

GENOMIC STUDIES OF OOCYTE PHYSIOLOGY REVEAL
MECHANISMS CRITICAL TO OOCYTE COMPETENCE

BY

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DISSERTATION

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ABSTRACT

In vitro maturation of porcine oocytes has been intensively studied for many years. However, the in vitro maturation system is still inefficient to produce competent oocytes that can develop to viable embryos. The studies discussed in this dissertation attempt to use different techniques and methods to characterize and improve the developmental competence of in vitro matured porcine oocytes. Medium environment impacts the metabolism and physiology of oocytes. Using chemically defined medium, we demonstrated the detrimental effects of ammonium on porcine oocyte maturation and developmental potential. This result suggested that using a more stable dipeptide, alanyl-glutamine, to replace the highly volatile glutamine can optimize the maturation medium. The microfluidic well system provides an easy-to-use physical platform that can successfully mature porcine oocytes and culture embryos individually without compromising their competence. Further optimization of the current system may result in a novel system that can also increase the development of oocytes and embryos. The main focus of this dissertation is to identify the specific genes whose function is related to oocyte competence. Functional studies were also conducted to further validate the relationship of selected genes with oocyte competence. In one experiment, we found genes related to cholesterol synthesis, fatty acid oxidation, glycolysis, and 4 other genes, *TNF*, *PERV*, *TL10* and *SRSF1*, were differentially expressed between adult (more competent) and prepubertal (less competent) porcine in vitro matured oocytes. These genes were further analyzed by comparing transcript

abundance in in vitro and in vivo matured prepubertal and adult oocytes. The different gene expression patterns between competent and less competent models suggested these pathways and genes may be functionally relevant to oocyte competence. The functional study of *TNF* demonstrated that TNF in the medium regulates *TNFAIP6* expression in cumulus cells, and thus influence oocyte development. The other experiment focused on the genes that are related to redox homeostasis. Using same oocyte competence models (prepubertal versus adult, in vivo versus in vitro) we found that *GLRX2*, *PDIA4*, *PDIA6* and *TRXR1* may be functionally relevant to oocyte competence. By maturing oocytes in different redox environments, we found that a balanced redox environment is critical for oocyte development. Elevated glutathione in prepubertal oocytes suggested the disrupted redox homeostasis exists in these cells. By further comparing *redox* gene expression patterns, we found aberrant expression patterns in prepubertal oocytes when extra antioxidant was supplemented to the medium. These results suggested that prepubertal oocytes are less competent in regulating redox balance compared to adult oocytes. The aberrant expression patterns of *GLRX2*, *PDIA4*, *PDIA6* and *TRXR1* may contribute to the decreased developmental competence in prepubertal porcine oocytes. In conclusion, the studies in this dissertation provide a molecular sketch of genes and metabolic pathways that may be important regulators of oocyte quality in pigs. Further studies of these genes and pathways may result in a better understanding of oocyte competence and how better to support optimal competence during in vitro maturation.

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TABLE OF CONTENTS

CHAPTER 1 REVIEW OF CURRENT LITERATURE	1
REFERENCES	21
CHAPTER 2 EFFECT OF AMMONIUM DURING IN VITRO MATURATION ON PORCINE OOCYTE NUCLEAR MATURATION AND SUBSEQUENT EMBRYONIC DEVELOPMENT	43
REFERENCES	58
CHAPTER 3 IDENTIFICATION OF DEVELOPMENTAL COMPETENCE RELATED GENES IN MATURE PORCINE OOCYTES	68
REFERENCES	92
CHAPTER 4 DISRUPTED REDOX HOMEOSTASIS AND ABERRANT REDOX GENE EXPRESSION PATTERNS IN PREPUBERTAL PORCINE OOCYTES CONTRIBUTE TO DECREASED DEVELOPMENTAL COMPETENCE.....	109
REFERENCES	131
CHAPTER 5 USE OF A MICROFLUIDIC WELL INSERT TO SUCCESSFULLY MATURE PORCINE OOCYTES AND CULTURE EMBRYOS INDIVIDUALLY IN VITRO	146
REFERENCES	160
VITAE.....	170

CHAPTER 1

REVIEW OF CURRENT LITERATURE

The research contained in this dissertation mainly focuses on characterizing oocyte developmental competence and exploring possible new ways to optimize the current porcine oocyte in vitro maturation (IVM) protocol. However, in vitro fertilization (IVF) and in vitro culture (IVC) are the other two indispensable techniques for in vitro embryo production (IVP). Therefore, the topics covered in this chapter will primarily focus on oocyte IVM, while important information regarding IVF and IVC is also discussed.

