo *et al.* 2003), cows (Pujol, López-Béjar *et al.* 2004), mice (Wu, Liu *et al.* 2007), and dogs (Rodrigues, Rodriguez *et al.* 2009) showed a negative association between G6PDH-activity and oocyte developmental competence. In this study we have demonstrated that oocytes derived from large follicles have lower G6PDH activity compared with oocytes derived from small follicles, indicating that a greater proportion of the oocytes from large follicles were more developed and have higher quality compared to oocytes from small follicles.

Previous studies showed that oocytes from large follicles have better embryo development rates after IVF in goats compared to small follicles (Crozet, Ahmed-Ali *et al.* 1995; Romaguera, Casanovas *et al.* 2010). In adult goats, Crozet et al. (1995) found a significant difference in the percentage of blastocysts obtained from oocytes recovered from follicles of 2−3 mm (6%), follicles of 3.1–5 mm (12%), follicles > 5 mm in diameter (26%) and from ovulated oocytes (41%) after IVF. In prepubertal goats, it was shown that oocytes derived from follicles larger than 3 mm have significantly greater rates of blastocyst development than oocytes from follicles smaller than 3 mm following the IVF procedure (Romaguera, Casanovas *et al.* 2010). Similar results have been found in other species including cattle, sheep and buffalo (Cognie, Benoit *et al.* 1998; Lequarre, Vigneron *et al.* 2005; Raghu, Nandi *et al.* 2002). However, data on the effect of follicle

size on developmental competence of SCNT embryos in goats are lacking. In cattle, Piedrahita et al. found that cytoplast donors obtained from follicles of 1−3 and 6−12 mm have similar developmental competence *in vitro* and *in vivo* when used in a nuclear transfer procedure (Piedrahita, Wells *et al.* 2002). However, in a study in pigs, embryonic development *in vitro* after SCNT was influenced by oocyte and follicle size (Kim, You *et al.* 2010). The question whether oocytes from small follicles would lead to a lower *in vivo* development following SCNT in goats has not been previously addressed. In this study, we demonstrated that oocytes derived from small follicles of prepubertal goats have similar ability to support embryonic development to term following SCNT as oocytes derived from large follicles. Our results showed that no significant difference in fusion rate occurred between oocytes derived from large and small follicles. Furthermore, no differences were observed in pregnancy and birth rates between these two oocyte groups following SCNT in goats. These results are consistent with the previous observation in cattle (Piedrahita, Wells *et al.* 2002) and demonstrate that while the effect of follicle size on oocyte maturational competence is present, once the oocytes reach metaphase II stage, they have similar ability to support full term *in vivo* development after SCNT regardless of the size of the follicles they derived from.

Unlike previous reports that used IVF procedures (Cognie, Benoit *et al.* 1998; Crozet, Ahmed-Ali *et al.* 1995; Lequarre, Vigneron *et al.* 2005; Raghu, Nandi *et al.* 2002; Romaguera, Casanovas *et al.* 2010), our results do not show any effect of follicle size on the developmental competence of oocytes after SCNT in goats. This inconsistency between the SCNT and IVF results is not fully elucidated. We postulate that artificial oocyte activation employed during SCNT could rescue developmental competence of

small follicle oocytes. The entrance of sperm triggers oocyte activation naturally during fertilization, which allows development to progress. One of the earliest events during this process is an increase in the intracellular calcium concentration. It is believed that calcium signals induce the fundamental events surrounding the activation of development (Miao and Williams 2012). The ability of normal calcium oscillation initiation during the oocyte activation in response to fertilization is gradually acquired during the oocyte growth and maturation (Damiani, Fissore *et al.* 1996; Herbert, Gillespie *et al.* 1997). Oocyte activation can also be artificially triggered by certain stimuli to facilitate embryonic development (Alberio, Zakhartchenko *et al.* 2001). In ART, three types of assisted oocyte activation are commonly used (mechanical, electrical and chemical) to elevate cytoplasmic free calcium in oocytes (Neri, Lee *et al.* 2014). In this study, oocytes were treated with ionomycin to raise the intracellular level of calcium. This procedure is commonly used to activate oocytes for ICSI, SCNT, and parthenogenesis. We speculate that oocytes from large follicles have greater competence in inducing calcium signaling during IVF than oocytes from small follicles. However, this deficiency might be counteracted by either artificially-induced calcium influx in the SCNT activation procedure or potentially mechanical activation as a result of the oolemmas piercing during micromanipulations. The artificial activation allows small follicle oocytes to achieve similar rate of development to term as their large follicle counterparts. This hypothesis is supported by a human study in which the decrease in pregnancy rate with oocytes from small follicles was only observed in the conventional IVF cycles, but not in the ICSI cycles (Bergh, Broden *et al.* 1998).

Cytoplasmic maturation (the processes that prepare oocytes for activation and

preimplantation development) plays a key role in oocyte and embryo developmental competence (Duranthon and Renard 2001; Eppig 1996; Neri, Lee *et al.* 2014; Salviano, Collares *et al.* 2015; Watson 2007). However, cytoplasmic maturation is poorly understood at the molecular level. Previous studies indicate that the follicle size affects the ability of oocytes to achieve completion of cytoplasmic maturation (De Bem, Adona *et al.* 2014; Eppig, Wigglesworth *et al.* 2002; Gougeon 1996; Hirshfield 1991). Cytoplasmic changes in the maturation process include several cellular processes such as accumulation of maternal messenger RNAs (mRNAs), proteins, substrates and nutrients. Since major maternal transcription ceases before final oocyte maturation, the quality of the stored maternal mRNA in the MII oocytes is generally accepted to determine the developmental competence by sustaining the meiotic maturation and embryonic development to the maternal-embryonic transition (MET) stage and possibly beyond (Fair, Murphy *et al.* 2004; Leoni, Bebbere *et al.* 2007; Mourot, Dufort *et al.* 2006; Sirard 2001). In this study, gene expression analysis was conducted on individual oocytes. Single cell gene expression analysis is demonstrated to reveal gene expression level with improved statistical power to model these effects on gene expression (Wills, Livak *et al.* 2013). Our results showed the mRNA levels of three genes (*MATER*, *IGF2R* and *GRB10*) were greater in MII oocytes derived from large follicles than those from small follicles. *MATER*, which was first identified in mice, is an oocyte-specific maternal effect gene required for embryonic development beyond the two-cell stage (Tong and Nelson 1999; Tong, Gold *et al.* 2000). While the precise function remains poorly understood, MATER protein is believed to play a role in embryonic genome activation (Pennetier, Perreau *et al.* 2006; Tong, Gold *et al.* 2000). The accumulation of *MATER* gene transcripts was

demonstrated during oogenesis (Tong, Gold *et al.* 2000). Due to the cessation of maternal mRNA transcription after reaching oocyte maturation, the stockpile of *MATER* transcripts is essential for the protein synthesis at the early embryo stage. In bovine, transcription of *MATER* decreased while the MATER protein levels increased in parallel with follicle development, which indicates a growing level of translation (Pennetier, Perreau *et al.* 2006). Interestingly, it is observed that the pattern of calcium oscillations was altered in *Mater* knockout oocytes after IVF. According to this study, *MATER* appears to be required for the proper storage of calcium in MII oocytes (Kim, Zhang *et al.* 2014). The fact that oocytes from small follicles have a lower level of expression of *MATER* gene indicates that they were not able to store as much *MATER* transcripts as oocytes from large follicles during the follicle development. This could lead to a deficient calcium oscillation following fertilization. Thus, the difference in levels of stored *MATER* mRNA might be one of the molecular markers of developmental competence that needs to be further investigated. The other two genes (*IGF2R* and *GRB10*) that expressed higher levels in oocytes from large follicles fall into the category of imprinted genes. Dysregulation of methylation and expression of imprinted genes in oocytes are believed to be one of the reasons for different developmental competence of oocytes from mice of different ages (Paczkowski, Schoolcraft *et al.* 2015). However, it was also demonstrated that the role of the transcript level of an imprinted gene in regulating aberrant development might be more important than its imprinting status (Paczkowski, Schoolcraft *et al.* 2015). *IGF2R* was demonstrated to be an important regulator of fetal growth and embryonic development (Lucifero, Mertineit *et al.* 2002). A few studies have demonstrated that *IGF2R* has a function in triggering an intracellular signaling pathway

that is associated with the regulation of a variety of physiological functions, such as calcium influx (Chu, Tzang *et al.* 2008; McKinnon, Chakraborty *et al.* 2001; Nishimoto, Hata *et al.* 1987). Studies in mice and cattle showed that *GRB10* has an important role in early embryonic development (Lim, Riedel *et al.* 2004). Abnormal expression of GRB10 leads to aberrant fetal development (Charalambous, Smith *et al.* 2003; Miyoshi, Kuroiwa *et al.* 1998). *GRB10* has also been described as a cellular partner of several receptor tyrosine kinases. Interestingly, calcium influx is demonstrated to be dependent on the activation of receptor tyrosine kinases in multiple cell types including oocytes (Fleming, Fisslthaler *et al.* 1995; Mergler, Dannowski *et al.* 2003; Sharma and Kinsey 2013; Tolloczko, Tao *et al.* 2000). Therefore, due to their direct or indirect role in calcium homeostasis in oocytes, the lower amount of transcripts of *MATER, IGF2R* and *GRB10* stored in MII oocytes derived from small follicles compared to those from large follicles might cause low activation efficiency after fertilization, which might be the reason for their low embryonic developmental competence. However, this deficiency appears to be counteracted by the artificial activation following the SCNT in goats.

In conclusions, our results demonstrate that oocytes derived from small follicles are able to support development to term after SCNT. Even though the maturational competence was lower in oocytes from small follicles, no difference in SCNT efficiency was observed between oocytes from small and large follicles. This is likely due to the activation procedure that overcomes the deficiency of calcium signaling in oocytes derived from small follicles. The difference observed in *MATER, IGF2R* and *GRB10* transcripts between oocytes from large and small follicles could be a contributing factor to the different developmental competence following IVF (Cognie, Benoit *et al.* 1998;

Crozet, Ahmed-Ali *et al.* 1995; Lequarre, Vigneron *et al.* 2005; Raghu, Nandi *et al.* 2002; Romaguera, Casanovas *et al.* 2010), but not SCNT.

Table 1. The sequence of primers for quantitative real-time PCR

Gene	5'- primer sequence-3' (forward, reverse)	Product size (bp)
ACTB	CCTGACGGACTACCTCATGAA, GATGTCACGGACGATTTCCC	85
GAPDH	GATGTCACGGACGATTTCCC, GTACTCAGCACCAGCATCAC	69
IGF2R	TGTCCCGACGAAGTGAAGAC, GAGAGGCTGGACAGGTTGAA	80
CDK2	TGCTGCACTACGACCCTAAC, TGGCTTGGTCACATCTTGGAA	80
FSHR	TACGCCATCTTCACCAGGAAC, TGGGCTTGCACTTCATAGCA	80
GDF9	TTGTGGCCCCACACAAATAC, AGAGCCATACCGATGTCCAA	77
LHR	TGAAAGCACAGCAAGGAGAC, AATCCCAGCCACTCAGTTCA	80
ZAR1	TGGCTACTATCACTGCAAGGAC, TGCCTTGTACACACCACACA	68
EGF	GCTCTTGGTGGTATGGAAGTGAA, GCCGCTTATCAAGCACATCC	85
ACADL	CTAGGAGAGCACACCATTATCC, ACTGCTCGCAAATAAAAGTCAC	88
ZP3	ATCTTCCTGGGGAAGGTGAA, ATCACAGTAGCCAGGCCTAA	92
SLBP	CCGACTTGGAGACGGATGAAA, ATAGGCGATGGTGTTCTTCCC	77
H2A	AGCGTATTACCCCTCGTCAC, TTGTAGCCTTGATGAGAGAGTCC	75
PKG1	AGGTGGTGAAAGCCACTTCC, GTGTTCCATTTGGCACAGCAA	82
CPEB1	TTGAGCTTCCTGCCTCTGG, CTCTAATGGAGGGTGCTGGAAA	70
ZNF198	ATGCTCTAGCTCTGCAGACA, CCGTACGAGCATGTTTTCCA	82
COX1	GCATCCAAACGAGAGGTCCTAA, GGGCATCCGTTCAGTCATTCTA	68
ALG9	CTGGCCAGCTGCATTTCA, CTCACAAAAGCCAGCAGACA	87
CCNA2	ACCCTGCATTTGGCTGTGAA, AGCAGTGCCCACAAGTTGAA	87
POFUT2	CCTCCGCAGAGATGTCTACA, CACCCACTCCTCTGTCTTCA	70
MSX1	CACCGAGACGCAGGTGAA, GGCCATCTTCAGCTTCTCCA	91
PLAT	GAGGCTCACGTCAGGTTGTA, ACACAGCATGTTGTTGGTGAC	84
FGF2	CCGGTCAAGGAAATACTCCAGT, TTCTGCCCAGGTCCTGTTTT	87
H19	CTGGTCTCTGAGCGTGTGTAA, GAGGAGGCCGACACATTCA	61
CX43	GGGCTTGCTGAGAACCTACA, TGTACCACTGGATCAGCAAGAA	80
PFKFB3	ATCTCCAGCCCGGATTACAA, TGGTAACTGGCCTCATAGCA	89
DDR1	CGACTGGTTCGCTTCTATCCC, TCCTTCCAGAGGCAGCCATA	83
DNMT3B	TCCTCAAAGAACTGGGCATCA, CTTAACGGTGCCAACAGCAA	83
GDNF	TCACCGCCGTGCATCTAAA, AGCCGCTGCAGTACCTAAAA	84
ATP1A1	ACGCCTTTCAGAACGCCTA, GCAGCATCAAGTGGCAGAAA	75
ACSL3	GTGCTCCACTTTCTGCAACC, GAGTCCATACCCCTGACCAAC	77
AMH	GGCTATGAGCAGGCCTTCC, CAGCGAAGGTGGTCAAGTCA	73
NALP5	CAGAGCACAAAGAGCCTGAC, TCACAAGGCCAAACAGGAAC	88
CCNB1	GGCAACACTTACACCAAGTTCC, TGCAGGGGTAGAAGTTGATCC	92
KITLG	CCAGGCAGTTTACTCCTGAGAA, AGAAGCCACTATCTCCAAGTCC	83
IGF2	TTCTACTTCAGCCGACCATCC, TCGGAAGCAACACTCTTCCA	72
INHA	GACTGGACAGACAGGAGACC, GACCCCTGGATGTCAGTACC	78
OCT4	TGCAGGCCCGAAAGAGAAA, ACTGCAGGAACATGCTCTCC	75
BMP15	GTTGGGCAAAAGCTCTGGAA, ACCTCTTGGCTGCTGACATA	75
IGF1	TGTGGAGACAGGGGCTTTTA, CAGCACTCATCCACGATTCC	92
PPIA	ACTGGTGGCAAGTCCATCTA, ACAAGATGCCAGGACCTGTA	79
CDK1	AAGTGTGGCCAGAAGTGGAA, GATGCTAAGCTTCCTGGTTTCC	76
BAX	TGGCGGCTGAAATGTTTTCC, CCAGTTTGCTGGCAAAGTAGAA	78
BCL2	ATGTGTGTGGAGAGCGTCAAGGTT, CAGGTACTCGGTCATCC	79
PTEN	AAAGCTGGAAAGGGACGAAC, TAGGGCCTCTTGTGCCTTTA	84
GRB10	GTGAAGGAGCTGGGAAGGAAA, AAGCTCCCTTTGTGGAGCAA	85
SOX2	ATGAAGGAGCACCCGGATTA, CCAGGCAGTGTGTACTTATCC	83
NANOG	CTGGGAACTGCTGGGGAAAA, TCAGTGATTTGCTGCTGGGAA	77

Table 2. Effect of follicle size on the G6PDH activity in GV stage oocytes subjected to BCB test

Source of oocytes	Total COCs	Oocyte classification	
		BCB+, n (%)	BCB−, n (%)
Large follicles (≥3mm)	336	181(54.9±8.5)[a]	155 (45.1±8.5)[a]
Small follicles (<3mm)	1194	454 (38.7±9.2)[b]	740 (61.3±9.2)[b]

Values in parentheses are means ± SEM of 9 experimental replicates expressed as percentages. Values in the same column with different superscripts differ significantly at $P < 0.05$([a, b]).

Table 3. Oocyte maturation and donor cell-cytoplast fusion rates using cytoplasts derived from large and small follicles

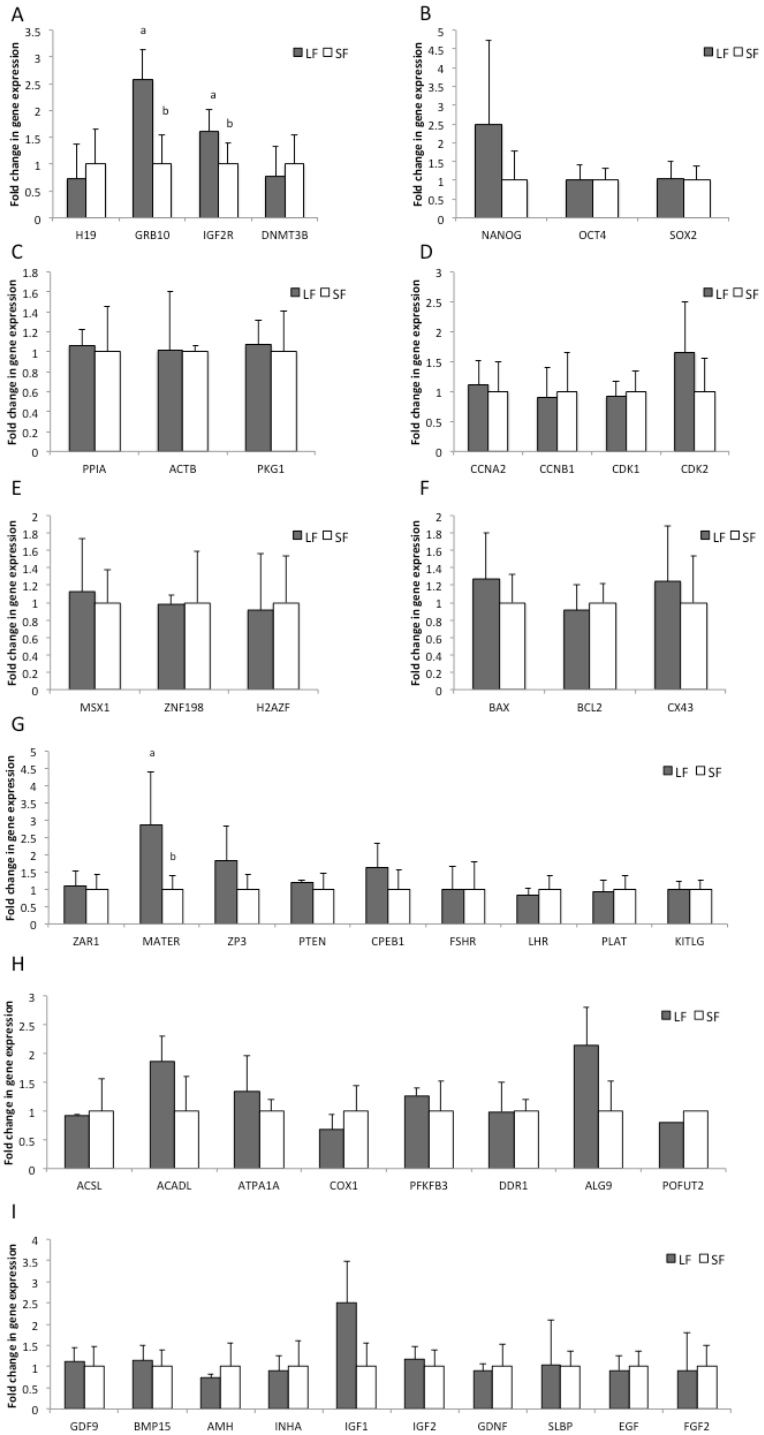
Source of oocytes	Ovaries, n	Oocytes, n	Oocyte/Ovary, n	Mature oocytes, n (%)	Fused embryos, n (%)
Large follicles (≥3mm)	121	452	3.00±0.33	233 (51.6±4.8)[a]	138 (71.9±5.4)
Small follicles (<3mm)	121	1451	8.45±0.86	488 (33.7±4.1)[b]	215 (66.4±1.8)

Values in parentheses are means ± SEM of 7 experimental replicates expressed as percentages. Values within columns with a different superscript are significantly different at $P < 0.05$([a, b]).