Porcine oocyte metabolism and IVM medium environment

Oocyte maturation involves important nuclear and cytoplasmic changes. It is a critical step for acquiring oocyte developmental competence which affects embryonic development, fetal development, and even the health of the offspring (Eppig and O'Brien 1998). In pigs, the IVP of porcine embryos, including oocyte IVM, IVF and embryo IVC, is historically difficult. Tremendous efforts have been made to optimize this system to allow more efficient production of viable embryos (Abeydeera and Day 1997; Funahashi and Day 1997; Nagai et al. 1984; Yoshida et al. 1990; Yoshioka et al. 2002). However, the competence of in vitro produced embryos is still low compared with in vivo derived embryos (Kikuchi et al. 1999), as well as compared with in vitro counterparts of other species such as mouse and cattle. Incomplete cytoplasmic maturation of IVM oocytes and suboptimal culture conditions for embryos are

thought to be responsible for the low efficiency (Nagai et al. 2006). In this section, the discussion of oocyte metabolism and its relationship to the maturation environment are mainly focused on pigs, although additional information from other species is also provided.

Nuclear maturation and hormonal supplementation

During maturation, oocyte needs to undergo meiosis and reach the metaphase II stage to be ready for fertilization. During the development of ovarian follicles, follicle stimulating hormone (FSH) produced by the pituitary can stimulate estrogen production in granulosa cells. The estrogen then acts via intracrine mechanisms through estrogen receptor type 2 to enhance granulosa cell responses to FSH (Richards 1994) This estrogen pathway is critical for development of follicles for the acquisition of responses to luteinizing hormone (LH) (Couse et al. 2005). In pig follicles, progesterone also impacts functional changes in cumulus and granulosa cells and plays an important role for cells responding to the ovulatory LH signal (Shimada et al. 2004). The LH surge induces production of prostaglandins (Floman and Zor 1977) and epidermal growth factor (EGF)-like growth factors that impact ovulation, cumulus expansion, and meiotic resumption (Hsieh et al. 2007; Park et al. 2004). The supplementation of various hormones and growth factors in IVM medium is required for oocyte meiotic progression. For porcine oocyte IVM, oocytes are typically collected from small antral follicles, which have limited responsiveness to

LH (Shimada et al. 2003) and EGF (Prochazka et al. 2003). Therefore, treatment with FSH is required for cumulus cells to obtain the ability to respond to LH. A combination of FSH and LH supplementation is commonly used in porcine IVM (Mattioli et al. 1989; Sun et al. 2004; Yuan and Krisher 2010). The other commonly used hormonal supplements for oocyte IVM are equine chorionic gonadotropin (eCG) and hCG (Iwamoto et al. 2005; Li et al. 2003; Yuan et al. 2008). However, no differences in oocyte nuclear maturation and subsequent developmental competence were observed between these two protocols (Silvestre et al. 2007). Another discrepancy among protocols is the time period of hormonal treatment. Studies demonstrated that removal of hormonal supplements (eCG, hCG and oestradiol) from maturation media after 20 h of culture enhances cytoplasmic maturation of porcine oocytes (Funahashi et al. 1994a; Funahashi and Day 1993b). Most of the maturation protocols that move oocytes to hormone free medium halfway through the IVM period were using eCG and hCG as hormonal supplements. Our laboratory found no difference in development whether oocytes were matured in one-step system or two-step system when using FSH and LH supplements, and the extra handling of the oocyte during IVM may cause negative effects on oocyte development. Kawashima et al. suggested a novel hormonal supplement protocol to mimic hormonal changes observed in vivo. They proposed a new IVM system that oocytes were firstly matured with 2 ng/ml FSH and 100 ng/ml estradiol. After 20 h culture, oocytes were removed to fresh medium with 1 µg/ml LH, 1 ng/ml EGF and 100 ng/ml progesterone for an

additional 24 h maturation. Using this protocol, they improve oocytes developmental competence to blastocyst stage (Kawashima et al. 2008).