Table 4. *In vivo* development of SCNT goat embryos derived from cytoplast donors of large and small follicles

Cytoplast Donors	Transferred embryos, n	Recipients n	Pregnancies, n (%) Day 35	Day 60	Day 90	Birth Rate (%)	Cloning Efficiency (%)
Large follicles (≥3mm)	124	9	3 (33.3)	3 (33.3)	2 (22.2)	2/9 (22.2)	2/124 (1.61)
Small follicles (<3mm)	193	12	5 (41.7)	5 (41.7)	4 (33.3)	2/12 (16.7)	2/193 (1.04)

Fig. 1. Fold change (FC) of gene expression of oocytes derived from large follicles (≥ 3 mm) and small follicles (< 3 mm) relative to the housekeeping gene assessed at the MII stage. Genes are grouped according to their function: (A) imprinting, (B) pluripotency, (C) housekeeping, (D) cell cycle, (E) transcription factors, (F) apoptosis, (G) oogenesis, (H) metabolism and (I) growth factors. Different superscripts ([a] and [b]) indicate a significant difference between oocytes from large follicles and small follicles ($P < 0.05$) and FC>1.5. LF= large follicle oocytes; SF= small follicle oocytes.



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CHAPTER III

ASSOCIATION OF G6PDH-ACTIVITY WITH OOCYTE DEVELOPMENTAL COMPETENCE AND MOLECULAR CHARACTERISTICS IN GOATS

Abstract

Glucose-6-phosphate dehydrogenase (G6PDH) activity is a marker for determining oocyte quality that can be detected by brilliant cresyl blue (BCB) staining. The aim of this study was to investigate the developmental competence of goat BCB+ (low G6PDH-activity) and BCB− (high G6PDH-activity) oocytes and to identify molecular markers associated with developmental competence. We hypothesized that oocytes selected based on G6PDH activity would differ in their developmental potential and have differential gene expression profiles both before (GV stage) and after (MII stage) *in vitro* maturation (IVM). Genes representing various functional categories (housekeeping genes, transcription factors, metabolism, growth factor, pluripotency, apoptosis, oogenesis, cell cycle and imprinting) were assessed. Real-time PCR was used to evaluate relative expression of 46 genes in individual BCB+ and BCB− oocytes (n=30) collected from five different dates before IVM. The same analysis was conducted on BCB+ and BCB− oocytes collected after IVM. Twelve genes (*CCNA2, CDK2, ZNF198, ACSL, ATP1A1, COX1, GDF9, BMP15, INHA, OCT4, BCL2* and *ZAR1*) were found to be expressed at a higher level in BCB+ oocytes than BCB- oocytes at GV stage ($P < 0.05$). Moreover, the relative expression was significantly higher ($P < 0.05$) for five genes (*CDK1, CCNB1, INHA, IGF2,* and *CPEB1*) and significantly lower for three genes (*MATER, ZP3,* and *BAX*) in BCB+ oocytes compared to BCB- oocytes at MII stage. This

study has demonstrated molecular differences of oocytes with different G6PDH activity. The identified genes could be used as biomarkers for oocyte quality.

Introduction

The quality of the oocyte is characterized by the ability of an oocyte to undergo maturation, fertilization and the later development to blastocyst stages or live offspring (Krisher 2004; Sirard and Coenen 2006). The developmental competence of oocyte is a determining factor for the efficiency of reproductive techniques in animal and human assisted reproductive technologies (ARTs). In addition to its role in supporting fertilization in normal development, an oocyte can also reprogram the nucleus of a somatic cell into an embryonic pluripotent state after somatic cell nuclear transfer (SCNT, (Jouneau 2010)). The low efficiency of ARTs could be attributed to the quality of the cytoplast donor (Krisher 2004). It is difficult to draw reliable criteria for oocyte selection due to the fact that the oocyte developmental competence depends on multiple factors. Oocyte selection is generally based on their morphological characteristics, which is vague and imprecise and makes it difficult to distinguish oocytes of good developmental competence. The results derived from using morphological evaluations are conflicting and insufficient due to their subjectivity (Coticchio, Sereni *et al.* 2004; Lonergan, Rizos *et al.* 2003). Therefore, there is an urgent need to have a noninvasive and non-perturbing approach for oocyte selection. A number of parameters have been identified to be associated with developmental competence. Glucose-6-phosphate dehydrogenase (G6PDH) is one of the enzymes is that synthesized in immature oocytes. While this enzyme is active in the growing oocytes, the activity of G6PDH decreases in fully-grown oocytes (Abazari-Kia, Mohammadi-Sangcheshmeh *et al.* 2014; Alm, Torner

et al. 2005; Sato, Iwata *et al.* 2007). Brilliant cresyl blue (BCB) dye can be reduced by the G6PDH activity. Therefore, BCB staining can differentiate between fully-grown, more developmentally competent oocytes and growing oocytes. Due to the high level of active G6PDH in oocytes undergoing growth, the dye is reduced to colorless (BCB−), while fully-grown oocytes of presumed higher quality remain blue (BCB+) due to the decreased amount of cytoplasmic G6PDH-activity. BCB staining has been used as a supravital stain method for oocyte selection in several species. Studies in pigs (El Shourbagy, Spikings *et al.* 2006; Santos, Sato *et al.* 2015), goats (Rodriguez-Gonzalez, Lopez-Bejar *et al.* 2003; Rodriguez-Gonzalez, Lopez-Bejar *et al.* 2002), cattle (Bhojwani, Alm *et al.* 2007; Sadeesh, Fozia *et al.* 2017), sheep (Wang, Lin *et al.* 2012), mice (Wu, Liu *et al.* 2007) and dogs (Rodrigues, Rodriguez *et al.* 2009) showed that the maturation and blastocyst developmental rates of BCB+ oocytes were significantly higher than BCB− oocytes after *in vitro* culture. A previously published bovine study has also explored the *in vitro* and full-term developmental competence of SCNT embryos derived from BCB+ and BCB− and found that BCB+ oocytes yielded higher cloning efficiency (Su, Wang *et al.* 2012). The activity of G6PDH in oocytes has been related to several intrinsic parameters such as chromatin configuration (Fu, Ren *et al.* 2015), mitochondrial activity (Egerszegi, Alm *et al.* 2010; Fu, Ren *et al.* 2015) and intracellular glutathione (GSH) level (Abazari-Kia, Mohammadi-Sangcheshmeh *et al.* 2014; Wu, Liu *et al.* 2007). Moreover, various oocyte quality markers such as cytoplasmic volume, oocyte diameter, and mitochondrial DNA copy number differ in oocytes of high (BCB+) and low (BCB−) G6PDH-activity (El Shourbagy, Spikings *et al.* 2006). During oogenesis, a high rate of gene transcription leads to the accumulation and storage of maternal mRNAs. It is largely

accepted that the quality of the stored mRNA in the matured oocyte dictates developmental competency (Fair, Murphy *et al.* 2004; Knijn, Wrenzycki *et al.* 2002; Lonergan, Gutierrez-Adan *et al.* 2003). The transcription levels of a number of genes that related to developmental competence were reported significantly different between the BCB+ and BCB− oocytes. However, the results of different studies were conflicting and controversial. So far, to our knowledge, little is known about molecular and the subcellular characteristics of the goat oocytes selected by BCB staining. The aim of this study was to investigate the *in vitro* developmental competence of the oocytes selected by BCB staining after parthenogenetic activation in goats.

In addition, with the aim of providing more fundamental evidence regarding the molecular and subcellular basis of oocyte developmental competence based on BCB staining, we investigated the association of the G6PDH-activity with the intracellular levels of GSH and reactive oxygen species (ROS) content. Furthermore, we assessed the expression patterns of a set of genes that are involved in oocyte developmental competence in BCB+ and BCB− oocytes at metaphase II (MII) stage.

Materials and Methods

All animal procedures were approved by and conducted according to the guidelines of the Utah State University Animal Care and Use Committee. All chemicals were from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise specified.

Oocyte collection and brilliant cresyl blue (BCB) staining

Ovaries from domestic goats (*Capra aegagrus hircus*) were obtained from a local abattoir (Springville, UT, USA) and transported to our laboratory in saline containing 100

U/mL penicillin/streptomycin at 20°C to 27°C within 4 h after collection. The ovaries were washed 3 times with saline and then three times in Dulbecco's phosphate-buffered saline (DPBS, Hyclone, Logan, UT, USA) containing 100 U/mL penicillin/streptomycin before oocyte collection. Oocyte collection was conducted as described previously (Yang, Hall *et al.* 2016). Briefly, cumulus-oocyte complexes were recovered from ovaries in modified TL-Hepes medium (Lonza, Walkersville, MD, USA) supplemented with 1% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 100 U/mL penicillin/streptomycin, and 30 mg/mL heparin. A modified slicing technique was used to recover the COCs, in which only the surface of the ovaries was sliced. Only the oocytes with three or more layers of compact cumulus cells and homogeneous cytoplasm were selected. After three washes in modified DPBS (mDPBS, PBS supplemented with 1 g/L glucose, 0.5 g/L BSA, 36 mg/L sodium pyruvate, and 0.05 g/L gentamicin), the oocytes were incubated with 26 mM BCB diluted in mDPBS at 38.5°C in 5% CO_2 for 30 min. The treated COCs were then washed two times in mDPBS and classified into BCB+ group (blue) and BCB- group (colorless) according to their cytoplasm coloration.

In vitro maturation

The BCB treated COCs were washed three times in maturation medium (TCM-199 [Gibco, Grand Island, NY, USA], containing 10% FBS, 10 mg/mL LH, 5 mg/mL FSH, 1-mg/mL estradiol-17b, and 0.05 g/L gentamicin) and then incubated in groups of 50 in 4-well plates containing 500 μL of maturation medium for 22 h in humidified air with 5% CO_2 at 38.5°C. After maturation, oocytes were transferred to TL-Hepes medium containing 1 mg/mL hyaluronidase. Cumulus cells were removed mechanically by gentle pipetting. Nuclear maturation status was evaluated by the extrusion of a first polar body.

Oocytes with a polar body are at the metaphase II stage, which is termed MII oocytes.

Parthenogenetic activation (PA) and in vitro culture of PA embryos

Parthenogenetic activation was performed as described previously (Fan, Yang *et al.* 2017). Briefly, after maturation, the MII oocytes were washed three times in synthetic oviductal fluid (SOF) medium (Tervit, Whittingham *et al.* 1972). We transferred 15-20 oocytes into 25 μL droplets of SOF supplemented with 7.5 μg/mL of cytochalasin B and incubated them in humidified air with 5% CO_2 at 38.5°C for 50 min. The oocytes were then transferred and activated for 5 min in SOF medium supplemented with 2 μM of ionomycin. Finally, oocytes were incubated for 4.5 h in SOF medium containing 2 mM 6-dimethylaminopurine and 10 μg/mL cycloheximide in humidified air with 5% CO_2 at 38.5°C.

After PA, the parthenogenetic embryos were washed three times in SOF and incubated in groups of 15-20 embryos in 25 μL droplets of SOF medium under mineral oil in a humidified air with 5% O_2, 5% CO_2, and 90% N_2 at 38.5°C. Culture dishes were pre-equilibrated for at least 5 h in an incubator before use. The efficiency of the *in vitro* development was evaluated by the percentage of cleaved embryos 2 days after PA and the percentage of blastocysts 8 days after PA.

Measurement of intracellular GSH and ROS levels in MII oocytes

The MII oocytes were collected to determine intracellular GSH and ROS levels by methods described previously (Fan, Yang *et al.* 2017). CellTracker Blue CMF2HC (4-chloromethyl-6.8-difluoro-7-hydroxycoumarin, Invitrogen, Eugene, OR) and H2DCFDA (2',7'-dichlorodihydrofluorescein diacetate, Invitrogen, Eugene, OR) were used to assess

the intracellular GSH and ROS concentrations in oocytes by the blue and green fluorescence, respectively. Briefly, The MII oocytes were incubated in H199 medium containing 20 μM CellTracker Blue CMF2HC or 10 μM H2DCFDA for 20 min at 38.5°C in the dark. Seven to ten oocytes from each group were then placed into 10 μL droplets respectively after three washes in DPBS containing 0.1% (w/v) PVA. The fluorescence was detected by a fluorescence microscope (Zeiss, Oberkochen, Germany) with ultraviolet filters (370 nm for GSH and 460 nm for ROS). Fluorescent images were captured and saved as TIFF files. ImageJ software (National Institutes of Health, Bethesda, MD) was used to quantify the intensity of fluorescent of the treated oocytes.

Gene expression analysis

Expression levels of 46 development-associated genes were analyzed in both GV and MII oocytes from BCB+ and BCB- groups. GV oocytes were collected before IVM. MII oocytes were collected after 22 h IVM. Denuded oocytes were washed three times and stored individually in 1 μL DNA suspension buffer. Gene-expression experiments were conducted on individual oocytes using Fluidigm's 48.48 quantitative PCR (qPCR) Dynamic Array Integrated Fluidic Circuits (IFC; Fluidigm, San Francisco, CA) as described previously (Yang, Hall *et al.* 2016). First, all 46 pairs of primers were diluted in the DNA suspension buffer to make a 200nM pooled primer mix in preparation of reverse transcription-specific target amplification (RT-STA). The RT- STA mixture solution was then made by adding each individual oocyte (stored in DNA suspension buffer) into 9 μL of RT-STA solution (5 μL of CellsDirect PCR mix (Invitrogen, Carlsbad, CA), 0.2 μL SuperScript-III RT/Platinum Taq (Invitrogen, Carlsbad, CA), 2.5 μL of 200 nM pooled primer mix and 1.3 μL DNA suspension buffer). Reverse

transcription reaction was done in the thermal cycler at 50°C for 15 min, 95°C for 2 min. The cDNA was subsequently pre-amplified for 20 cycles (each cycle: 95°C for 15 s, 60°C for 4 min), which enriched cDNA of the target genes. Subsequently, 4 μL ExoSAP-IT (ExoI; New England Biolabs, Beverly, MA) was added to each RT-STA reaction and then the reactions were incubated at 37°C for 20 min to eliminate unincorporated primers, which was followed by 15 min at 80°C cycle to inactivate the enzyme. After ExoSap-IT treatment, 36 μL of DNA suspension buffer were added to each reaction to achieve 5-fold dilution. The Fluidigm chip was primed before use according to manufacturer's instructions (Fluidigm PN 100–7717 B1). A 5 μL sample mix solution (2 μL diluted sample, 2.5 μL of the 2X TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA), 0.25 μL of 20X DNA Binding Dye Sample Loading Reagent (Fluidigm, San Francisco, CA) and 0.25 μLEvaGreen DNA Binding Dye (Biotium, Hayward, CA)) were loaded into one of the sample inlets of the primed chip. A 5 μL aliquot of the assay (primer mix solution (2.5 μL of the 2X Assay Loading Reagent (Fluidigm), 1.25 μL DNA suspension buffer, and 1.25 μL of 20 μM forward and reverse primer mix of each gene)) was inserted into "assay" inlets of the primed chip. The chip was then returned to the IFC Controller for chip loading. Subsequently, the chip was placed in the BioMark real-time PCR machine for quantitative PCR following manufacturer's instructions. Cycling parameters included a 5 min initial enzyme activation at 95°C, 35 denaturation/extension cycles (95°C for 15 s followed by 60°C for 60 s), and a 3 min final extension cycle at 60°C. Primer efficiency for each analyzed gene was calculated by the standard curve using the formula equation $E=10^{-1/slope}$. Only primers that had the amplification efficiency of more than 90% were qualified for the

gene expression analysis. The real-time PCR data were analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The expression level of target genes was normalized to the internal control gene (PPIA). The gene expression data are presented in fold change compared to control values.

Experimental design

Experiment 1. Effect of G6PHD activity on oocyte selection and IVM.

Each round of oocyte collection (different dates) was considered an experimental replicate. Thirty rounds of collections were conducted. Collected oocytes were divided into BCB+ group and BCB− group. Oocyte maturation rates were assessed after IVM for 22 h.

Experiment 2. Effects of G6PDH activity on *in vitro* embryo developmental competence following parthenogenetic activation.

Matured COCs obtained from BCB+ and BCB− groups were denuded from cumulus cells and parthenogenetically activated. The cleavage and blastocyst development rates were recorded during *in vitro* culture at day 2 and day 8 after PA, respectively. The experiment was replicated for 4 times.

Experiment 3. Effect of G6PDH activity on intracellular GSH and ROS levels in MII oocytes.

The intracellular GSH and ROS levels were assessed using specific fluorescence assays. After IVM, MII oocytes from BCB+ and BCB− groups were collected and stained. In total, 61 BCB+ and 55 BCB− MII oocytes from 4 rounds of IVM were used

for intracellular GSH and ROS detection.

Experiment 4: G6PDH-activity in relation to the expression of candidate genes in oocytes at GV and MII stage.

Immature GV oocytes and MII oocytes were collected and denuded for gene expression after BCB staining and IVM, respectively. The association between G6PDH-activity and expression of developmentally important genes (Table 1) were examined in both GV and MII oocytes. The selected genes fell into one of nine functional categories: housekeeping genes, transcription factors, metabolism, growth factor, pluripotency, apoptosis, oogenesis, cell cycle and imprinting. The relative gene expression levels of the selected genes were compared between the oocytes from BCB+ and BCB− groups at the two stages, respectively. Both BCB+ and BCB− group consisted of 30 oocytes collected from five separate rounds of oocyte collection. Real-time PCR was conducted on individual oocytes.

Statistical analysis

Each round of *in vitro* culture was considered a replication. The cleavage rate and blastocyst rate (data were converted to percentages) of BCB+ and BCB− control group were compared and analyzed by ANOVA in SAS 9.4 (SAS Institute Inc.). A difference was considered significant when the *P* value was less than 0.05. Data on gene expression and levels of GSH or ROS were analyzed using the Student t-test. Differences were considered to be significant if $P < 0.05$ for GSH or ROS levels. For gene expression data, the difference was considered to be significant when the following requirements were met: at least 1.5-fold change in gene expression was observed and *P* value was less

than 0.05 (FDR- adjusted).

Results

Experiment 1. Effect of G6PHD activity on oocytes selection and IVM.

In total, 2909 oocytes were collected from 16 rounds of oocyte collection. Among them, 1287 oocytes (44.2%) were BCB+ and 1622 (55.8%) were BCB− (Fig. 1.). Oocytes with lower G6PDH activity (BCB+) had significantly greater maturation rates than oocytes with higher G6PDH activity (Table 2; $P < 0.05$).

Experiment 2. Effects of G6PDH activity on *in vitro* embryo developmental competence following parthenogenetic activation.

The matured oocytes were parthenogenetically activated, then cultured in SOF medium for 8 days. As shown in Table 3, the cleavage rate at day 2 of *in vitro* culture was significantly higher in BCB+ group compared to BCB− group ($P < 0.05$). No significant difference was found in morula rates among the different treatments. Blastocyst rate recorded at day 8 was significantly greater in BCB+ group than BCB− group.

Experiment 3. Effect of G6PDH activity on intracellular GSH and ROS levels in matured MII oocytes.

The results for the intracellular GSH and ROS levels are shown in Fig. 2. After IVM, significantly greater ($P < 0.05$) intracellular GSH level was observed in MII oocytes of BCB+ group than those of BCB− group. On the contrary, intracellular ROS level was significantly lower in MII oocytes of BCB+ group compared to those of BCB− group ($P < 0.05$).

Experiment 4: G6PDH-activity in relation to the expression of candidate genes in oocytes at GV and MII stage.