One historical problem for oocyte IVM is the desynchronization of nuclear and cytoplasmic maturation. Many in vitro matured oocytes can successfully complete meiosis but fail to complete embryonic development indicating that the cytoplasmic maturation is incomplete (Krisher 2004). Efforts have been made to synchronize the progress of maturation to improve the oocyte developmental competence after IVM. Cell cycle-dependent kinase inhibitors butyrolactone-1 (Wu et al. 2002), roscovitine (Romar and Funahashi 2006), protein-synthesis inhibitors cycloheximide (Ye et al. 2005) and dibutyryl cyclic adenosine monophosphate (cAMP) (Somfai et al. 2003) have been supplemented into the IVM medium to inhibit meiotic maturation. The delayed meiotic resumption allows accumulation of more nutrients and growth factors through the cumulus-oocyte communication to promote cytoplasmic maturation. However, the improved developmental competence of oocyte was only observed with the supplementation of cycloheximide and dibutyryl cAMP.

Energy metabolism and carbohydrate supplementation

Oocyte cytoplasmic maturation involves a series of cellular events. Several metabolic pathways play important roles during oocyte maturation and affect oocyte developmental competence. Glucose is an essential energy substrate for most mammalian cells. It plays fundamental roles during oocyte maturation. Mouse and

bovine oocytes have a very poor capacity to metabolize glucose directly and rely on the cumulus cells to convert glucose into substrates that can be readily utilized by oocytes (Biggers et al. 1967; Sutton-McDowall et al. 2010). In many species, pyruvate is the preferred substrate of in vitro matured oocyte (O'Brien et al. 1996; Rieger and Loskutoff 1994; Spindler et al. 2000). However, porcine oocyte tends to use glucose as their primary energy source (Krisher et al. 2007). The glucose can be metabolized by oocytes through glycolysis and the pentose phosphate pathway (PPP). Many studies demonstrated that the oocyte glycolytic and the PPP activities are closely related to its developmental competence. The enhanced glycolysis is associated with increased oocyte developmental competence in cats and cattle (Krisher and Bavister 1999; Spindler et al. 2000). In vivo matured porcine oocytes utilized significantly more glucose via glycolysis and the PPP compared to porcine oocytes matured in vitro (Krisher et al. 2007). Chemical inhibition of glycolysis and the PPP cause decreased oocyte maturation and developmental competence (Herrick et al. 2006a).

Lipid contains several folds more energy compared with glucose. However, the role of lipid metabolism in oocytes is not as well studied as that of glucose metabolism. A recent study in mice demonstrated that lipid β -oxidation is essential for oocyte developmental competence and early embryo development (Dunning et al. 2010). Porcine oocytes, compared with other mammalian species, are characterized by high lipid content, stored mainly as lipid droplets in the cytoplasm that are co-localized

with mitochondria (Sturmey and Leese 2003). The amount of endogenous lipid decreases in porcine oocytes during IVM, and there is a corresponding fall in the oxygen consumption sufficient to account for this depletion (Sturmey and Leese 2003). Exposure to inhibitors of lipid β -oxidation during porcine oocyte maturation results in developmental failure post IVF (Sturmey et al. 2006). These results suggest that lipid metabolism during porcine oocyte maturation is critical for developmental competence.

It is important that the maturation environment is able to meet the metabolic needs to support oocyte development. Carbohydrates including glucose, pyruvate, and lactate, are very important components in the culture medium to provide the energy needs for oocyte maturation. The concentrations of glucose, pyruvate and lactate in culture medium can greatly influence the metabolism of these substrates (Downs and Utech 1999; Khurana and Niemann 2000; Krisher and Bavister 1999). To determine the optimal concentrations of the substrates in culture medium, the physiological concentration in follicular fluid should be determined. A study in goat oocytes demonstrated that although supra physiological concentrations of glucose and lactate did not influence the oocyte developmental competence to blastocyst stage, the glycolytic activity of oocytes were significantly decreased (Herrick et al. 2006b). The follicular glucose concentrations in pig were different in different size follicles: ranging from 1.81 mM in 7-9 mm follicles to 1.03 mM in 3-5 mm follicles (Brad et al. 2003). The dosing experiment of glucose and lactate for porcine oocyte IVM revealed

that physiological concentrations of energy substrates (2 mM glucose and 6 mM lactate) during maturation increased glycolytic and PPP activity. Higher concentrations of glucose and lactate decreased glucose metabolism, although no differences were observed in cleavage and blastocyst formation (Brad et al. 2003). Due to the unique energy metabolism patterns in porcine oocytes, the maturation medium needs to be specifically designed to meet the metabolic requirement of porcine oocyte development.

Comparison of undefined medium and defined medium

Various maturation media have been used for maturation of pig oocytes including Waymouth's medium (Yoshida et al. 1992a), NCSU solutions (NCSU-23 and -37) (Petters and Wells 1993), TCM-199 (Yoshida et al. 1990), and modified Tyrode's medium containing lactate and pyruvate (TLP) (Yoshida et al. 1993). These media are often supplemented with serum or porcine follicular fluid (pFF). Numerous studies confirmed that pFF can improve oocyte maturation, normal fertilization and embryo development in pigs (Funahashi and Day 1993a; Naito et al. 1988; Yoshida et al. 1990; Yoshida et al. 1992b). Moreover, pFF in the IVM medium was found to support normal distribution of mitochondria and had a positive effect on the cytoplasmic microtubule network that controls mitochondrial reorganization (Brevini et al. 2005). The effects of pFF on oocyte maturation and development are influenced by the size of the follicle from which pFF is harvested. Snap-frozen pFF can increase maturation

and normal fertilization compared to that in fresh pFF (Vatzias and Hagen 1999).

Currently, pFF cannot be obtained commercially, every laboratory has to prepare it from abattoir-derived ovaries. There is a potential problem in that the properties of the pFF may be variable in different batches and influence the experimental results. Therefore, commercially available bovine serum has been used for oocyte maturation. Compared to pFF, oocytes matured with serum supplementation have decreased maturation rates (Vatzias and Hagen 1999). However, further studies proved that, once oocytes have matured, oocytes matured with serum have the same competence to complete IVF and embryo transfer (Suzuki et al. 2006).

Although undefined medium supplemented with serum or pFF is beneficial to oocyte development, it contains many unknown factors that may regulate the maturation process, making it difficult to analyze the metabolic requirements of the oocytes. Supplementing pFF also may lead to variability in the experiments and has the potential risk of introducing occult viruses in to maturation environment (Stringfellow and Givens 2000). A defined IVM medium (containing no serum or pFF) can help to identify the specific metabolic requirements of oocytes and support oocyte development with no potential risk. In addition, it provides more consistent and comparable results between laboratories. pFF can be replaced by macromolecular polymers, such as poly-vinyl-pyrrolidone (PVP) or poly-vinyl-alcohol (PVA) to produce a chemically defined medium, such as modified TCM-199(Abeydeera et al. 1998b) and modified NCSU-23(Hong et al. 2004). However, the basic medium TCM-