Real-time PCR was used to evaluate relative expression of 46 genes in individual BCB+ and BCB- oocytes (n=30) at GV stage. The same analysis was conducted on MII BCB+ and BCB- oocytes collected after IVM. Twelve genes (*CCNA2, CDK2, ZNF198, ACSL, ATP1A1, COX1, GDF9, BMP15, INHA, OCT4, BCL2* and *ZAR1*) were found to be expressed at a higher level in BCB+ oocytes compared to BCB- oocytes at GV stage ($P < 0.05$, Fig. 3.). Moreover, the relative expression was significantly higher ($P < 0.05$) for five genes (*CDK1, CCNB1, INHA, IGF2,* and *CPEB1*) and significantly lower for three genes (*MATER, ZP3,* and *BAX*) in BCB+ oocytes compared to BCB- oocytes at MII stage (Fig. 4.).

Discussion

The developmental competence of the oocyte donor is one of the most important limiting factors affecting efficiency of the assisted reproductive technologies including SCNT. Due to the limited recourses of goat ovaries, COCs are often recovered by slicing the ovary surface to maximize the amount of oocytes obtained from one ovary. These recovered oocytes are commonly heterogeneous in quality and developmental competence. Selection of developmentally competent oocytes is vital for the success of various embryo technologies. In this study, we used BCB staining, a noninvasive and non-perturbing approach, to separate oocytes by its G6PDH activity. BCB staining is efficient in selecting developmentally competent oocytes in various species, including goat (Rodriguez-Gonzalez, Lopez-Bejar *et al.* 2003; Rodriguez-Gonzalez, Lopez-Bejar *et al.* 2002). The results of the present study further prove the difference of the

developmental potential and molecular characteristics between the two oocyte groups separated by BCB staining. Our results demonstrated that the maturation rate, as well as the cleavage and blastocyst rates after PA, of BCB+ oocytes, were higher compared to those of BCB− oocytes. The better performance of oocytes with lower G6PDH activity might have been associated with the more advanced growing extent. Low G6PDH-activity prior to IVM can be regarded as a marker for the competence of the oocytes to accomplish cytoplasmic maturation and to obtain good maturation rate. The fact that BCB+ oocytes yielded higher *in vitro* development rates indicates that oocyte with low G6PDH is intrinsically more competent to complete developmental processes successfully. Our study reinforced that BCB staining can be used as an oocyte selection approach to improve the *in vitro* culture efficiency of goat oocytes.

The cellular mechanisms by which an oocyte acquires developmental competence remain unclear. In this study, we also aimed to elucidate the differences in oocyte quality as determined by G6PDH activity. Comparing the molecular characteristics of oocytes with different developmental competence could provide insightful information of how oocytes acquire developmental competence. Oocyte cytoplasmic maturation consists of a series of complex cellular processes and molecular reactions including the synthesis of fatty acid, steroids and nucleotides and the regulation of numerous metabolic pathways. G6PDH was demonstrated to be crucial in these oocyte cellular processes and plays a key role in protection against reactive oxygen species (ROS) and reduction of glutathione (GSH) content (Berg, Tymoczko *et al.* 2002).

In oocytes, the energy production in the metabolic regulation and the level of ROS are closely connected (Babayev and Seli 2015). Intracellular ROS contributes to

numerous oocyte physiological functions including meiosis resumption and cytoplasmic maturation. High level of ROS is related to DNA damage by breaking the DNA strands and could eventually result in oocyte and embryo apoptosis. GSH is an antioxidant that prevents the oocyte from being poisoned by ROS. Intracellular GSH content in oocytes is crucial for oocyte competence by maintaining the meiotic spindle forms and protecting the spindle from oxidative damage. Previous studies have shown that GSH accumulates during oocyte maturation and the accumulation of GSH can reduce oxidative damage during cytoplasmic maturation and the developmental process of embryos after fertilization, which therefore improves the developmental competence of oocytes (De Matos and Furnus 2000; Ozawa, Nagai *et al.* 2010). Low levels of GSH are associated with incomplete cytoplasmic maturation, and therefore intracellular GSH has commonly been proposed as a molecular marker of oocyte cytoplasmic maturation. Our study examined GSH and ROS content in oocytes of different developmental competence based on BCB selection. To our knowledge, this is the first time that the association between G6PDH-activity and intracellular GSH and ROS content in goat oocytes has been demonstrated. We found that, compared to BCB− oocytes, BCB+ oocytes have higher intracellular GSH level and lower ROS level. This result indicates that G6PDH activity of the oocyte not only reflects the ability of oocyte to accomplish nuclear maturation but also is associated with cytoplasmic maturation. The higher competence of BCB+ oocytes is likely caused by the better cytoplasmic maturation of these oocytes.

Unlike nuclear maturation, cytoplasmic maturation is not as visible and clearly defined. As we know, transcription events cease after the germinal vesicle undergoes breakdown during meiosis. Therefore, the mRNAs stored during the oocyte growth and

maturation phase are crucial for early embryonic development before embryonic genome is activated (Krisher 2004). In this study, gene expression was conducted on both immature (GV stage) and mature oocytes (MII stage) of two oocyte groups classified by G6PDH activity. The 46 selected genes were previously implicated in oocyte maturation and early embryonic development. Our results showed that 12 out of 46 genes were differentially expressed between BCB+ and BCB− oocytes at GV stage. All of these 12 differentially expressed genes were expressed at a higher level in the BCB+ oocytes, and they fell in the function categories of cell cycle (*CCNA2* and *CDK2*), transcription (*ZNF198*), metabolism (*ACSL, ATP1A1* and *COX1*), growth factors (*GDF9, BMP15*, and *INHA*), pluripotency (*OCT4*), apoptosis (BCL2) and oogenesis (*ZAR1*). We found only 8 genes were differentially expressed between BCB+ and BCB− oocytes at MII stage. Five out of the 8 genes were expressed at higher levels in the BCB+ oocytes and they fell in the categories of cell cycle (*CCNB1* and *CDK1*), growth factors (*INHA* and *IGF2*), oogenesis (*CPEB1*); and 3 genes were expressed at higher levels in BCB− oocytes and they fell in the categories of apoptosis (*BAX*) and oogenesis (*MATER* and *ZP3*). The difference in gene expression profiles reinforced the fact that BCB+ and BCB− oocytes compose two distinct populations. The transcripts of the majority of the selected genes are more abundant in the BCB+ oocytes at both GV stage and MII stage. The lower abundance of transcripts of these developmentally important genes in BCB− is very likely to be caused by the inefficiency of a number of mechanisms, including dysregulation of the post-transcriptional control. Oocyte meiotic maturation, fertilization, and later embryo development are all influenced by cell-cycle regulation (Parrish, Kim *et al.* 1992). Our results showed that the transcripts of cell cycle regulator genes *CCNA2*

and CDK2 were more abundant in BCB+ oocytes than BCB− oocytes at GV stage and the transcripts of *CCNB1* and *CDK1* were more abundant in BCB+ oocytes at MII stage. Cyclins (CCN) genes code for a family of proteins that control the cell cycle by activating cyclin-dependent kinase (CDK) proteins. Different phases of the cell cycle were regulated by different cell cycle proteins. It is not surprising that several cell cycle regulator genes were highly expressed in BCB+ oocytes. In goats, the embryo has to divide three times to reach maternal to zygotic transition (MZT) in the condition of transcriptional silence (Barnes and First 1991). Thus, the more competent oocyte must have stored more mRNA coding for the cell cycle proteins to efficiently sustain the cell cycle untill the activation of the zygotic genome (Tremblay, Vigneault *et al.* 2005). The fact that the transcripts of several cell cycle regulator genes are more abundant in BCB+ oocytes indicates more cell cycle proteins could be translated, which might contribute to the higher efficiency of the BCB+ oocytes in completing oocyte maturation and early embryonic development. Numerous studies have reported the association of growth factors with oocyte maturation and embryo developmental progression (Patel, Bettegowda *et al.* 2007; Ruvolo, Fattouh *et al.* 2013; Torner, Ghanem *et al.* 2008; Wang and Sun 2007). *BMP15* and *GDF9* are oocyte-specific growth factors that have been implicated to play key roles in follicular development and to determine the fertility of the oocyte in most mammalian species (Su, Wu *et al.* 2004). Our observation that *BMP15* and *GDF9* were expressed at a higher level in more competent BCB+ oocytes compared to BCB− oocytes is well in line with a report that has also identified these two factors as being important for oocyte competence (Hussein, Thompson *et al.* 2006) and suggests the positive correlation between the transcript storage of these two genes and oocyte

developmental competence. Previous studies demonstrated that the expression profile of INHA is associated with the follicle growth and development and may represent a marker of the oocyte quality (Bartosz, Marta *et al.* 2012). Our result indicate that *INHA* mRNA transcripts were more abundant in BCB+ oocytes is consistent with the previous study in which the expression of *INHA* as well as expression of other members of TGFβ superfamily, including *BMP15*, was down-regulated in *in vitro* cultured COCs with compromised competence compared to *in vivo* (Parrish, Siletz *et al.* 2011). Our gene expression data also showed that the gene expression of transcription factor *ZFN198* was greater in BCB+ oocytes. The fact that transcripts of transcription factor *ZNF198* and pluripotent transcription factor *OCT4* were more abundant in BCB+ oocytes than BCB− oocytes at GV stage highlights the importance of mRNA synthesis and turnover before the transcriptional quiescent stage in oocyte developmental competence. We also observed that genes involved in metabolism (*ACSL*, *ATP1A1*, and *COX1*) were highly expressed in BCB+ oocytes. Gene *ACSL* codes for the protein that has a role in mediating fatty lipid metabolism for ATP production in oocytes. This process is important for the oocyte to acquire developmental competence during oocyte maturation. The other two metabolism genes *ATP1A1* and *COX1* are also related to the ATP production in oocytes (Angèle, FranOis *et al.* 2009; Catala, Izquierdo *et al.* 2011). Our results indicate that abundantly stored transcripts of metabolism genes might ensure the adequate energy supply during oocyte maturation and even later embryonic development, which thus contributes to the high developmental competence of oocytes. Our data also revealed the association of apoptosis genes and oocyte developmental competence; anti-apoptotic *BCL2* mRNA transcripts were more abundant in BCB+ oocytes at GV stage and pro-

apoptotic *BAX* mRNA transcripts were more abundant in BCB− oocytes at MII stage. This result also indicates the interaction of the expression of these two genes with G6PDH-activity in oocytes. *MATER* and *ZAR1* are two essential oocyte-specific proteins that are gradually accumulated during oogenesis (Bebbere, Bogliolo *et al.* 2008; Tong, Gold *et al.* 2004; Uzbekova, Roy-Sabau *et al.* 2006). Previous knockout studies showed that the embryo could not grow beyond 2-cell stage without these two genes (Tong, Gold *et al.* 2000; Wu, Viveiros *et al.* 2003). In this study, we found that the expression level of *ZAR1* was greater in BCB+ oocytes at the GV stage. However, the expression level of *MATER* was detected to be lower in BCB+ oocytes at the MII stage. The reason for this decrease of the *MATER* gene transcprits in BCB+ oocyte is unclear. One of the possible explanations is that more stored *MATER* transcripts have been translated into functional proteins during maturation in BCB+ oocytes while no new transcripts were produced. We also found the transcript level of *ZP3* gene is lower in BCB+ oocyte at MII stage, which is possibly due to a similar reason as it is to the *MATER* gene. The *ZP3* gene codes for the protein that functions as the sperm receptor during fertilization and contributes to the oocyte developmental competence by ensuring the efficiency of the fertilization process (Canosa, Adriaenssens *et al.* 2017). Last but not least, we found the expression level of the *CPEB1* gene was significantly greater in BCB+ oocytes compared to BCB− oocytes after maturation. *CPEB1* is a sequence-specific RNA-binding protein that regulates polyadenylation-induced translation. *CPEB1* controls the oocyte growth by meditating the translations of a number of maternal mRNAs (Racki and Richter 2006; Setoyama, Yamashita *et al.* 2007). Overall, our gene expression results followed the published trend in the sense that many of the differentially expressed transcripts were more abundant in

BCB+ oocytes at GV and MII stage. Fewer genes were differentially expressed between BCB+ and BCB- group after *in vitro* maturation, which is possibly attributed to the reduced difference between the two oocyte population upon the completion of oocyte meiotic maturation.

In summary, we examined the effects of oocyte G6PDH-activity on their developmental potential using parthenogenetical activation (PA) and the *in vitro* embryo production system in goat oocytes. Based on the results that BCB+ oocytes had greater ability to complete maturation and reach to blastocyst stage after PA compared to BCB−oocytes, we confirmed that the BCB staining that reflects G6PDH activity could serve as a screening tool to differentiate oocyte quality in goats. In this study, our data also demonstrated that the more competent oocytes (BCB+) presented higher intracellular GSH level and lower intracellular ROS level after *in vitro* maturation. Also, this study provides the gene expression profile of a panel of genes that could be associated with the acquisition of developmental competence in oocytes. The difference in transcript levels observed between the two oocyte populations could provide information of the contributing factors in determining oocyte developmental competence. Further investigations based on these data might be able to identify additional biomarkers for oocyte quality.

Fig. 1. Representative photographs of differentially stained bovine COCs after exposure to BCB stain. BCB+ (blue cytoplasm) and BCB- oocytes (colorless cytoplasm) were directed by black and white arrows, respectively. Bar = 100 μm.

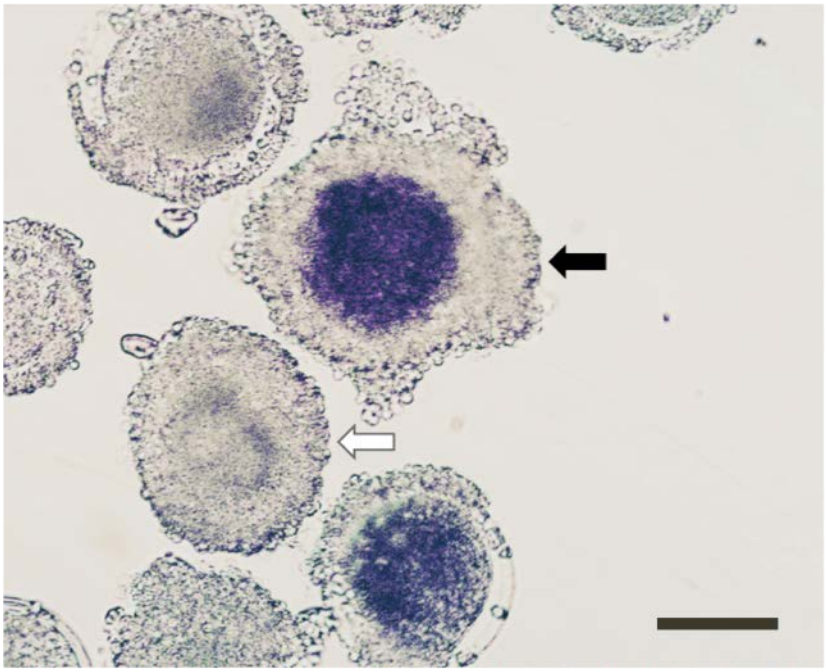

Fig. 2. Fluorescent photomicrographic images of *in vitro*-matured goat oocytes. (A) BCB- oocytes (a and c) and BCB+ (b and d) oocytes were stained with CellTrackeBlue (a and b) and 20,70-dichlorodihydrofluorescein diacetate (c and d) to detect intracellular levels of GSH and ROS, respectively. (B) Relative levels of intracellular GSH and ROS in MII goat oocytes. Values with different superscripts differed significantly ($P < 0.05$). GSH: glutathione; ROS: reactive oxygen species.

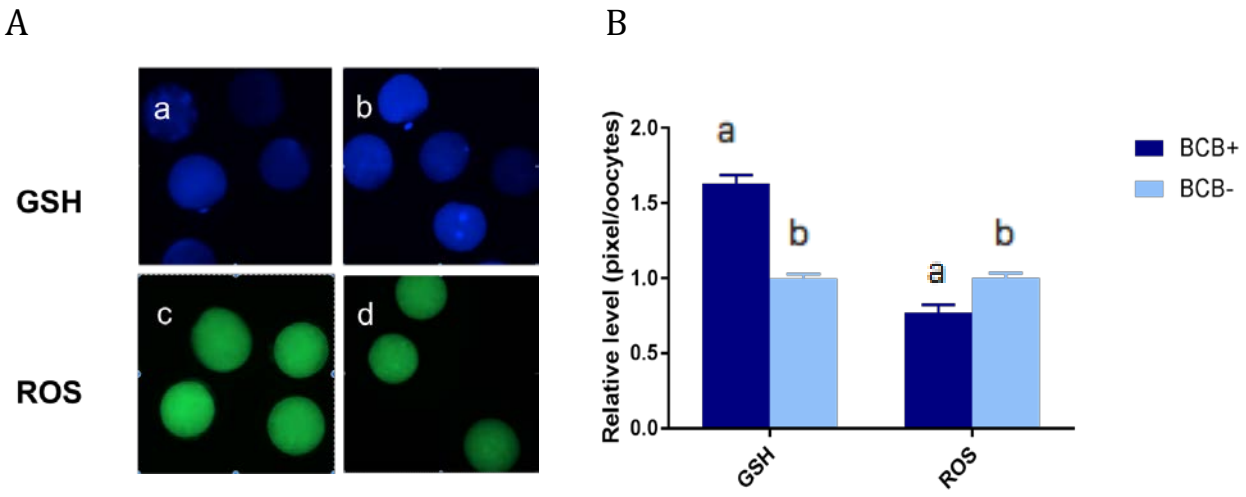

Fig. 3. Fold change of gene expression of BCB+ and BCB− oocytes relative to the housekeeping gene assessed at the GV stage. Genes are grouped according to their function: (A) imprinting, (B) pluripotency, (C) housekeeping, (D) cell cycle, (E) transcription factors, (F) apoptosis, (G) oogenesis, (H) metabolism, and (I) growth factors. [a, b]Different superscripts indicate a significant difference between two groups of oocytes ($P < 0.05$) and FC > 1.5.

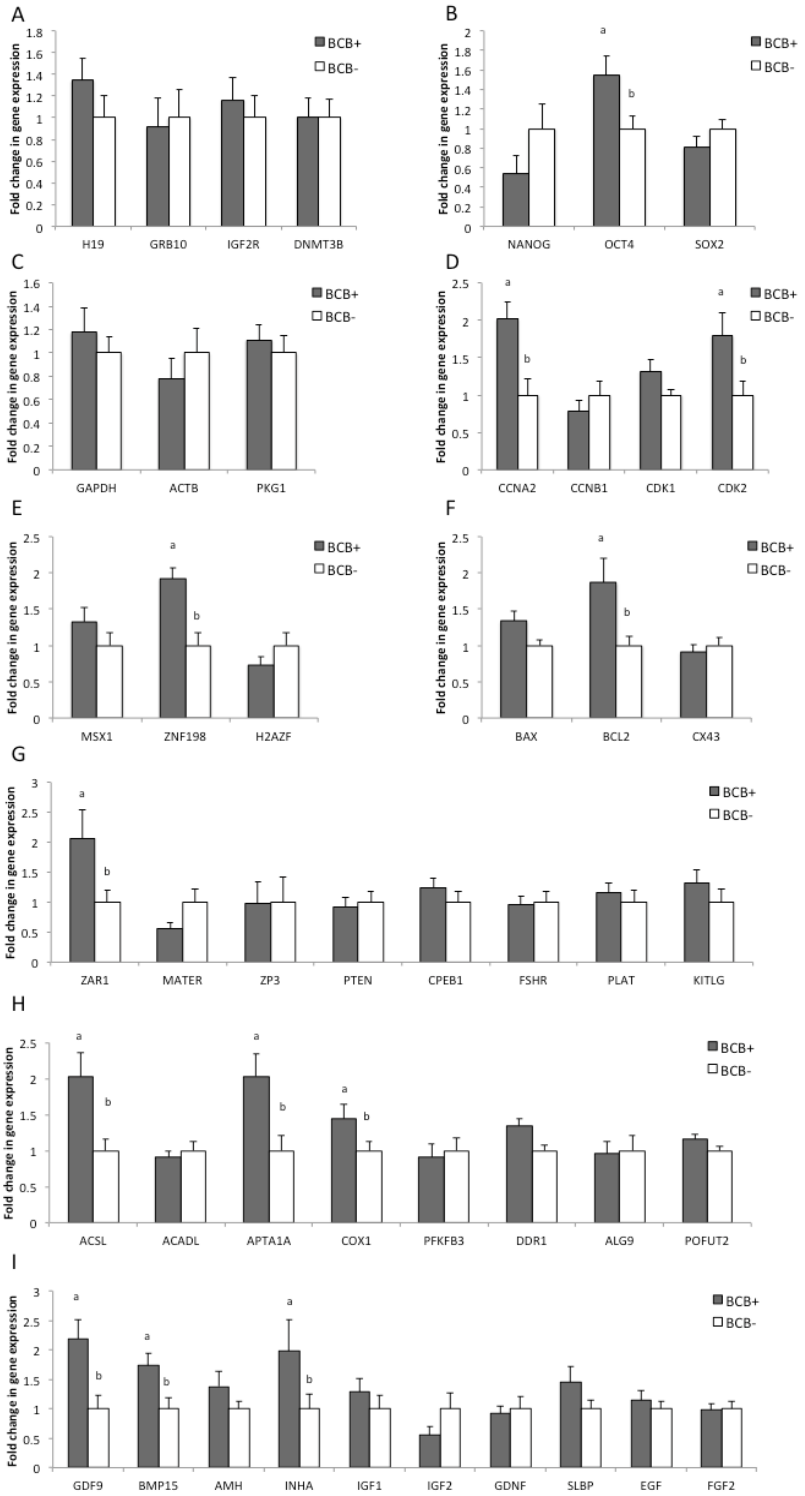

Fig. 4. Fold change of gene expression of BCB+ and BCB− oocytes relative to the housekeeping gene assessed at the MII stage. Genes are grouped according to their function: (A) imprinting, (B) pluripotency, (C) housekeeping, (D) cell cycle, (E) transcription factors, (F) apoptosis, (G) oogenesis, (H) metabolism, and (I) growth factors. [a, b]Different superscripts indicate a significant difference between two groups of oocytes ($P < 0.05$) and FC > 1.5.

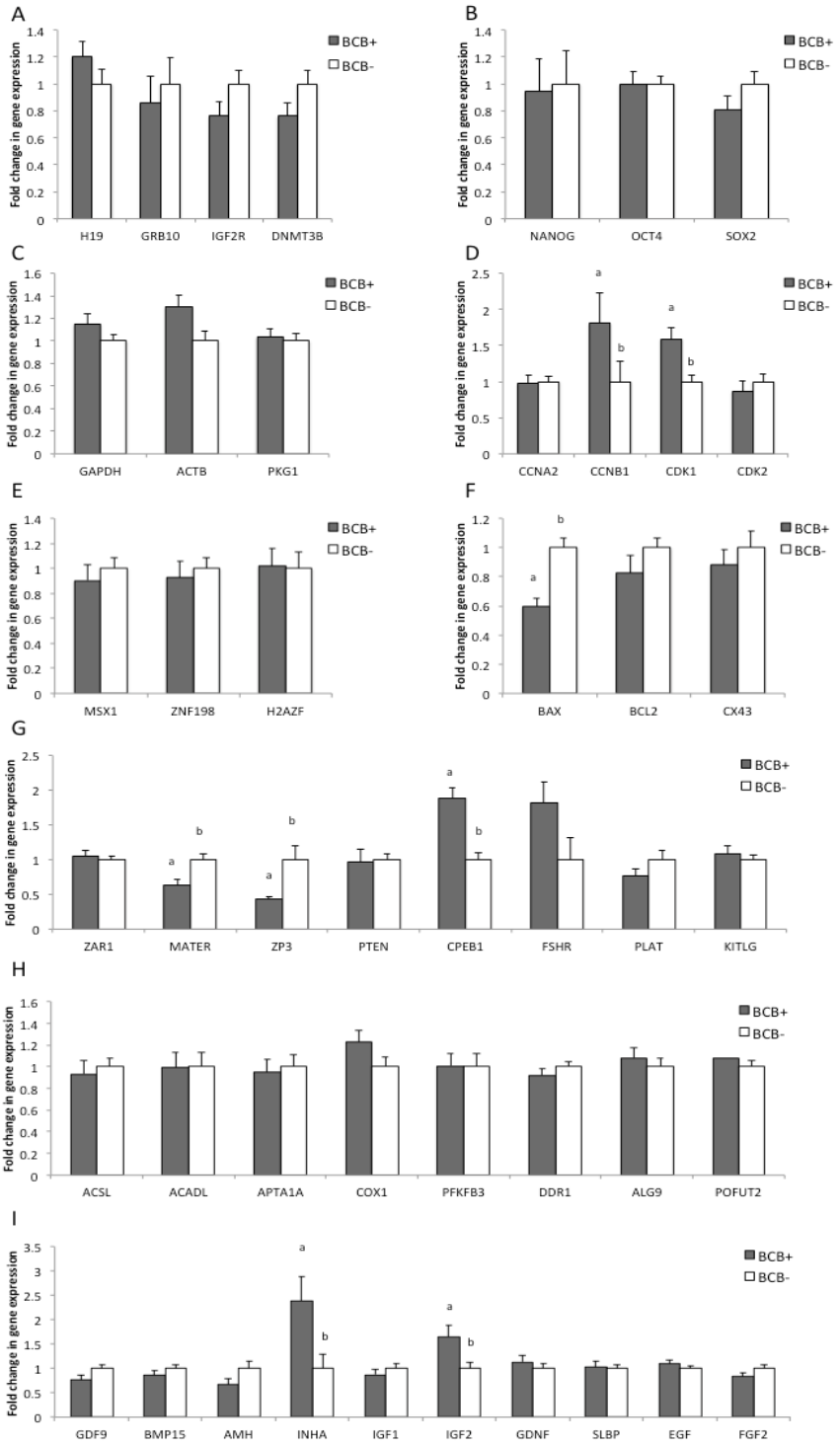

Table 1. The sequence of primers for quantitative real-time PCR

Gene	5'- primer sequence-3' (forward, reverse)	Product size (bp)
ACTB	CCTGACGGACTACCTCATGAA, GATGTCACGGACGATTTCCC	85
GAPDH	GATGTCACGGACGATTTCCC, GTACTCAGCACCAGCATCAC	69
IGF2R	TGTCCCGACGAAGTGAAGAC, GAGAGGCTGGACAGGTTGAA	80
CDK2	TGCTGCACTACGACCCTAAC, TGGCTTGGTCACATCTTGGAA	80
FSHR	TACGCCATCTTCACCAGGAAC, TGGGCTTGCACTTCATAGCA	80
GDF9	TTGTGGCCCCACACAAATAC, AGAGCCATACCGATGTCCAA	77
LHR	TGAAAGCACAGCAAGGAGAC, AATCCCAGCCACTCAGTTCA	80
ZAR1	TGGCTACTATCACTGCAAGGAC, TGCCTTGTACACACCACACA	68
EGF	GCTCTTGGTGGTATGGAAGTGAA, GCCGCTTATCAAGCACATCC	85
ACADL	CTAGGAGAGCACACCATTATCC, ACTGCTCGCAAATAAAAGTCAC	88
ZP3	ATCTTCCTGGGGAAGGTGAA, ATCACAGTAGCCAGGCCTAA	92
SLBP	CCGACTTGGAGACGGATGAAA, ATAGGCGATGGTGTTCTTCCC	77
H2A	AGCGTATTACCCCTCGTCAC, TTGTAGCCTTGATGAGAGAGTCC	75
PKG1	AGGTGGTGAAAGCCACTTCC, GTGTTCCATTTGGCACAGCAA	82
CPEB1	TTGAGCTTCCTGCCTCTGG, CTCTAATGGAGGGTGCTGGAAA	70
ZNF198	ATGCTCTAGCTCTGCAGACA, CCGTACGAGCATGTTTTCCA	82
COX1	GCATCCAAACGAGAGGTCCTAA, GGGCATCCGTTCAGTCATTCTA	68
ALG9	CTGGCCAGCTGCATTTCA, CTCACAAAAGCCAGCAGACA	87
CCNA2	ACCCTGCATTTGGCTGTGAA, AGCAGTGCCCACAAGTTGAA	87
POFUT2	CCTCCGCAGAGATGTCTACA, CACCCACTCCTCTGTCTTCA	70
MSX1	CACCGAGACGCAGGTGAA, GGCCATCTTCAGCTTCTCCA	91
PLAT	GAGGCTCACGTCAGGTTGTA, ACACAGCATGTTGTTGGTGAC	84
FGF2	CCGGTCAAGGAAATACTCCAGT, TTCTGCCCAGGTCCTGTTTT	87
H19	CTGGTCTCTGAGCGTGTGTAA, GAGGAGGCCGACACATTCA	61
CX43	GGGCTTGCTGAGAACCTACA, TGTACCACTGGATCAGCAAGAA	80
PFKFB3	ATCTCCAGCCCGGATTACAA, TGGTAACTGGCCTCATAGCA	89
DDR1	CGACTGGTTCGCTTCTATCCC, TCCTTCCAGAGGCAGCCATA	83
DNMT3B	TCCTCAAAGAACTGGGCATCA, CTTAACGGTGCCAACAGCAA	83
GDNF	TCACCGCCGTGCATCTAAA, AGCCGCTGCAGTACCTAAAA	84
ATP1A1	ACGCCTTTCAGAACGCCTA, GCAGCATCAAGTGGCAGAAA	75
ACSL3	GTGCTCCACTTTCTGCAACC, GAGTCCATACCCCTGACCAAC	77
AMH	GGCTATGAGCAGGCCTTCC, CAGCGAAGGTGGTCAAGTCA	73
NALP5	CAGAGCACAAAGAGCCTGAC, TCACAAGGCCAAACAGGAAC	88
CCNB1	GGCAACACTTACACCAAGTTCC, TGCAGGGGTAGAAGTTGATCC	92
KITLG	CCAGGCAGTTTACTCCTGAGAA, AGAAGCCACTATCTCCAAGTCC	83
IGF2	TTCTACTTCAGCCGACCATCC, TCGGAAGCAACACTCTTCCA	72
INHA	GACTGGACAGACAGGAGACC, GACCCCTGGATGTCAGTACC	78
OCT4	TGCAGGCCCGAAAGAGAAA, ACTGCAGGAACATGCTCTCC	75
BMP15	GTTGGGCAAAAGCTCTGGAA, ACCTCTTGGCTGCTGACATA	75
IGF1	TGTGGAGACAGGGGCTTTTA, CAGCACTCATCCACGATTCC	92
PPIA	ACTGGTGGCAAGTCCATCTA, ACAAGATGCCAGGACCTGTA	79
CDK1	AAGTGTGGCCAGAAGTGGAA, GATGCTAAGCTTCCTGGTTTCC	76
BAX	TGGCGGCTGAAATGTTTTCC, CCAGTTTGCTGGCAAAGTAGAA	78
BCL2	ATGTGTGTGGAGAGCGTCAAGGTT, CAGGTACTCGGTCATCC	79
PTEN	AAAGCTGGAAAGGGACGAAC, TAGGGCCTCTTGTGCCTTTA	84
GRB10	GTGAAGGAGCTGGGAAGGAAA, AAGCTCCCTTTGTGGAGCAA	85
SOX2	ATGAAGGAGCACCCGGATTA, CCAGGCAGTGTGTACTTATCC	83
NANOG	CTGGGAACTGCTGGGGAAAA, TCAGTGATTTGCTGCTGGGAA	77

Table 2. The effect of G6PDH activity on *in vitro* maturation.

Groups of oocytes	Oocytes, n	MII oocytes, n	Maturation rate, n(%)
BCB+	1287	702	53.7 ± 11.9^{a}
BCB−	1622	311	20.36 ± 9.1^{b}
Total	2909	1033	

Values of maturation rate are means ± SEM of 16 experimental replicates expressed as percentages. Values within columns with a different superscript are significantly different at $P < 0.05$ (a, b).

Table 3. The effect of G6PDH activity on embryonic development of parthogenetically activated oocytes.

Groups of oocytes	Oocytes for PA, n	Cleaved oocytes, n (%)	Morula/cleaved oocytes, n (%)	Blastocyst/cleaved oocytes, n (%)
BCB+	136	108 (79.4±5.6)[a]	44 (40.7±0.8)	32 (23.5±4.2)[a]
BCB−	76	44 (57.9±4.0)[b]	12 (27.3±3.9)	4 (5.3±2.0)[b]

Values in parentheses are means ± SEM of 4 experimental replicates expressed as percentages.

Values within columns with a different superscript are significantly different at $P < 0.05$ ([a, b]).

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CHAPTER IV

EFFECTS OF THREE DIFFERENT MEDIA ON IN VITRO MATURATION AND DEVELOPMENT, INTRACELLULAR GLUTATHIONE AND REACTIVE OXYGEN SPECIES LEVELS, AND MATERNAL GENE EXPRESSION OF ABATTOIR-DERIVED GOAT OOCYTES[1]

Abstract

This study was designed to compare the behavior of abattoir-derived goat oocytes submitted to different *in vitro* maturation (IVM) treatments. Cumulus-oocyte complexes collected from abattoir ovaries were subjected to IVM in three maturation media, which consisted of TCM199 supplemented either with: (1) 10 ng/ml EGF and 100 μM cysteamine (Defined medium); (2) 10% FCS, 5 μg/ml FSH, 10 μg/ml LH, 1 μg/ml estradiol (FCS medium); or (3) combined components of both Defined and FCS media (Combined medium). After 22 hours of IVM, oocyte maturation rate was significantly higher in Combined medium compared to FCS medium (52.0 ± 2.0% vs. 37.7 ± 3.2%, $P < 0.05$); however, no significant difference ($P > 0.05$) was observed in maturation rates between Defined (43.4 ± 4.7%) and FCS or Combined medium. There was no significant difference in the rates of cleavage, blastocyst, or blastocyst cell number among different maturation conditions. The intracellular glutathione (GSH) level was observed to be significantly higher ($P < 0.05$) in oocytes matured in Combined medium compared to

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either Defined or FCS medium. The level of intracellular reactive oxygen species (ROS) was lower ($P < 0.05$) in Defined medium compared to both FCS and Combined media. Relative mRNA expression analysis of 39 developmentally associated genes revealed that four genes (MATER, PLAT, NANOG, and CCNB1) were differentially expressed between the groups. In summary, our results indicate that similar *in vitro* maturation rates and developmental ability can be obtained when Defined and Combined maturation media were used and the supplementation of FCS medium with EGF and Cysteamine improves IVM efficiency of goat oocytes.

Introduction

In vitro maturation (IVM) has become a routine technique performed to produce matured oocytes for embryonic research because a large number of oocytes can be recovered from abattoir-derived ovaries with minimal cost. During IVM, oocytes resume meiosis after their release from the follicular meiotic inhibiting environment and culture in suitable conditions (Pincus and Enzmann, 1935). However, it is recognized that the developmental capacity of IVM oocytes is compromised in comparison with that of oocytes matured in vivo (Khatun, Bhuiyan *et al.* 2011). Previous studies demonstrated that oocyte IVM media could affect embryo development, blastocyst cell number, and apoptosis (Watson et al., 2007). The lower competence of IVM oocytes could be related to culture medium components and culture conditions used in IVM (Cognie, Baril *et al.* 2003), therefore, optimizing culture media for IVM remains a necessity for enhancing IVM rates and obtaining good quality embryos.

Over the years, researchers have developed different maturation media for goat oocytes trying to enhance IVM efficiency. The generally used system is a tissue culture

medium (TCM 199) supplemented with follicle stimulating hormone (FSH), luteinizing hormone (LH), estradiol, and 10% serum (Cognie, Baril *et al.* 2003). The oocytes matured in this system have been successfully used in the production of cloned goats (Keefer, Baldassarre *et al.* 2001). However, serum in the media contains many unknown factors and often has considerable variability among sources or even among batches from the same source, leading to lack of reproducibility and a risk of pathogen contamination. A simplified and defined (without serum or follicular fluid) maturation medium, TCM 199 supplemented with epidermal growth factor (EGF) and cysteamine, has been previously developed for IVM of abattoir-derived oocytes (Souza-Fabjan, Locatelli *et al.* 2014). Though the use of defined media could eliminate variability of IVM results, the information concerning the quality of IVM oocytes after treatment with defined media, especially compared with other media, is still incipient.

Several methods are commonly used to assess the quality of IVM oocytes such as evaluation of *in vitro* development of oocytes after *in vitro* fertilization (IVF) or parthenogenetic activation (PA), detection of intracellular glutathione (GSH) and reactive oxygen species (ROS) content (Abazari-Kia, Mohammadi-Sangcheshmeh *et al.* 2014), and expression analysis of genes associated with oocyte maturation (Silva, Lazzarotto *et al.* 2014). It has been reported that the cleavage and blastocyst rates of PA embryos were higher than IVF counterparts (Ongeri, Bormann *et al.* 2001; Souza-Fabjan, Locatelli *et al.* 2014). In most studies, lower intracellular ROS and higher GSH content implies better developmental competence of IVM oocytes (Abazari-Kia, Mohammadi-Sangcheshmeh *et al.* 2014; Mukherjee, Malik *et al.* 2014). Meanwhile, the change of GSH/ROS content and developmental potential could be associated with differential expression of genes

involved in proliferation, metabolism, oxidative stress, and apoptosis (Silva, Lazzarotto *et al.* 2014). However, to our knowledge, no systematic study has been performed to compare gene expression profiles of goat oocytes after IVM using different maturation media. Therefore, the present study was designed.

Materials and Methods

Chemicals

Unless otherwise stated, all chemicals and reagents used in the present study were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

Collection and *in vitro* maturation (IVM)

Ovaries from domestic goats (*Capra aegagrus hircus*) at the age of less than or equal to 6 months, were obtained from a local abattoir (Springville, UT). The ovaries were transported to the laboratory at 25 ± 2°C in saline solution containing 100 IU/ml penicillin G and 100 μg/ml streptomycin sulphate. After thorough washing in warm saline solution, the surface of the ovaries were sliced to release immature cumulus-oocyte complexes (COCs) in TL-Hepes medium (Sirard, Parrish *et al.* 1988). The COCs with at least three layers of compact cumulus cells were selected and washed three times in one of three maturation media. The maturation medium consisted of TCM 199 supplemented either with: (1) 10 ng/ml EGF and 100 μM cysteamine (Defined medium; (Rodriguez-Dorta, Cognie *et al.* 2007; Souza-Fabjan, Locatelli *et al.* 2014); (2) 10% fetal calf serum (FCS), 5 μg/ml FSH, 10 μg/ml LH, 1 μg/ml estradiol (FCS medium; (Reggio, James *et al.* 2001); or (3) combined components of both defined and FCS media. Groups of 50 COCs were transferred into 500 μl of maturation media and subjected to IVM in a humidified atmosphere of 5% CO_2 in air at 38.5°C in an incubator. Nuclear maturation status was

assessed 22 h after maturation culture by the presence of the first polar body in the perivitelline space, which is the key feature of the metaphase II stage (MII) oocytes.

Parthenogenetic activation (PA)

Twenty-two hours after maturation, oocytes from each of the medium groups were submitted to PA. Cumulus cells were removed from matured oocytes by vortexing the COCs for 1-2 min in TL-Hepes containing 1 mg/ml hyaluronidase. After vortexing, the oocytes were washed three times in synthetic oviductal fluid (SOF) medium (Tervit, Whittingham *et al.* 1972). The MII oocytes with apparent first polar bodies were transferred into 25 µl droplets of SOF supplemented with 7.5 µg/ml of cytochalasin B and incubated for 50 min. Then, they were activated for 5 min in SOF containing 10 µM of ionomycin. Finally, oocytes were transferred into SOF containing 2 mM 6-dimethylaminopurine and 10 µg/ml cyclohexamide and incubated for 4.5 h.