199 was originally developed for chick embryo fibroblasts culture, and NCSU was developed for porcine embryo culture, therefore they may not be optimal for oocyte maturation. Based on the composition of pig oviductal fluid, a chemically defined medium (porcine zygote medium, PZM) was developed for embryo IVC (Yoshioka et al. 2002). The same group also established a chemical defined system for IVF (porcine gamete medium, PGM) (Yoshioka et al. 2003) and IVM (porcine oocyte medium, POM) (Yoshioka et al. 2008) with modifications from PZM as a basic medium. Our laboratory developed a defined medium system for porcine IVM, IVF and IVC (Purdue porcine medium, PPM) (Herrick et al. 2006a; Herrick et al. 2003; Stroble et al. 2002). By analyzing concentrations of energy substrates in porcine follicular fluid, the concentrations of substances in modified PPM for maturation (PPMmat) are more similar to physiological levels. Oocytes matured in PPMmat have improved metabolism and increased normal fertilization and cleavage rates (Brad et al. 2003; Stroble et al. 2002)

Redox balance and oocyte maturation

During oocyte maturation, the balanced intracellular redox status is crucial for successful development (Agarwal et al. 2008; Dumollard et al. 2007). Oocyte cellular redox status regulates metabolic and enzyme activity and is maintained by a number of parameters including thiol/disulfide ratios and the intracellular level of reactive oxygen species (ROS). In this section, the mechanism of redox balance regulation and its effects on oocyte maturation are discussed.

ROS and oxidative stress

ROS such as hydrogen peroxide (H_2O_2), hydroxyl radicals ($\text{OH}^\cdot$), superoxide anions ($\text{O}_2^\cdot$) and nitric oxide (NO) are produced during normal cellular metabolism. Like any other cell, oocytes generate ATP through mitochondrial oxidative phosphorylation and glycolysis (Thompson et al. 2000). Under normal physiological conditions, these aerobic and anaerobic metabolism processes lead to the production of ROS. ROS in oocytes may also accumulate indirectly from the surrounding environment. ROS are highly reactive and are known to cause widespread damage to proteins, nucleic acids, lipids, and other biomolecules (Storz and Imlay 1999). Cells have intrinsic antioxidant mechanisms to counteract this problem. However, when ROS concentrations rise above a certain level, the antioxidant mechanisms are not sufficient to prevent oxidative damage, and increased ROS can directly oxidize protein thiols and rapidly form disulfide bonds (Claiborne et al. 1999), putting the cell into a state that is defined as oxidative stress (Sies 1991). Oxidative stress has broad impacts on the reproductive system. It has detrimental effects on ovarian steroidogenesis and ovulation (Agarwal et al. 2005), embryogenesis (Bedaiwy et al. 2004), and implantation and maintenance of pregnancy (Myatt and Cui 2004). It also directly impairs oocyte and embryo development (Hashimoto et al. 2000; Yang et al. 1998). Several studies demonstrated negative correlations between follicular fluid ROS levels and successful IVF and pregnancy in humans as well (Bedaiwy et al. 2005; Pasqualotto et al. 2004). However, ROS at physiological levels also plays important roles in regulating proper cellular

functions (Agarwal et al. 2004; Rhee 2006). It regulates various reproductive processes, including maintaining the proper function of sperm (Gagnon et al. 1991) as well as being involved in sperm-oocyte interaction (deLamirande et al. 1997). A transient increase of intracellular ROS level may signal meiotic resumption from diplotene arrest in mammalian oocytes (Pandey et al. 2010). In addition, ROS is indispensable for cumulus cell expansion, and ovarian production of ROS is an essential preovulatory signaling event (Shkolnik et al. 2011).

Defense mechanism against oxidative stress

Antioxidants are molecules that retard the oxidation of another. They can prevent oxidation by gaining an electron and can be referred to as a reducing agent.

Antioxidants are able to alleviate the buildup of ROS by removing free radicals.