In vitro culture (IVC) of parthenogenetic embryos

After PA, the embryos were washed three times in SOF and cultured in groups of 15-20 embryos in 25 µl droplets of SOF covered by mineral oil. The embryos were cultured at 38.5°C in a humidified atmosphere of 5% O_2, 5% CO_2, and 90% N_2. Culture dishes were pre-equilibrated for at least 5 h in an incubator before use. The efficiency of development was evaluated as the percentage of cleaved embryos 2 days after PA and the percentage of blastocysts 8 days after PA on the basis of the number of cleaved embryos at Day 2. On Day 8, all expanded or hatched blastocysts were transferred into washing plates, washed to remove the mineral oil, fixed and stained with Hoechst to count their total cell number. Cell counting was conducted under a fluorescence microscope.

Measurement of intracellular GSH and ROS levels in matured oocytes

The oocytes matured in three maturation media were collected separately to assess the intracellular GSH and ROS levels using a previously described fluorescence assay (Kwak, Cheong *et al.* 2012). Briefly, CellTracker Blue CMF2HC (4-chloromethyl-6.8-difluoro-7-hydroxycoumarin, Invitrogen, Eugene, OR) and H2DCFDA (2',7'-dichlorodihydrofluorescein diacetate, Invitrogen, Eugene, OR) were used to detect the intracellular GSH and ROS concentrations in oocytes under the blue and green fluorescence, respectively. Seven to 10 MII oocytes from each treatment were incubated in H199 medium supplemented with 20 μM CellTracker Blue CMF2HC or 10 μM H2DCFDA for 20 min at 38.5°C in the dark and then washed three times with D-PBS containing 0.1% (w/v) PVA. The oocytes were then placed into 10 μl droplets. The fluorescence was observed with ultraviolet filters (370 nm for GSH and 460 nm for ROS) and images were captured using a fluorescence microscope. The fluorescence intensity of oocytes was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD).

Gene expression analysis of matured oocytes by the Single-oocyte Real-time PCR

Expression levels of 39 development-associated genes were analyzed in oocytes matured in three maturation media. Denuded MII oocytes were washed three times and stored individually in 1 μl DNA suspension buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0). Luciferase RNA (0.1 pg/oocyte, Promega, Madison, WI) was added as exogenous standard to each sample. Single-oocyte gene-expression experiments were performed using Fluidigm's 48.48 quantitative PCR (qPCR) Dynamic Array Integrated Fluidic Circuits (IFC; Fluidigm, San Francisco, CA) as per the manufacturer's recommended

protocol. The specific primers were designed and synthesized for qPCR amplification of the luciferase gene as well as the other 39 development-associated genes. Reverse transcription-specific target amplification (RT-STA) was performed for each single oocyte sample to convert the RNA to cDNA and enrich target-specific cDNA prior to quantitative PCR. In the preparation for RT-STA, a 200 nM pooled STA primer mix was made by diluting all of the 39 pairs of primers in the DNA suspension buffer. Each individual oocyte (stored in DNA suspension buffer) was added with 9 μl of RT-STA solution (5 μl of CellsDirect PCR mix (Invitrogen, Carlsbad, CA), 0.2 μl SuperScript-III RT/Platinum Taq (Invitrogen, Carlsbad, CA), 2.5 μl of 200 nM pooled primer mix and 1.3 μl DNA suspension buffer). The RNA from the oocyte was then extracted and reverse transcribed into cDNA (50°C for 15 min, 95°C for 2 min). The cDNA was subsequently pre-amplified for 20 cycles (each cycle: 95°C for 15 s, 60°C for 4 min). After the RT-STA cycling, 4 μl ExoSAP-IT (ExoI; New England Biolabs, Beverly, MA) was added to every RT-STA reaction in order to remove any unincorporated primers and then placed in the thermal cycler at 37°C for digestion, followed by 15 min inactivation at 80°C. After ExoSAp-IT treatment, each reaction was diluted 5-fold by adding 36 μl of DNA suspension buffer and stored at −20°C until use. The Fluidigm 48.48 chip was primed before use according to the manufacturer's directions. A 2 μl aliquot of diluted reaction sample was mixed with 2.5 μl of the 2x TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA), 0.25 μl of 20x DNA Binding Dye Sample Loading Reagent (Fluidigm, San Francisco, CA), and 0.25 μl EvaGreen DNA Binding Dye (Biotium, Hayward, CA). The 5 μl sample pre-mix solution was then inserted into one of "sample" inlets of the primed chip. An assay (primer) mix solution was made for every

primer set by using 2.5 µl of the 2x Assay Loading Reagent (Fluidigm, San Francisco, CA), 1.25 µl DNA suspension buffer, and 1.25 µl of each 20 µM Forward and Reverse Primer Mix and then individually inserted into one of the "assay" inlets of the primed chip. The chip was then returned into the IFC Controller Chip Loading (Load Mix (113x) script) and placed in the BioMark real-time PCR reader for quantitative PCR. Cycling parameters included a 5 min initial enzyme activation at 95°C, 35 denaturation/extension cycles (95 for 15 s followed by 60°C for 60 s), and a 3 min final extension cycle at 60°C. The real-time PCR data were presented as the fold change based on Ct values of three treatment groups in target gene expression and previously described $2^{-\Delta\Delta Ct}$ methodology (Livak and Schmittgen 2001) was used for relative quantification.

Experimental design

Experiment 1. Effect of different maturation media on IVM and *in vitro* development of goat oocytes

The COCs from abattoir ovaries were divided into three groups and subjected to IVM in three different maturation media. Nuclear maturation status was assessed by the presence of the first polar body in the perivitelline space of oocytes. Twenty-two hours after maturation culture, maturation rates were recorded for each medium group. The matured oocytes were parthenogenetically activated and cultured *in vitro* in SOF media. Cleavage rates and blastocyst developmental rates were determined for each group at day 2 and day 8 after PA, respectively. Totally, 4 rounds of oocyte collection were carried out and 608 COCs were used for IVM followed by *in vitro* development detection. Each round of oocyte collection was considered as an experimental replicate.

Experiment 2. Effect of different maturation media on intracellular GSH and ROS levels in matured MII oocytes

The intracellular GSH and ROS levels, considered to be closely related to developmental competence of oocytes, were evaluated by using specific fluorescence assays. After IVM, MII oocytes matured in different media were collected and stained. Totally, 193 MII oocytes from 3 rounds of IVM were used for intracellular GSH and ROS detection.

Experiment 3. Effect of different maturation media on maternal mRNA levels of matured MII oocytes

The association between maturation medium composition and mRNA levels of 39 development-associated genes in MII oocytes was examined using real-time PCR. These selected genes fell into one of nine functional categories: imprinting (H19 and IGF2R), metabolism (ACSL, ATP1A1, ACADL, ALG9, and POFUT2), pluripotency (OCT4 and NANOG), growth factor (GDF9, BMP15, INHA, IGF1, GDNF, SLBP, EGF, and FGF2), housekeeping (GAPDH, ACTB, PPIA, PKG1; (Cheng, Chang *et al.* 2011; O'Connor, Wilmut *et al.* 2013), cell cycle (CCNA2, CCNB1, CDK1, and CDK2), transcription (MSX1,H2AFZ, and ZNF198), apoptosis (BCL2, BAX, and CX43) and oogenesis (ZAR1, MATER, ZP3, PTEN, CPEB1, KITLG, FSHR, and PLAT). The relative gene expression was compared among oocytes originated from different media treatments. Each treatment consisted of 30 oocytes collected from 3 separate rounds of IVM. Real-time PCR was conducted on individual oocyte.

Statistical analysis

The data from IVM and IVC were subjected to arcsine transformation and analyzed by One-way ANOVA. A *P* value of < 0.05 for the effect of the factor (maturation media) was considered significant. When the factor had significant effect on the parameters, a post hoc procedure with LSD tests was used for multiple comparisons between the groups. Data on gene expression and levels of GSH or ROS were analyzed using the Student *t*-test. Differences were considered to be significant if $P < 0.05$ for GSH or ROS data. For the gene expression data, the following requirements have to be met: $P < 0.05$ and the fold change > 2 to be considered as a significant difference for up-regulated or down-regulated genes. All data were analyzed with a computer program, SPSS for Windows (Version 16.0).

Results

Experiment 1.

The effect of different maturation media was assessed on maturation rates of goat oocytes and results were summarized in Table 1. No difference was detected in nuclear maturation rates between Defined and FCS or Defined and Combined media; however, oocyte maturation rate was significantly higher using Combined medium compared to FCS medium (52.0 ± 2.0% vs. 37.7 ± 3.2%, $P < 0.05$).

The oocytes matured in different maturation media were parthenogenetically activated, then cultured in SOF media for 8 days. As shown in Table 2, no significant difference was found in cleavage rates among the different treatments (91.3% on average). Though not significantly different, slightly higher blastocyst rates were

observed in oocytes matured in Defined and Combined medium (19.7 ± 5.0% or 17.8 ± 6.3%, respectively) than those in FCS medium (13.5 ± 4.6%). Furthermore, no significant effect of maturation media was observed on the total blastocyst cell number among the groups (Table 2). Representative parthenogenetically derived blastocysts and a Hoechst stained blastocyst were shown in Fig. 1.

Experiment 2.

The results for the intracellular GSH and ROS levels were shown in Fig. 2. After IVM, significantly higher ($P < 0.05$) intracellular GSH level was detected in MII oocytes matured in Combined medium than Defined or FCS medium. The lowest intracellular ROS level was observed in MII oocytes matured in Defined medium compared to those in FCS or Combined medium ($P < 0.05$).

Experiment 3.

When the mRNA levels of 39 genes were analyzed, 35 genes showed no significant difference among the three media and only four genes were differentially expressed including MATER and PLAT genes involved in oogenesis, NANOG in pluripotency, and CCNB1 in cell cycle (Fig. 3). The mRNA of the two oogenesis associated genes were significantly higher in oocytes matured in FCS medium compared to those matured in Defined medium for MATER ($P < 0.05$) and in Combined medium for PLAT ($P < 0.01$). The expression of NANOG was similar in FCS and Combined groups, while significantly higher in Defined medium group ($P < 0.05$). The expression of CCNB1 was significantly lower in Combined group ($P < 0.01$) compared to that in Defined group. Table 3 summarized the primers for gene expression analysis including

luciferase and 39 developmentally associated genes.

Discussion

The main objective of the present study was to compare the behavior of goat oocytes recovered from abattoir ovaries subjected to three different IVM treatments. The data in our study indicate that it is possible to achieve similar nuclear maturation rates and blastocyst development using defined maturation media compared to undefined media. Our results are in agreement with a previous report in goats showing that defined, semi-defined and undefined maturation media promoted similar maturation, cleavage and embryo development (Souza-Fabjan, Locatelli *et al.* 2014). The use of a defined maturation medium is helpful to compare maturation data among different laboratories and to further elucidate the metabolic and nutritional requirements of mammalian oocytes and embryos produced *in vitro*. However, the present results are not consistent with the report in which a significant difference was observed in cleavage and blastocyst development rates for ovine oocytes matured in defined versus undefined media (Shabankareh and Zandi 2010). Serum or follicular fluid contains many components, including proteins, fatty acids, vitamins, trace elements, hormones, and growth factors, which may be responsible for enhanced viability of oocytes (Harper and Brackett 1993). Other studies also indicated that FCS had a beneficial effect on *in vitro* fertilization of bovine IVM oocytes (Im and Park 1995) and possibly some serum components were necessary for the completion of cytoplasmic maturation of bovine and ovine oocytes *in vitro* (Sakaguchi, Dominko *et al.* 2000; Shabankareh and Zandi 2010). In addition, in our study lower GSH level was observed in goat oocytes matured in Defined medium compared to that in Combined medium. Intracellular GSH is a molecular marker of

cytoplasmic maturation of oocytes and low level of GSH is closely associated with incomplete cytoplasmic maturation (Abazari-Kia, Mohammadi-Sangcheshmeh *et al.* 2014; Lee, Jin *et al.* 2015). It is noteworthy that lower intracellular GSH level did not lead to damaged development capacity in both Defined and FCS medium groups (Table 2). The intracellular GSH detection may be more sensitive for assessing the quality of IVM oocytes than the *in vitro* development assay and could discriminate subtle changes of GSH levels among different treatments (Lee, Jin *et al.* 2015). Thus, we suggest that there is still a need for optimizing the Defined medium even though no difference was found in nuclear maturation rates and blastocyst development between Defined and undefined media.

In our study the Combined medium (FCS medium supplemented with EGF and cysteamine) promoted oocyte maturation and enhanced the intracellular GSH level compared to the FCS medium. It has been shown in other studies that EGF induces not only nuclear maturation but also cytoplasmic maturation of cumulus-enclosed oocytes (Coskun, Sanbuissho *et al.* 1991; Guler, Poulin *et al.* 2000; Park, Iga *et al.* 1997). The EGF is considered to accelerate the meiotic cell cycle of oocytes, possibly by increasing histone and mitogen-activated protein kinase activities during the early stages of IVM (Purohit, Brady *et al.* 2005). The benefit of supplementing maturation media with EGF has been found in sheep (Guler, Poulin *et al.* 2000; Shabankareh and Zandi 2010), pig (Abeydeera, Wang *et al.* 1998), bovine (Lorenzo, Illera *et al.* 1994) and buffalo (Purohit, Brady *et al.* 2005). The addition of cysteamine in maturation medium has also shown to have a positive influence on embryo development (Gasparrini, Neglia *et al.* 2000; Gasparrini, Sayoud *et al.* 2003). Several studies have shown that cysteamine increases

cytoplasmic GSH level in bovine (de Matos, Furnus *et al.* 1997; de Matos, Furnus *et al.* 1996), and ovine (de Matos, Gasparrini *et al.* 2002) oocytes. It is likely that the enhanced GSH concentration promotes the completion of oocyte cytoplasmic maturation (Gasparrini, Boccia *et al.* 2006; Gasparrini, Sayoud *et al.* 2003), which consequently facilitates early embryonic development. The positive effects of combining both EGF and cysteamine in the basic maturation medium on cleavage and development have been reported in sheep oocytes (Shabankareh and Zandi 2010). Moreover, a maturation medium, in which main components are similar to those in the Combined medium in our study, has been used successfully for the production of cloned goats (Hosseini, Hajian *et al.* 2015). Therefore, we presume that the Combined medium should be more suitable for IVM of goat oocytes than the commonly used FCS medium.

It has been widely accepted that IVM and developmental competence of oocytes are greatly influenced by the levels of intracellular GSH and ROS (Jiao, Cao *et al.* 2013; Luberda 2005; Mukherjee, Malik *et al.* 2014). The intracellular GSH of oocytes can be regulated by multiple modulators (Luberda 2005). It has been proved that the addition of cysteamine (de Matos, Furnus *et al.* 1995) or cysteine and EGF (Kishida, Lee *et al.* 2004) to IVM medium increases GSH level in oocytes. In our study, oocytes cultured in Combined medium had the highest intracellular GSH level after IVM, indicating that adding cysteamine and EGF to the FCS medium enhances *in vitro* cytoplasmic maturation in goats. The GSH is a natural antioxidant in oocytes and plays an important role in protecting the oocyte from oxidative damage (Ali, Bilodeau *et al.* 2003). In most cases, the increase of intracellular GSH level is accompanied by the decrease of the intracellular ROS level in oocytes after IVM, and vice versa (Kwak, Cheong *et al.* 2012;

Mukherjee, Malik *et al.* 2014). However, in our study oocytes from Combined medium have both high GSH and ROS levels, while oocytes from Defined medium have both low GSH and ROS levels. The ROS levels were significantly higher in oocytes cultured in FCS and Combined media compared to Defined medium. This finding indicates that one or more components in the FCS medium lead to the up-regulation of ROS during IVM. A study in rats showed that LH treatment was associated with an increase in ROS in follicles (Yacobi, Tsafriri *et al.* 2007). We assume that supplementation with LH might cause the increased level of intracellular ROS in oocytes cultured in FCS and Combined media. In oocytes, high concentration of ROS may impair intracellular environment and lead to disturbed metabolism, which may cause oocyte apoptosis (Yang, Hwang *et al.* 1998) , meiotic arrest (Nakamura, Yamagata *et al.* 2002), embryonic block and death (Hashimoto, Minami *et al.* 2000). On the other hand, previous studies have documented that variation in ROS production might be attributed to the difference of oocyte and early embryo metabolic activities during IVM and embryo development (Dalvit, Cetica *et al.* 2005; Morado, Cetica *et al.* 2009). The production of ROS in the ovary is an essential preovulatory signaling event probably triggered by LH surge (Shkolnik, Tadmor *et al.* 2011). Whether relatively higher level of ROS is negatively associated with oocyte developmental competence is still unclear. We speculate that the intracellular ROS concentration might need to pass a threshold to lead to initiation of apoptosis.

The developmental competence of *in vitro* matured oocytes has been reported to be lower than that matured in vivo and the low fertilization and developmental rates have been attributed to defective cytoplasmic maturation (Leoni, Bebbere *et al.* 2007). The difference in gene expression profile of oocytes matured in various media might give us

an indication of what causes the difference in the maturational competence and indicate intrinsic properties of those oocytes. In our study, the majority of the genes (35 of 39) stayed consistently expressed among oocytes matured in three maturation media except for these four genes: MATER, NANOG, CCNB1, and PLAT. MATER is identified as a maternal effect gene required for embryonic development beyond the two-cell stage (Pennetier, Perreau *et al.* 2006; Tong, Gold *et al.* 2000). The mRNA of MATER has been observed to decrease strongly during maturation, and progressively during embryo cleavage stages (Pennetier, Perreau *et al.* 2006). MATER protein is first detected in the cytoplasm of growing oocytes and remains present through the late blastocyst stage (Tong, Gold *et al.* 2000). Our results showed the MATER mRNA was higher in FCS group, but the maturation rate was lower. It is possible that EGF and cysteamine (the common components in Defined and Combined media) promote earlier maturation of oocytes, thus, more MATER mRNAs were translated into protein. The lower maturation rates also account for the higher expression of PLAT in FCS group. The PLAT encodes a serine protease, tissue-type plasminogen activator (tPA), which plays roles in various reproductive processes including ovulation and implantation (Bartlett and Menino 1993; Park, Choi *et al.* 1999). Huarte et al. (1985) found that the PLAT mRNA was present in primary oocytes and resumption of meiosis in mouse and rat oocytes triggered the production of tPA. Therefore, higher expression of MATER and PLAT in the FCS medium might be related to incomplete cytoplasmic maturation of oocytes. The oocytes matured in Combined medium had lower mRNA level of CCNB1 (Cyclin B1) in our study. In mammalian oocytes, resumption of cell cycle is stimulated by the change of maturation promoting factor, a heterodimer consisting of kinase, cdk1 and its regulatory

partner cyclin (Marangos and Carroll 2004). Cyclin synthesis during mitotic cell cycles is driven by ubiquitin-dependent, proteasome-mediated destruction of Cyclin B1 (Glotzer, Murray *et al.* 1991). Kotani et al. (2013) provided the evidence that cyclin B1 mRNA translation was temporally regulated through the formation and disassembly of RNA granules during oocyte maturation. Cyclin B1 RNA destruction is an important requirement in the progression from metaphase I to MII or oocyte maturation (Herbert, Levasseur *et al.* 2003), and is the most likely explanation of lower CCNB1 mRNA in Combined medium group. According to our data, we detected very low expression of NANOG in MII goat oocytes in FCS and Combined medium groups. NANOG is a transcription factor expressed in the inner cell mass, archaeocytes and embryonic stem cells (Mitsui, Tokuzawa *et al.* 2003). Our finding is not in agreement with that by (Khan, Dube *et al.* 2012), which showed the absence of NANOG mRNA in the germinal vesicle and MII stage bovine oocytes. The difference in species (goat vs. cattle) or sensitivity of detection methods might account for the discrepancy. More investigation should be conducted to elucidate possible effects of NANOG expression on the IVM of goat oocytes.

Conclusions

Our results indicate that similar maturation, cleavage, and blastocyst development rates can be obtained when Defined and Combined maturation media were used for the IVM of goat abattoir-derived oocytes. The supplementation of FCS medium with EGF and Cysteamine (Combined medium) improves both nuclear and cytoplasmic maturation of goat oocytes even though no enhanced blastocyst development was observed. The differences in GSH and ROS levels and differential expression of four developmentally

associated genes among three tested groups suggest that further research are required for optimizing maturation medium in order to improve IVM of goat abattoir-derived oocytes.

Table 1. Effect of different maturation media on the nuclear maturation of abattoir-derived goat oocytes submitted to IVM for 22 hours.

Treatment	No. of oocytes	No. of matured	Maturation rate (%) [1]
Defined [2]	196	89	43.4 ± 4.7 [a, b]
FCS [3]	199	77	37.7 ± 3.2 [a]
Combined [4]	213	112	52.0 ± 2.0 [b]

[1] The percentages were calculated based on the data from 4 replicates and described as mean ± SEM.

[2] Defined: TCM199 supplemented with 10 ng/ml EGF and 100 μM cysteamine.

[3] FCS: TCM199 supplemented with 10% FCS, 5 μg/ml FSH, 10 μg/ml LH, and 1 μg/ml estradiol.

[4] Combined: TCM199 supplemented with components of both defined and FCS media.

[a,b] Within a column, values with different superscripts differed significantly ($P < 0.05$). Data were analyzed by One-way ANOVA.

Table 2. Effect of different maturation media on the *in vitro* development of abattoir-derived goat oocytes after parthenogenetic activation

Treatment	MII oocytes, n	Cleaved embryos, n	Cleaved / matured (%)[1]	Blastocysts, n	Blastocysts/ cleaved (%)	Total blastocyst cell number, n
Defined [2]	89	80	88.4 ± 4.6 [a]	16	19.7 ± 5.0 [a]	94.4 ± 14.1 [a]
FCS [3]	77	70	93.1 ± 4.7 [a]	9	13.5 ± 4.6 [a]	105.8 ± 30.4 [a]
Combined [4]	112	103	92.5 ± 2.2 [a]	17	17.8 ± 6.3 [a]	88.1 ± 13.7 [a]

[1] The percentages were calculated based on the data from 4 replicates and described as mean ± SEM.

[2] Defined: TCM199 supplemented with 10 ng/ml EGF and 100 μM cysteamine.

[3] FCS: TCM199 supplemented with 10% FCS, 5 μg/ml FSH, 10 μg/ml LH, and 1 μg/ml estradiol.

Table 3. Sequence of primers, GenBank accession numbers, and expected product sizes of genes used for expression analysis of MII oocytes

Gene	5'- primer sequence-3' (forward, reverse)	Product size (bp)	Accession No.
H19	CTGGTCTCTGAGCGTGTGTAA, GAGGAGGCCGACACATTCA	61	AC_000186.1
IGF2R	TGTCCCGACGAAGTGAAGAC, GAGAGGCTGGACAGGTTGAA	80	NC_022301.1
ACSL3	GTGCTCCACTTTCTGCAACC, GAGTCCATACCCCTGACCAAC	77	NC_022294.1
ACADL	CTAGGAGAGCACACCATTATCC, ACTGCTCGCAAATAAAAGTCAC	88	NC_022294.1
ATP1A1	ACGCCTTTCAGAACGCCTA, GCAGCATCAAGTGGCAGAAA	75	NC_022295.1
ALG9	CTGGCCAGCTGCATTTCA, CTCACAAAAGCCAGCAGACA	87	NC_022307.1
POFUT2	CCTCCGCAGAGATGTCTACA, CACCCACTCCTCTGTCTTCA	70	NC_022293.1
NANOG	CTGGGAACTGCTGGGGAAAA, TCAGTGATTTGCTGCTGGGAA	77	NC_022297.1
OCT4	TGCAGGCCCGAAAGAGAAA, ACTGCAGGAACATGCTCTCC	75	NW_005101845.1
GDF9	TTGTGGCCCCACACAAATAC, AGAGCCATACCGATGTCCAA	77	NC_022299.1
BMP15	GTTGGGCAAAAGCTCTGGAA, ACCTCTTGGCTGCTGACATA	75	NC_000023.11
INHA	GACTGGACAGACAGGAGACC, GACCCCTGGATGTCAGTACC	78	NC_022294.1
IGF1	TGTGGAGACAGGGGCTTTTA, CAGCACTCATCCACGATTCC	92	NC_022297.1
GDNF	TCACCGCCGTGCATCTAAA, AGCCGCTGCAGTACCTAAAA	84	NC_022312.1
SLBP	CCGACTTGGAGACGGATGAAA, ATAGGCGATGGTGTTCTTCCC	77	NW_005104454.1
EGF	GCTCTTGGTGGTATGGAAGTGAA, GCCGCTTATCAAGCACATCC	85	NC_022298.1
FGF2	CCGGTCAAGGAAATACTCCAGT, TTCTGCCCAGGTCCTGTTTT	87	NC_022309.1
PPIA	ACTGGTGGCAAGTCCATCTA, ACAAGATGCCAGGACCTGTA	79	NC_022296.1
ACTB	CCTGACGGACTACCTCATGAA, GATGTCACGGACGATTTCCC	85	NC_022317.1
PKG1	AGGTGGTGAAAGCCACTTCC, GTGTTCCATTTGGCACAGCAA	82	NC_019479.1
GAPDH	GATGTCACGGACGATTTCCC, GTACTCAGCACCAGCATCAC	69	NC_022297.1
CCNA2	ACCCTGCATTTGGCTGTGAA, AGCAGTGCCCACAAGTTGAA	87	NC_022298.1
CCNB1	GGCAACACTTACACCAAGTTCC, TGCAGGGGTAGAAGTTGATCC	92	NC_022312.1
CDK1	AAGTGTGGCCAGAAGTGGAA, GATGCTAAGCTTCCTGGTTTCC	76	NC_022320.1
CDK2	TGCTGCACTACGACCCTAAC, TGGCTTGGTCACATCTTGGAA	80	NC_022297.1
MSX1	CACCGAGACGCAGGTGAA,	91	NC_022298.1

	GGCCATCTTCAGCTTCTCCA		
ZNF198	ATGCTCTAGCTCTGCAGACA,	82	NC_022304.1
	CCGTACGAGCATGTTTTCCA		
H2AFZ	AGCGTATTACCCCTCGTCAC,	75	NC_022298.1
	TTGTAGCCTTGATGAGAGAGTCC		
BAX	TGGCGGCTGAAATGTTTTCC,	78	NC_022310.1
	CCAGTTTGCTGGCAAAGTAGAA		
BCL2	ATGTGTGTGGAGAGCGTCAAGGTT,	79	NC_022316.1
	CAGGTACTCGGTCATCC		
CX43	GGGCTTGCTGAGAACCTACA,	80	NC_022301.1
	TGTACCACTGGATCAGCAAGAA		
ZAR1	TGGCTACTATCACTGCAAGGAC,	68	NC_022298.1
	TGCCTTGTACACACCACACA		
MATER	CAGAGCACAAAGAGCCTGAC,	88	AC_000175.1
	TCACAAGGCCAAACAGGAAC		
ZP3	ATCTTCCTGGGGAAGGTGAA,	92	NC_022317.1
	ATCACAGTAGCCAGGCCTAA		
PTEN	AAAGCTGGAAAGGGACGAAC,	84	NC_022318.1
	TAGGGCCTCTTGTGCCTTTA		
CPEB1	TTGAGCTTCCTGCCTCTGG,	70	NC_022313.1
	CTCTAATGGAGGGTGCTGGAAA		
FSHR	TACGCCATCTTCACCAGGAAC,	80	NC_022303.1
	TGGGCTTGCACTTCATAGCA		
PLAT	GAGGCTCACGTCAGGTTGTA,	84	NC_022319.1
	ACACAGCATGTTGTTGGTGAC		
KITLG	CCAGGCAGTTTACTCCTGAGAA,	83	NC_000012.12
	AGAAGCCACTATCTCCAAGTCC		
Luciferase[1]	AGAAGGGCGGAAAGTCCAAA,	81	
	CCCTCGGAGGATTACAATAGCTAA		

[1]The primers were designed according to the RNA sequence (L4561) provided by the Promega Corporation, Madison, WI, USA.

Fig. 1. Representative goat blastocysts obtained after *in vitro* maturation following by parthenogenetic activation. (A) Expanded (arrowheads) and hatching (arrow) blastocysts derived from goat oocytes matured and parthenogenetically activated *in vitro*. (B) A representative blastocyst stained with Hoechst for total cell counting (total cell number: 95). Scale bar equals 40 μm.

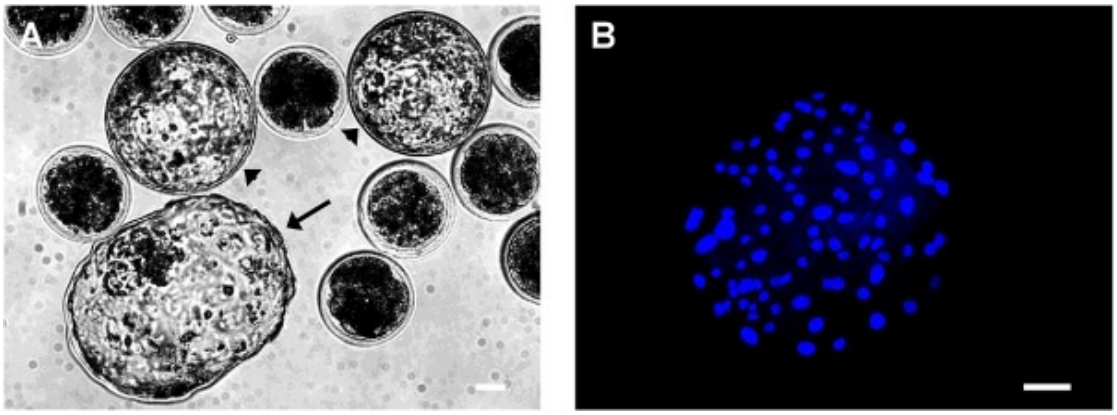


Fig. 2. Epifluorescent photomicrographic images of *in vitro* matured goat oocytes. (A) Oocytes were stained with CellTracker Blue CMF2HC (a, b, and c) and 20, 70-dichlorodihydrofluorescein diacetate (d, e, and f) to detect intracellular levels of GSH and ROS, respectively. Metaphase II oocytes were derived from Defined (a and d), FCS (b and e) and Combined (c and f) media. (B) Relative levels of intracellular GSH and ROS in MII goat oocytes matured in different maturation media. Values with different superscripts differed significantly ($P < 0.05$). GSH: glutathione; ROS: reactive oxygen species.

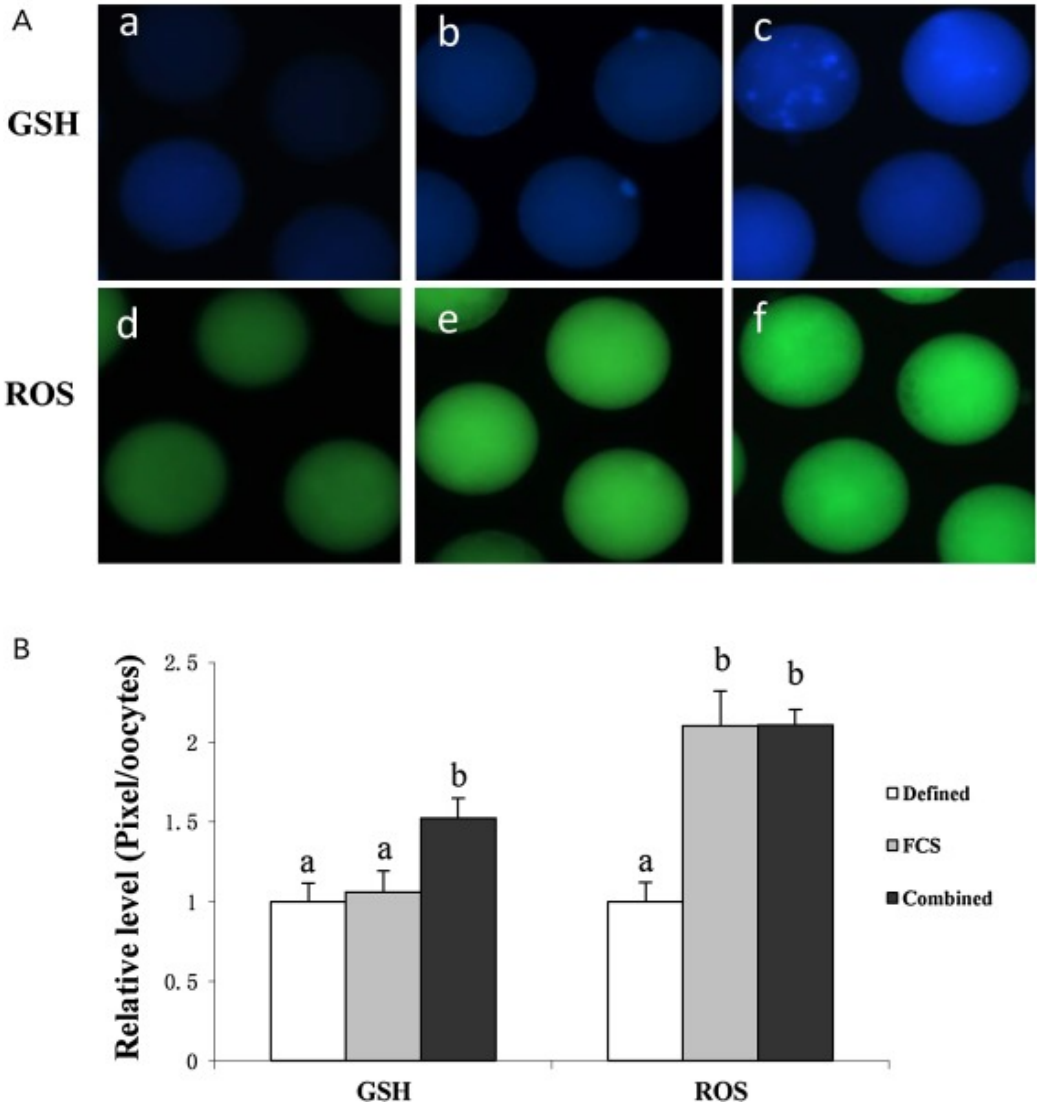
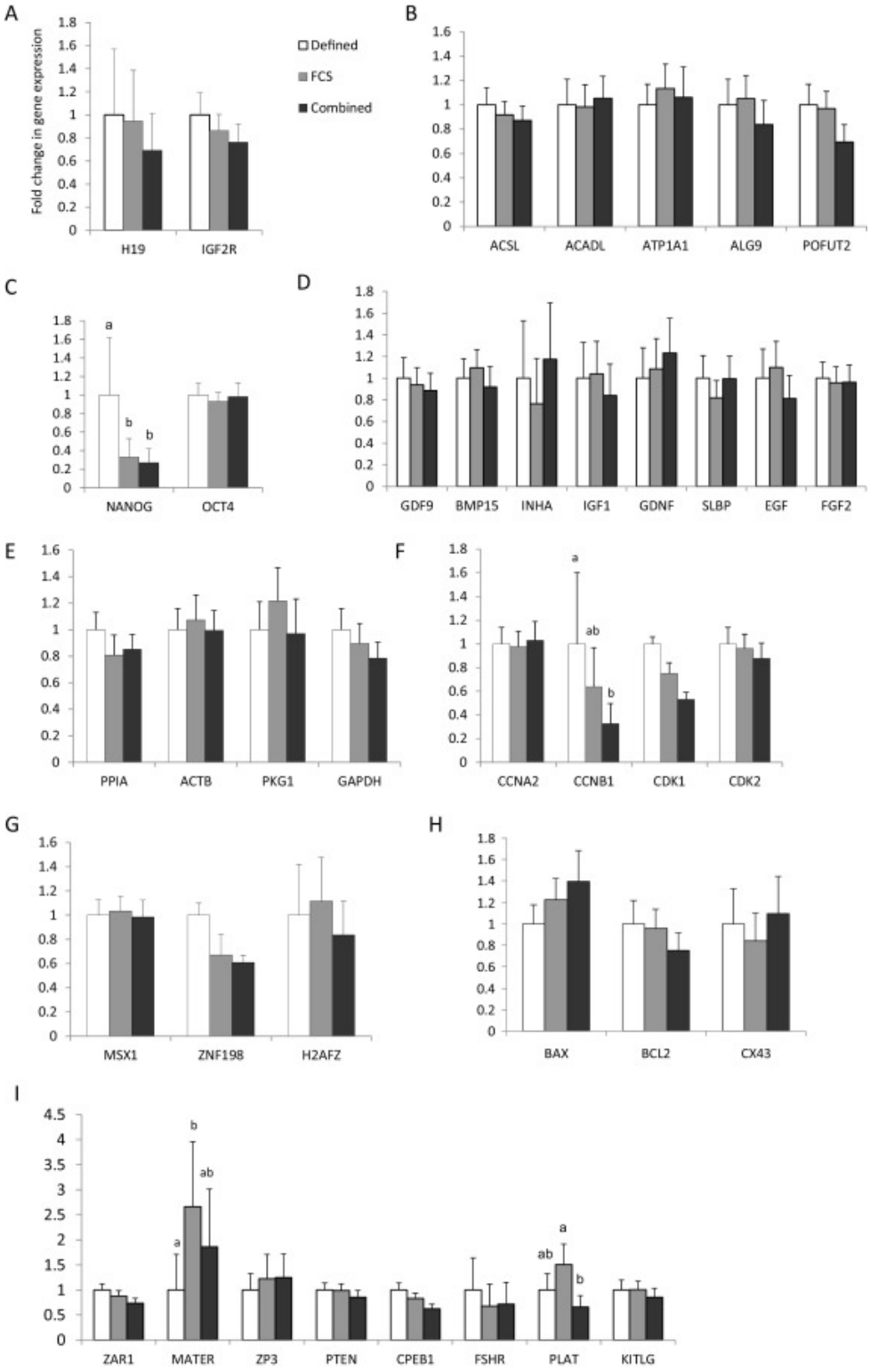


Fig. 3. Fold change (FC) of gene expression of oocytes derived from three maturation media (Defined, FCS and Combined) relative to the exogenous control luciferase gene assessed at the metaphase II stage oocytes. Genes were grouped according to their function: (A) imprinting, (B) metabolism, (C) pluripotency, (D) growth factors, (E) housekeeping, (F) cell cycle, (G) transcription factors, (H) apoptosis, and (I) oogenesis. Different superscripts (a and b) indicated a significant difference in gene expression among oocytes cultured in different maturation media ($P < 0.05$) and FC > 2.



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CHAPTER V

MICROINJECTION OF *CPEB* POLYADENYLATED mRNA INCREASED DEVELOPMENTAL COMPETENCE OF BOVINE OOCYTES

Abstract

Developmental competence is acquired during oocyte growth and maturation while oocytes undergo both nuclear and cytoplasmic changes. Completion of oocyte maturation and subsequent embryo development relies mostly on maternally synthesized and stored mRNAs at the transcriptionally quiescent phase. The temporal and spatial post-transcriptional and translational regulation of the stored mRNA in mammalian oocyte cytoplasm is essential for the developmental competence of oocytes and often controlled via cytoplasmic polyadenylation. Cytoplasmic polyadenylation element binding protein (CPEB) is required for polyadenylation of most mRNAs during oocyte maturation. It has been reported that *in vitro* matured oocytes with high developmental competence show an increased level of *CPEB1* mRNA in oocyte cytoplasm. Thus, we hypothesize that the introduction of exogenous *CPEB1* mRNA into *in vitro* matured oocytes could increase their developmental capability. In this study, we first synthesized polyadenylated *CPEB1* mRNAs by *in vitro* transcription. Cumulus-oocyte complexes were recovered from slaughterhouse bovine ovaries and subjected to *in vitro* maturation. After the removal of cumulus cells, matured oocytes were parthenogenetically activated. Each activated oocyte was injected with 5-10 pL of poly(A)–RNA solution (400ng/ µL; *CPEB1* mRNA and GFP mRNA for the injection group or GFP mRNA for the control group) using a micromanipulator. After injection, the oocytes were cultured in SOF

medium supplemented with amino acids for eight days. No difference was observed in cleavage rate between CPEB and control group. However, the blastocyst rate was significantly higher in the CPEB group than the control (24.9 ± 2.9% vs. 15.0 ± 4.5%; $P < 0.05$). We also compared the gene expression profile of blastocysts derived from both groups. The blastocysts were collected individually and analyzed by single-embryo RT-PCR. Twenty genes were selected for the analysis based on their roles in genomic reprogramming and embryonic development and fell into six functional categories: growth regulatory factors, cell cycle regulation, imprinting, apoptosis, pluripotency, and DNA methyltransferase. We found six genes (*H19*, *GRB10*, *DNMT1*, *CCNB1*, *CDK2* and *SOX2*) were up-regulated and three genes were down-regulated (DNMT2, BAX, and *P53*) along with the increased level of the *CPEB1* transcripts ($P < 0.05$). Our results demonstrate that developmental competence can be improved by injecting exogenous *CPEB1* mRNA into *in vitro* matured MII cattle oocytes, which reaffirmed the role of *CPEB1* in early embryonic development.

Introduction

A number of women fail to achieve pregnancy due to either fertilization failure or embryo arrest during early development. This often results from low oocyte developmental competence. Oocyte developmental competence is acquired during oocyte growth and maturation, which is a process that includes not only nuclear (meiotic) maturation but also cytoplasmic maturation. Cytoplasmic maturation plays a key role in oocyte and embryo developmental competence and is a complex process that consists of numerous cellular and molecular changes (Watson 2007). Oocytes are arrested at the diplotene stage of prophase I (the prophase of the first meiotic division) during the

growth phase. Both transcription and translation in oocyte are highly active before the immature (germinal vesicle; GV) oocytes reach the fully-grown stage. Once the oocyte is fully-grown, the nucleus (GV) of mammalian oocytes becomes transcriptionally quiescent and stays inactive until the new embryonic genome is activated during the maternal-to-zygotic transition (MZT), which happens during the 4-8-cell stage in human and 8-16 cell stage in bovine (Zhang and Smith 2015b). In the absence of transcription, embryo survival relies almost exclusively on mRNA, proteins, and other nutrients that are accumulated in the cytoplasm during oocyte growth and maturation. Cytoplasmic transfer (ooplasm transfer) from fertile donor oocytes into oocytes from patients with poor embryo development and recurrent implantation failure has successfully led to the birth of more than 30 healthy babies worldwide, which confirmed the fundamental role of oocyte cytoplasm in embryonic development (Barritt, Willadsen *et al.* 2001). Ooplasm transfer takes cytoplasm from donor oocytes and transfers it to recipient oocytes to restore some unknown defects through the transfusion of unidentified factors (including mRNA, proteins and mitochondrial DNA) in the cytoplasm. Oocyte quality can also be rescued by mitochondrial transfer (mitochondrial replacement therapy), in which the oocyte of compromised competence is injected with active mitochondria from competent donor oocytes (Wolf, Mitalipov *et al.* 2015). However, in both cases, extensive research is lacking, which has aroused the concern of potential dangers and unpredictable outcomes of this procedure (Cummins 2001; De Rycke, Liebaers *et al.* 2002; Templeton 2002). Also, due to the genetic material mitochondria possess, ooplasm and mitochondrial transfers cause severe ethical debate and have not been approved in the United States.

Since one major component of the oocyte cytoplasm is the stored maternal transcripts, we assume, by injection of the essential mRNAs into the developmentally compromised oocytes, we could possibly achieve similar results as the cytoplasmic transfer. During the period of transcriptional silence, oocytes and early embryos depend enormously on the post-transcriptional regulation of maternally synthesized and stored mRNA to sustain the rapid development (Kang and Han 2011). Temporal and spatial regulation of translation in the mammalian oocyte cytoplasm is essential for completion of oocyte growth, maturation and early embryo development, which is often regulated via increases in the poly(A) length (Dickson, Thompson *et al.* 2001). Cytoplasmic polyadenylation (CP) regulates translation of stored mRNAs and enables the spatiotemporal utilization of stored mRNA by the RNA-protein and protein-protein interactions by the direction of 3'UTR of the transcripts (Reyes and Ross 2016). In particular, cytoplasmic polyadenylation is generally associated with activation of translation and deadenylation with repression of translation. Cytoplasmic polyadenylation element binding protein (CPEB) is one of the essential factors that regulates this process by promoting lengthening of poly(A) tails on transcripts that contain cytoplasmic polyadenylation element (CPE) motifs (Gebauer and Richter 1996; Hake and Richter 1994). CPEB is a bifunctional translational regulator that not only contributes to translational activation by promoting transcript CP but also translational repression by binding transcripts' 3′ untranslated regions, which is essential for mRNA stability (Cao and Richter 2002; Charlesworth, Welk *et al.* 2000; Stebbins-Boaz, Hake *et al.* 1996). Stability of the large amounts of mRNAs accumulated during the growth phase is vital to sustain the following transcriptionally quiescent phase. Therefore, CPEB is a key

player during oocyte maturation and early embryonic development. There are four types of CPEB protein in vertebrates; *CPEB1* has been reported to be important in oocyte maturation and early embryonic development (Reyes and Ross 2016). Our previous data showed that oocytes of higher developmental competence had a higher level of CPEB transcripts at the MII stage.

In this study, we investigated whether increasing the *CPEB1* transcript level could increase oocyte developmental ability in cattle. Bovine *CPEB1* cDNA was cloned by RT-PCR from total RNA extracted from the whole bovine ovary tissue. We aimed to assess the effect of the increase in transcript level (overexpression) of *CPEB1* by mRNA injection into MII oocytes on oocyte developmental competence. In addition, expression levels of 20 developmental important genes were assessed in the blastocysts that were derived from the oocytes injected with exogenous *CPEB1* transcripts. The selected genes were previously implicated in embryonic and fetal development.

Materials and Methods

All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise specified.

Cloning of bovine CPEB1 cDNA

RNA was extracted from whole bovine ovaries using PureLink® RNA Mini kit (Thermo Fisher Scientific San Jose, CA, USA) according to the manufacturer's instructions including an on-column DNase treatment. First strand cDNA was then synthesized from 1µg total RNA using SuperScript® III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen, Carlsbad, CA, USA) following the manufacturer's

instructions. Then the full-length cDNA encoding bovine *CPEB1* (GeneBank accession no. XM_010824838.2) was amplified by standard PCR. Primer sequences were as follows: forward primer, 5'-CAGAAGAAACAGTCACAAGCAG-3'; reverse primer, 5' CTACGATTTCCCTTGTCCTCG 3'. The PCR product was then cloned into a pGEM-T Vector (Promega, Madison, WI, USA). The reconstructed vector was then sequenced to confirm the cDNA fidelity.

In vitro synthesis of RNAs and polyadenylation

The constructed plasmids were linearized with the restriction enzyme NotI at the site downstream of the *CPEB1* cDNA insert and then purified by phenol-chloroform extraction as previously described (Sambrook and Russell 2006) to eliminate protein and especially RNA contamination from the template DNA. The linearized cDNA was transcribed *in vitro* with T7 RNA polymerase using mMESSAGE mMACHINE T7 Ultra kit (Thermo Fisher Scientific San Jose, CA, USA) following the manufacturer's instructions. The transcripts were then polyadenylated by adding the tailing reagents (10 µl ATP solution (10 mM), 10 µl 25 mM $MnCl_2$, 20 µl 5X E-PAP Buffer and 36 µl nuclease-free water; Thermo Fisher Scientific San Jose, CA, USA) to the 20 µl mMESSAGE mMACHINE® T7 ultra reaction through 1 h incubation at 37°C. The polyadenylation, estimated to be 200 A, was confirmed by agarose gel electrophoresis.

Oocyte collection and maturation

Heifer and cow ovaries were collected from the local abattoir and transported to the laboratory within three hours after collection in saline containing 100-U/mL penicillin/ streptomycin. Cumulus-oocyte complexes (COCs) were aspirated from 3-8

mm follicles into 50-mL centrifuge tubes using an 18-gauge needle. The COCs were recovered in phosphate buffered medium (PB1) supplemented with 3 mg/mL BSA. Only oocytes with uniform cytoplasm and compact cumulus cells of multiple layers were selected. The COCS were washed three times in supplemented PB1, three times in maturation medium (TCM-199 [Gibco, Grand Island, NY, USA], containing 10% FBS, 0.5 μg/mL FSH, 5 μg/mL LH, and 100 U/mL penicillin/ streptomycin) and then incubated in maturation medium for 18-22 h at 38.5 °C in 5% CO_2 in the humidifier air. The COCs were cultured in groups of 50 in 4-well plates containing 500 μL of maturation medium. After maturation, cumulus cells were removed from oocytes by vortexing the COCs for 1–2 min in TL-Hepes containing 1 mg/ml hyaluronidase. Maturation status was assessed by the presence of a first polar body. Oocytes at this stage are termed MII oocytes.

Parthenogenetic activation and injection of RNA

The MII oocytes were activated for 5 min in synthetic oviductal fluid (SOF) medium (Tervit, Whittingham *et al.* 1972) supplemented 10 μM of ionomycin and then transferred into 25 μl droplets containing 2 mM 6-dimethylaminopurine and 10 μg/ml cycloheximide and incubated for 4 h at 38.5 °C with 5% CO_2 in air. After activation, oocytes were washed three times and transferred to SOF medium before RNA microinjection. Oocytes were randomly separated into two even groups: CPEB group and control group. Oocytes were then injected with 5–10 pL of RNA solution (400 ng/μL; *CPEB1* mRNA and GFP mRNA for the injection group or GFP mRNA for the control group) using a micromanipulator. In each experiment, 50-100 oocytes were injected for each group within 1h of activation.

In vitro culture (IVC)

After microinjection, the embryos were then washed 3 times in SOF and cultured in groups of 15–20 embryos in 25 μL droplets of SOF covered by mineral oil. Culture dishes were equilibrated for at least 5 h in an incubator before use. The embryos were cultured at 38.5 °C with 5% CO_2 in the air. Fluorescence was observed with ultraviolet filters (370 nm) using a fluorescence microscope at Day 2 after injection and embryos without fluorescence were discarded. The efficiency of development was evaluated as cleavage rate (the percentage of cleaved embryos) 2 days after PA and blastocyst rate (the percentage of blastocysts on the basis of the number of cleaved embryos at Day 2) 8 days after PA.

Real-time PCR

Blastocysts at day 8 were washed three times and stored individually in 1-μL DNA suspension buffer at −80 °C before gene expression analysis. Gene expression experiments were performed using Fluidigm's Flex Six Integrated Fluidic Circuits (IFC; Fluidigm, San Francisco, CA). Fluidigm BioMark system was used following the manufacturer's recommended protocol. As required by the Fluidigm protocol, a reverse transcription and specific target amplification (RT-STA) step were performed to each blastocyst sample to convert the RNA to cDNA and to enrich target-specific cDNA before qPCR. Briefly, a 200-nM pooled STA primer mix was made by diluting all the 21 pairs of primers in DNA suspension buffer. Each individual blastocyst (stored in DNA suspension buffer) was added with 9 μL of RT-STA solution (5 μL of CellsDirect PCR

mix (Invitrogen, Carlsbad, CA, USA), 0.2-μL SuperScript-III RT/Platinum Taq (Invitrogen, Carlsbad, CA, USA), 2.5 μL of 200-nM pooled primer mix and 1.3-μL DNA suspension buffer). Reverse transcription reaction was then done at 50 °C for 15 minutes, 95 °C for 2 min and cDNA was subsequently preamplified for 20 cycles under the following conditions: 95 °C for 15 seconds, 60 °C for 4 minutes. After the reaction, 4-μL ExoSAP-IT (ExoI; New England Biolabs, Beverly, MA, USA) was added to every RT-STA reaction and placed in the thermal cycler at 37 °C for digestion, followed by 15 minutes inactivation at 80 °C. The Fluidigm Flex Six chip was primed before use according to manufacturer's instructions (Fluidigm PN 100–7717 B1). 5 μL sample mix solution (2 μL diluted sample, 2.5 μL of the 2X TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA), 0.25 μL of 20X DNA Binding Dye Sample Loading Reagent (Fluidigm, San Francisco, CA) and 0.25 μL EvaGreen DNA Binding Dye (Biotium, Hayward, CA)) were loaded into one of the sample inlets of the primed chip. A 5 μL aliquot of the assay (primer mix solution (2.5 μL of the 2X Assay Loading Reagent (Fluidigm), 1.25 μL DNA suspension buffer, and 1.25 μL of 20 μM forward and reverse primer mix of each gene) was inserted into "assay" inlets of the primed chip. The chip was returned to the IFC Controller for chip loading. Subsequently, the chip was placed in the BioMark real-time PCR machine for quantitative PCR following manufacturer's instructions. Cycling parameters included a 5 min enzyme activation at 95°C, 35 denaturation/extension cycles (95°C for 15 s followed by 60°C for 60 s), and a 3 min final extension cycle at 60°C. The real-time PCR data were analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The expression level of target genes was normalized to the internal control gene (GAPDH). The gene expression data are

presented in fold change compared to control values.

Statistical analysis

Each round of *in vitro* culture was considered a replication. The cleavage rate and blastocyst rate (data were converted to percentages) between CPEB and control group were determined by ANOVA in SAS 9.4 (SAS Institute Inc.). A difference was considered significant when the *P* value was less than 0.05. For gene expression data, the difference was analyzed by student t-test and considered to be significant when the following requirements were met: at least 2-fold change in gene expression was observed and *P* value was less than 0.05 (FDR- adjusted)..

Results

Effect of overexpression of CPEB1 gene on developmental competence following parthenogenetically activation

The oocytes were parthenogenetically activated and then injected with mRNAs. The injected oocytes were then cultured in SOF media for 8 days. As shown in Table 2, in total, 124 and 193 CPEB oocytes and control oocytes were collected for *in vitro* culture after injection, respectively. No significant difference was observed in cleavage rates between two groups. However, the blastocyst rate at day 8 was significantly higher in CPEB group compared to the control ($P < 0.05$).

Effect of overexpression of CPEB1 gene on expression levels of developmentally important genes at the blastocyst stage

The association of *CPEB1* overexpression and expression of developmentally important genes (Table 1) was examined in the bovine blastocysts using real-time PCR. The selected genes fell into one of six functional categories: growth regulatory factors, cell cycle regulation, imprinted genes, apoptosis, pluripotent transcription factors and DNA methyltransferase. The relative gene expression results are presented in Fig 1. Eleven of the analyzed genes showed no statistical difference in relative transcript level between the blastocysts derived from oocytes of CPEB injected and control groups. The expression levels of six genes (*H19, GRB10, DNMT1, CCNB1, CDK2,* and *SOX2*) were significantly higher in the blastocysts from oocytes injected with *CPEB1* mRNA compared to control group ($P < 0.05$). The mRNA levels of DNMT2, BAX and P53 were more abundant in control blastocysts ($P < 0.05$).

Discussion

The oocyte and early embryo inherit a batch of mRNA transcripts synthesized and stored during oogenesis before the transcriptionally quiescent stage. The correct translation of these maternal mRNAs in a temporal and spatial manner contributes to oocyte maturation and drives the cleavage divisions of the early embryos. Cytoplasmic polyadenylation plays a key role in regulating maternal mRNA translation and is driven by multifuctional CPEB protein. Previous studies have demonstrated that inadequate cytoplasmic maturation is associated with inefficient regulation of the mRNA polyadenylation process, which results in compromised developmental competence (Brevini- Gandolfi, Favetta *et al.* 1999; Brevini, Lonergan *et al.* 2002). It is believed that assisting with the process of polyadenylation might rescue incompetent oocytes by injecting cytoplasmic polyadenylation element (CPE) and adenosine 3'-5' biphosphate

(PAP), or by elongating the polyA tail of some maternal mRNA (Levy, Elder *et al.* 2004). Due to the crucial role *CPEB1* has in cytoplasmic polyadenylation, we assume that adequate *CPEB1* transcripts are beneficial to assist this process. Dysregulation of gene expression was generally observed in oocytes with low developmental competence. A previous study in monkey oocytes demonstrated that the failure of *in vitro* matured oocytes to undergo a normal pattern of transcript silencing results in their reduced developmental competence (Zheng, Patel *et al.* 2005). Abnormal post-transcriptional regulation is one of the explanations of the relatively poor developmental competence of human oocytes matured *in vitro* (Jones, Cram et al. 2008). Our previous data (Chapter III) showed that *CPEB1* gene was expressed at a higher level in MII oocytes with higher glucose-6-phosphate dehydrogenase activity, which have also been confirmed to have higher developmental competence. This result suggests that the low developmental competence could be attributed to the inefficient post-transcriptional regulation caused by the reduced amount of *CPEB1* protein. Another study in pigs has demonstrated that overexpression of *CPEB1* gene in immature oocytes by injection of exogenous mRNAs significantly increased the rates of oocyte germinal vesicle breakdown (GVBD) after *in vitro* maturation (Nishimura, Kano *et al.* 2010). However, to our knowledge, *CPEB1* overexpression has never been examined in matured oocytes and no reports have been published to investigate the effect of increasing the level of *CPEB1* transcripts on further oocyte developmental competence.

In the present study, we found that oocytes injected with exogenous *CPEB1* transcripts have improved blastocyst rates following parthenogenetic activation compared to the control group. This result suggests that *CPEB1* overexpression could increase

oocyte developmental competence *in vitro*. A developmental block is observed in many species at various stages, and has been proved to be concurrent with the maternal-to-embryonic transition, the developmental phase when embryos start the major genome activation (De Sousa, Watson *et al.* 1998), which is highly dependent on the translation of stored maternal mRNAs (Zhang and Smith 2015a). Adequate *CPEB1* transcripts that result in sufficient supply of *CPEB1* protein in oocyte cytoplasm might increase cytoplasmic polyadenylation efficiency and, therefore, assist oocytes to achieve higher developmental competence via more efficient post-transcriptional regulation of the maternal mRNAs. Thus, oocytes injected with exogenous *CPEB1* transcripts were more capable to overcome the developmental block and reach the blastocyst stage. However, the mechanism and pathways by which CPEB1 regulates the genome activation remain unclear. Since the CPEB1 protein is involved in the translational control of various maternal factors, the proper translation of multiple critical transcriptional factors under the assistance of the CPEB1 protein might eventually result in the activation of the embryonic genome. Further studies are needed to elucidate the role CPEB1 plays in early embryonic development, which could improve our knowledge about how the oocyte acquires developmental competence.

In bovine, embryonic transcriptional activation begins at the 8-cell stage; embryos beyond this stage must rely on the mRNAs transcribed from the embryonic genome to continue development (Meirelles, Caetano *et al.* 2004). Inability to activate transcription of important developmental genes is believed to be one of the major mechanisms that cause embryo developmental failure. It has been demonstrated that embryonic development is regulated by the intricate cooperation of various developmentally

important genes in the context of signaling pathways. A precise gene expression profile at the blastocyst stage, which characterizes the embryo's capability to successfully implant and establish fetal development, is crucial for the viability of the pre-implantation embryos (Parks, McCallie *et al.* 2011). On the other hand, an analysis of the gene expression pattern could reflect insightful information of the embryo developmental competence.

Our data showed that, among the 20 developmentally important genes that were analyzed, nine genes were differentially expressed in the blastocysts derived from the oocytes injected with exogenous *CPEB* compared to those obtained from the control oocytes. *DNMT1, H19, GRB10, CCNB1, CDK2, and SOX2* were up-regulated, while *DNMT2, BAX* and *P53* were down-regulated along with the overexpression of the *CPEB1* gene. These genes all have crucial roles in early embryogenesis. High levels of DNA methyltransferase (*DNMT1*) are often present in normal development during embryogenesis; it is essential in maintaining methylation patterns of the embryonic genome, especially in imprinted regions (Hirasawa, Chiba *et al.* 2008). A higher level of *DNMT1* transcripts in the *CPEB* injected blastocyst group indicates that overexpression of *CPEB1* triggered the more active transcription of *DNMT1*. Previous studies demonstrated that embryos lacking *DNMT1* were not able to maintain proper methylation patterns and showed genome-wide demethylation, which led to loss of monoallelic expression of the imprinted genes (Hirasawa, Chiba *et al.* 2008; Li, Beard *et al.* 1993). This result is consistent with our data that the expression levels of imprinted genes *H19* and *GRB10* were lower along with the lower *DNMT1* transcript level. *In vitro* culture conditions affect both methylation status and the expression of imprinted genes in

preimplantation embryos and can lead to abnormalities. Thus, our gene expression data of *DNMT1, H19* and *GRB10* indicates that *CPEB1* overexpression might have rescued the epigenetic abnormalities that *in vitro* culture conditions cause to embryos. The role of *DNMT2* in development has not been studied as well as that of *DNMT1* and *DNMT3* families. *Unlike DNMT1, DNMT3A and DNMT3B, DNMT2* did not reveal methyltransferase activity specific for cytosine (Yoder and Bestor 1998), and a previously published study showed that a knock-out of the Dnmt2 gene in the mouse did not result in detectable abnormality (Okano, Xie *et al.* 1998). During Drosophila embryogenesis, the expression level of *DNMT2* decreases dramatically throughout development. It is believed that most of the *DNMT2* mRNAs are maternal components that are deposited in embryos but probably not translated (Kunert, Marhold *et al.* 2003). Thus, according to our gene expression results that have shown that *DNMT2* expression level was lower in CPEB blastocysts, we speculate that adequate amount of *CPEB1* protein might assist the proper degradation of *DNMT2* transcripts, which might be necessary for normal embryo development. SOX2 is a critical transcription factor for maintaining pluripotency of the embryo and has an essential function in preimplantation embryos to facilitate cell differentiation at the blastocyst stage. A *SOX2* knock-down in mouse embryos results in embryo arrest at morula stage and the rescue experiment showed that using cell-permeant SOX2 increased blastocyst formation. Previous studies also demonstrated that lack of SOX2 seemed to increase apoptosis in embryos (Keramari, Razavi *et al.* 2010). Our results have shown that an increased level of *CPEB1* transcript in oocytes led to an increase in *SOX2* transcripts and decrease in the apoptosis gene *BAX* transcripts indicating that adequate *CPEB1* transcripts might be critical for pluripotency

of the embryo and help to prevent apoptosis. The other two genes that were up-regulated in CPEB blastocysts, *CCNB1*, and *CDK2*, have an important role in cell division (Sánchez and Dynlacht 2005). In the blastocyst stage, embryos undergo rapid DNA replication and extensive proliferation, which is at a higher risk of acquiring DNA damage. *P53* is an important factor in DNA repairing process (Oren 1999). It has been demonstrated that the *p53* gene is not essential for the normal performance of cells; *P53* remains latent during normal cellular communications. However, DNA damage and other stress lead to the rapid induction of *P53* activity, which subsequently pauses the cell division until the damage is repaired. Previous studies showed that oocytes and embryos suffer from DNA damage under *in vitro* culture conditions (Mukherjee, Kumar *et al.* 2010). The presence of higher levels of *P53* mRNA in control blastocysts reflect the higher damage repair activity, which indicates that an increased level of *CPEB1* is negatively correlated with DNA damage during early embryonic cell division.

In conclusion, this study confirmed an important role that *CPEB1* plays in early embryo development. The microinjection of *CPEB1* mRNA into MII oocytes increased developmental competence *in vitro* and thus is looking promising as a novel potential treatment for patients with low oocyte quality. However, the mechanism for increased developmental competence by overexpression of *CPEB1* in mature oocytes remains unclear and will require further investigation. Even though our gene expression data provided some insightful information for the potential pathways and mechanism underneath, continued studies are necessary to further our understanding of *CPEB1* function in the oocyte and its role in embryonic development.

Table 1. The sequence of primers for quantitative real-time PCR

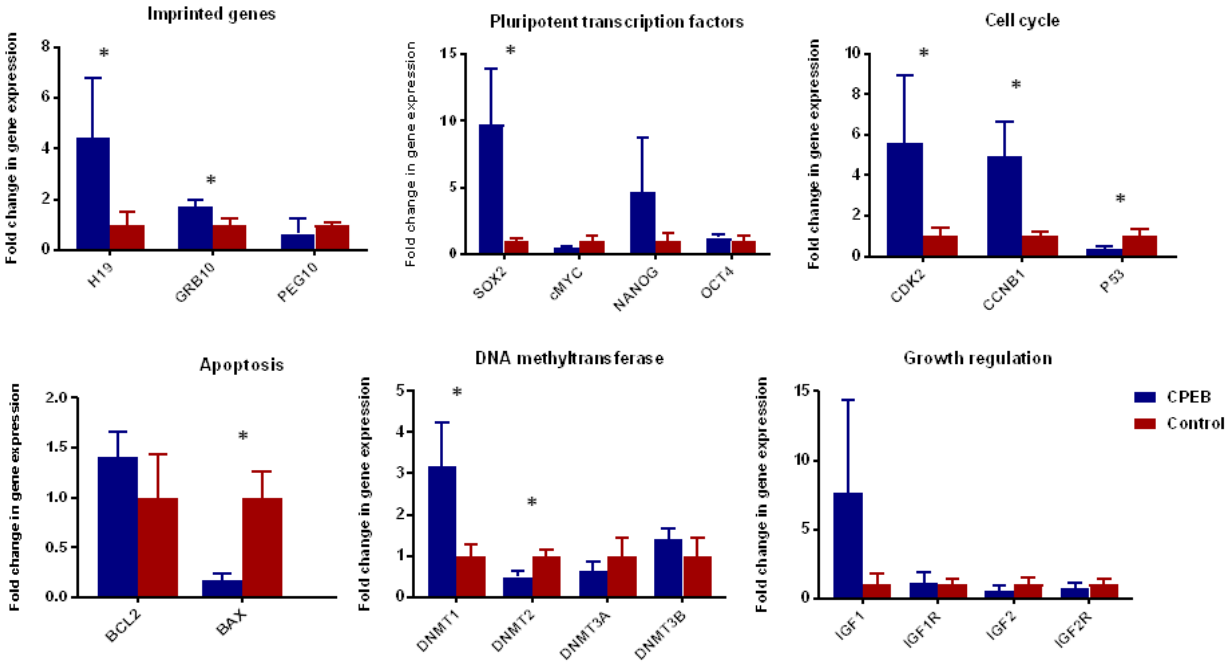
Gene	5'- primer sequence-3' (forward, reverse)		Product size (bp)
IGF1	GAGTTGGTGGATGCTCTCCA,	GACTGCTCGAGCCATACCC	82
IGF1R	GAACCTGCGCCAGATCCTA,	GCTGCAAGTTCTGGTTGTCA	80
IGF2	GATCCCCACCTGCTCAGAA,	CGACTTTCCTGCTGTGATCC	77
IGF2R	GGCAGATTCCACTCAAGTCA,	AGATCAAGGTGAGGTCTCCA	83
H19	CTTGGAACACGGACTTCTTCAA,	AGGGTGTGTAGTGGTTCCAA	76
GRB10	TTGCTGGCAGGAAGCAGTA,	GCCTCGTTCCTGACTCTGTTA	76
PEG10	GTGGGACCCCGTCTTTCC,	GCGCGTAGTAGCTTCACTCC	138
DNMT1	AGAGACGTCGAGTTACATCCA,	GTGTTCCTGGTCTTACTCTTCC	81
DNMT2	TTAATGAGCCCACCCTGTCA,	GTCCTTGGATCAGTCACATCAC	81
DNMT3A	CCATGTACCGCAAGGCTATCTA,	GCTGTCATGGCACATTGGAA	83
DNMT3B	GCCAAAGCTCTTCCGAGAAA,	GGGTGGAGGTACTGCTGTTA	80
BCL2	ATGTGTGTGGAGAGCGTCAA,	GGTTCAGGTACTCGGTCATCC	79
BAX	CGGGTTGTCGCCCTTTTCTA,	GCCCATGATGGTCCTGATCAA	90
CDK2	TTTTGGGGTCCCTGTTCGTAC,	TGCAGCCCAGAAGGATTTCC	80
CCNB1	TTTAGTCTGGGTCGCCCTCTA	GGCCAGAGTATGTAGCTCAACA	83
SOX2	CCCAAGAGAACCCTAAGATGCA,	CCGTCTCGGACAAAAGTTTCC	72
CMYC	GCGCTCCATGAGGAGACC,	GGCTGCCTCTTTTCCACAGAA	101
NANOG	AACTGGCCGAGGAATAGCAA,	ACCCCTGGTGGTAGGAATAGAA	82
OCT4	AGAAGCTGGAGCCGAACC,	CTGCTTTAGGAGCTTGGCAAA	86
P53	CCCATCCTCACCATCATCACA,	GCACAAACACGCACCTCAA	80

Table 2. Effect of *CPEB1* gene overexpression on the *in vitro* development of bovine oocytes after parthenogenetic activation

Treatment	MII oocytes, n	Cleaved embryos, n	Cleaved / matured (%)	Blastocysts, n	Blastocysts/ cleaved (%)
CPEB	209	173	82.8 ± 1.5	43	24.9 ± 2.9 [a]
Control	152	133	87.5 ± 4.0	20	15.0 ± 4.5 [b]

Values in parentheses are means ± SEM of 5 experimental replicates expressed as percentages. Data were analyzed by One-way ANOVA. Values within columns with a different superscript are significant.

Fig. 1. Fold change (FC) of gene expression of blastocysts derived from CPEB oocytes (injected with CPEB and GPF mRNA)and control oocytes (injected with GFP mRNA). Genes are grouped according to their function: (A) imprinted genes, (B) pluripotent transcription factors, (C) cell cycle, (D) apoptosis, (E) DNA methyltransferase, and (F) growth regulation factors. The data were analyzed by one-way ANOVA, Symbol * indicate a significant difference is observed in gene expression of blastocysts between two groups ($P < 0.05$ and FC>2).



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CHAPTER VI

SUMMARY

Assisted reproductive technologies (ARTs) have now been extensively used not only to promote reproductive efficiency as a fertility treatment, but also to preserve valuable genetics and advance the fundamental knowledge of reproductive processes. Improving the efficiency of ARTs holds great potential for its application in the clinical, biomedical and agricultural fields. Among the multiple causes that contribute to failures in the applications of ARTs, oocyte developmental competence is often regarded as a limiting factor (Zhang and Smith 2015). The quality of oocyte donors collected for ARTs varies and there is a lack of an efficient and rapid method for oocyte selection. The ability to select oocytes that predicts their developmental competence more efficiently could improve the overall efficiency of ARTs. Oocyte developmental competence is the ability of an oocyte to undergo maturation, fertilization, and development to the blastocyst stage or live offspring and is determined by various factors. Any alteration that happens during the oocyte growth and maturation process can result in decreased oocyte quality, which could have long-lasting effects on development. Improving the developmental efficiency of the oocyte is quite challenging due to the limited knowledge on how oocytes acquire their competence. Oocyte cytoplasmic maturation is the process that prepares oocytes for activation and preimplantation development, which plays a key role in oocyte and embryo developmental competence (Duranthon and Renard 2001; Eppig 1996; Neri, Lee *et al.* 2014; Salviano, Collares *et al.* 2015; Watson 2007). Cytoplasmic maturation is a complex progression, including various cellular processes such as accumulation of maternal messenger RNAs (mRNAs), proteins, substrates, and nutrients, which is poorly

understood at the molecular level. We believe that better understanding of the underlying mechanism of cytoplasmic maturation is beneficial for improving the developmental efficiency of oocytes and ART outcomes.

Comparing oocytes with high and low developmental competence has been used as a method to detect the key players in determining oocyte quality and identify potential biochemical markers that are associated with oocyte developmental competence. This approach helps to elucidate how the possible mechanism is related to oocyte developmental potentials. To date, numerous studies have added to the growing information regarding differences between two populations of oocytes with different developmental competence. For example, several previous studies have reported the differences in gene expression profiles as well as other molecular or cellular characteristics between *in vivo*- and *in vitro*-derived oocytes (Jones, Cram *et al.* 2008; Rath, Töpferpetersen *et al.* 2005; Rizos, Ward *et al.* 2002; Wrenzycki, Herrmann *et al.* 1996), Zhou, 2008. Advances are being made to understand the mechanisms of how oocytes acquire developmental competence and to identify the key players that determine oocyte quality, but the progress has been incremental.

In this work, we investigated the efficiency of two oocyte-selecting criteria (follicle size and brilliant cresyl blue staining) and confirmed their ability to differentiate oocytes according to their developmental competence. The experiments reported in this dissertation also characterized the cellular and molecular differences between oocytes with different developmental competence based on the these two selection methods.

Previous data have already indicated that size of the follicles from which the oocytes are collected is one of the factors that influence the maturational and

developmental competence using ARTs (Bagg MA 2004; Otoi, Yamamoto *et al.* 1997). Oocytes from large follicles have been demonstrated to have greater developmental competence than oocytes from small follicles in various species including goats. For the first time, the data in this dissertation determined the effect of follicle size of the cytoplast donor on the *in vivo* developmental competence following somatic cell nuclear transfer (SCNT) in goats. Our results have demonstrated that oocytes derived from large follicles (≥3mm) yield a significantly greater maturation rate than oocytes derived from small follicles (<3mm) reinforcing the fact that the oocytes derived from large and small follicles compose two distinct populations. However, there is no observable effect of follicle size on oocyte developmental efficiency after the SCNT procedure in goats. This is contradictory to the results from previous studies in which significantly greater rates of blastocyst development were observed in oocytes from large follicles than from smaller following the IVF procedure in goats (Ali and Mehrabian 1995). One possible reason for this inconsistency in the oocyte developmental efficiency between the two populations of oocytes is that artificial oocyte activation employed during SCNT might be able to rescue developmental competence of small follicle oocytes. Our gene expression results of the oocytes backed up this hypothesis. The stored transcript levels of three genes (MATER, IGF2R, and GRB1) were significantly greater in oocytes derived from large follicles compared to oocytes from small follicles. They all appear to be required for the proper calcium oscillation following fertilization (Fleming, Fisslthaler *et al.* 1995; Kim, Zhang *et al.* 2014; Mckinnon, Chakraborty *et al.* 2001; Mergler, Dannowski *et al.* 2003; Nishimoto, Hata *et al.* 1987). Therefore, the low developmental efficiency in the IVF procedure exhibited by the small-follicle oocyte is possibly due to their inefficient ability

to start calcium oscillation to activate oocytes following fertilization and this inefficiency could be overcomed by an artificial activation in the SCNT procedure.

Brilliant cresyl blue staining separates oocytes by its G6PDH activity and is efficient in selecting developmentally competent oocytes in various species (Manjunatha, Gupta *et al.* 2007; Otoi, Yamasaki *et al.* 2006; Wang, Jiang *et al.* 2016), including goat (Rodríguezgonzález, Lópezbéjar *et al.* 2002). In this dissertation work, we evidenced the difference of the developmental potential between the two oocyte groups separated by BCB staining in respect of maturation rate after *in vitro* maturation (IVM) as well as the cleavage and blastocyst rates followed parthenogenetic activation, which reinforced that BCB staining is an efficient non-invasive method of oocyte selection. Glutathione (GSH) is an essential compound for reducing harmful reactive oxygen species (ROS). We investigated the intracellular level of GSH and ROS in oocytes after IVM and found the more competent oocytes (BCB+) demonstrated higher intracellular GSH levels and lower intracellular ROS levels than those of BCB− oocytes. This is the first time that the association between G6PDH-activity and intracellular GSH and ROS content has been demonstrated in goat oocytes. In addition, real-time PCR was used to evaluate relative expression of a panel of genes that are associated with developmental competence in individual BCB+ and BCB− oocytes at both GV and MII stage. Many transcripts were determined to be differentially expressed and these data confirmed that G6PDH activity is associated with gene expression profiles of oocytes. The majority of the transcripts of the differentially expressed genes found to be more abundant in the BCB+ oocytes, which might directly contribute to their higher developmental competence compared to BCB− oocytes.

The cellular mechanism of oocytes acquiring their developmental competence is poorly understood. The first two studies of this dissertation (chapter II and chapter III) used the comparative approach to study several factors involved in oocyte developmental potentials. We believe the information obtained from these studies will also help to identify additional biomarkers for oocyte quality and would lead to strategies that directly increase oocyte development efficiency.

The third facet of the research reported in this dissertation was designed to compare the behavior of goat oocytes submitted to different *in vitro* maturation (IVM) treatments. The culture environment of oocytes plays a critical role in the development process (Behboodi, Anderson *et al.* 1995; Farin and Farin 1995; McEvoy 2003; Wilson, Williams *et al.* 1995). *In vitro* culture conditions have constantly been enhanced by optimizing the medium formulations in the attempts of improving the *in vitro* development efficiency of goat ARTs, which have led to a variety of culture media and cultural environments. We compared three types of medium and investigated the effect of maturation medium composition on: (1) oocyte nuclear maturation rates and development competence after PA; (2) the intracellular GSH and ROS levels; and (3) the gene expression profiles. Based on the results, we assumed that supplementation with LH might cause the increased level of intracellular ROS in oocytes. Overall, the differences in GSH and ROS levels and differential expression of four developmentally associated genes (MATER, PLAT, NANOG, and CCNB1) among the oocytes from these three tested medium groups provided us some information about how the environmental factors affect oocyte developmental competence. However, more studies are needed for further optimization of maturation medium to improve IVM of goat oocytes through a

more thorough understanding of the underlying mechanism.

This dissertation aimed to elucidate mechanisms involved in oocyte development with the ultimate goal of improving the efficiency of ARTs by increasing oocyte quality. In human medicine, cytoplasmic transfer was successfully used to rescue the development of compromised oocytes. One major component of the oocyte cytoplasm is the stored maternal transcripts. In the last study of this dissertation, we successfully achieved an improvement in the bovine oocyte *in vitro* developmental competence by a direct injection of the exogenous transcripts of CPEB1 gene. The CPEB1 gene codes for the cytoplasmic polyadenylation element binding protein, which is an essential factor involved in the post-transcriptional regulation in the oocyte (Gebauer and Richter 1996; Hake and Richter 1994). Through our comparative study, we found that the CPEB1 gene was expressed at a higher level in BCB+ oocytes, which has been confirmed to have higher developmental competence. This result indicates that the low developmental competence could be attributed to the inefficient post-transcriptional regulation caused by a reduced amount of the CPEB1 protein. A previous study in pigs has demonstrated that overexpression of CPEB1 increased oocyte maturation ability (Nishimura, Kano *et al.* 2010). Our study revealed the effect of an increased level of CPEB1 transcripts on the oocyte developmental competence. We also analyzed 20 developmentally important genes at the blastocyst stage and found that nine genes were differentially expressed in the blastocysts derived from the oocytes injected with an exogenous CPEB compared to the control oocytes. This difference in the gene expression profile of the blastocysts provided some insightful information for potential pathways and confirmed the important role of CPEB1 in the early embryonic development. Continued studies are needed to

further our understanding of CPEB1 function in the oocyte and embryonic development.

Together these data provide a better understanding of how oocytes acquire their developmental competence and represent a contribution to the improvement of ART efficiency by improving oocyte quality. We have identified the factors that are important in the process of oocyte maturation and embryo development, which we believe will explain what caused the decrease in oocyte developmental ability and contribute to the identification of appropriate markers of oocyte quality. This dissertation also provides information that benefits the understanding of how the nutrients in culture medium facilitate oocyte maturation, which will eventually help optimize the condition for oocyte culture. In addition, we also proposed a potential strategy for improving oocyte quality. The knowledge obtained from this research offers promise for many applications in the field of infertility treatment and potential improvements of ART efficiency.

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- Development of induced pluripotent stem cells
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PUBLICATIONS

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MANUSCRIPTS UNDER REVIEW

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MANUSCRIPTS IN PREPARATION

Yang, M., et al. Association of G6PDH-activity with oocyte developmental and reprogramming competence and molecular characteristics in goats (Data collection completed)

ABSTRACTS

Yang, M., Webster, D., Meng, Q., Carlson, D., Fan, Z., Fahrenkrug, S., Regouski, M., Bidwell, C., Polejaeva, I. "Introduction of Callipyge mutation into the goat genome

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Poster presentation at 43rd Annual Conference of International Embryo Transfer Society (IETS). Serial somatic cell nuclear transfer increases pregnancy losses in goats Jan 2017 Austin, USA

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