Numerous compounds have antioxidant functions. A series of enzymatic antioxidants including catalases (CAT) (Chelikani et al. 2004), superoxide dismutases (SOD) (Johnson and Giulivi 2005), and peroxidases (GPx) (Brigelius-Flohe 1999) are expressed in cells that act to neutralize ROS. Cu, Zn-SOD, located in the cytosol, and Mn-SOD located in the mitochondria scavenge the O_2^- , and this is the first reaction that protects cells against toxic ROS. H_2O_2 , the end-product of SOD action, is then converted into H_2O and O_2 either by CAT or by GPx. The transcripts of these enzymes are expressed during oocyte and embryo development, suggesting that these enzymes may be regulated depending on developmental stages. Transcripts encoding Cu,Zn-

SOD are present in oocytes at all stages of maturation, suggesting that it plays a pivotal role in protecting oocytes against oxidative stress (El Mouatassim et al. 1999). Mn-SOD and GPx are expressed in mouse and human oocytes at MII stage. CAT is absent in MII oocytes but are present in mouse blastocysts (El Mouatassim et al. 1999). In addition, CAT is present in bovine, porcine, and human oviductal fluid (Lapointe et al. 1998), suggesting that the reproductive tract has antioxidant properties to protect gametes and embryos against oxidative stress. The transcription of these antioxidants can be regulated by intracellular redox status. Suboptimal culture environments may result in altered metabolism and ROS production, therefore, modulating the antioxidant activity (El Mouatassim et al. 1999).

There are small thiol molecules that also act to maintain the redox balance of cells. The two major thiol-redox systems (thioredoxin and glutaredoxin) maintain the reducing capacity of the cytosol and prevent unwanted disulfide bond formation in proteins (Prinz et al. 1997). The thioredoxin system consists of thioredoxin reductase (*TRXR1*) and thioredoxin (*TRx*). The glutaredoxin system is mainly composed of glutathione reductase (*GSR*), glutathione (GSH), and three glutaredoxins (*GLRX1-3*) (Prinz et al. 1997). GSH is the major non-enzymatic defense system against ROS in the cytoplasm. It is responsible for reducing disulfide bonds in cytosolic proteins, providing cells with a reduced environment and protecting against oxidative stress (Aslund and Beckwith 1999; Meister and Tate 1976). Numerous studies suggested the importance of GSH in supporting oocyte maturation and further development. GSH in porcine oocytes is

associated with higher rates of male pronuclear formation (Yoshida et al. 1993). GSH concentrations in porcine oocytes are also correlated to the success of oocyte maturation and embryonic development (Tatemoto et al. 2000). Inhibiting glucose metabolism via PPP and glycolysis can decrease GSH concentrations and reduce oocyte developmental potential (Herrick et al. 2006a). GSH is suggested as an index of ooplasmic maturation in many studies (Abeydeera et al. 1998a; de Matos and Furnus 2000b; de Matos et al. 2002a; Funahashi et al. 1994b). However, a study in goat oocytes found that by supplementing a high level of cysteamine and cysteine in maturation medium, intracellular GSH was significantly increased, but the blastocyst formation was decreased. They speculated that too high a level of GSH upset redox homeostasis and interrupted certain cell functions (Zhou et al. 2008). During IVM, oocytes are exposed to a different redox environment compared to their in vivo counterparts. To balance the aberrant redox environment in vitro, several small thiol compounds can be added to the maturation medium to stimulate GSH synthesis, such as cysteine and cystine, cysteamine, or β -mercaptoethanol. Supplementation of these small thiols during oocyte IVM can stimulate GSH synthesis and thus may improve embryo development to the blastocyst stage (de Matos and Furnus 2000a; de Matos et al. 2002b).

Antioxidant supplementation of culture medium

Several compounds that have antioxidant functions are regularly supplemented in the culture medium for oocyte maturation and embryo culture. The most common ones in the culture medium are cysteine, cysteamine, pyruvate, taurine and hypotaurine. Their antioxidant properties are discussed here.

Cysteine is a crucial component amino acid of GSH. Synthesis of GSH is highly dependent on the availability of cysteine in the medium (Meister and Tate 1976).

Under in vitro conditions, cysteine is highly unstable and easy oxidized into cystine (Bannai 1984). The concentration of cysteine in TCM-199 is very low (0.6 μ M), it is possible that the cystine is converted into cysteine by cumulus cells and then participates in GSH synthesis (Yoshida et al. 1993). In porcine oocytes, a relatively high concentration of cysteine during in vitro maturation appears to be required to increase oocyte GSH concentrations at the end of maturation, and promote male pronuclear formation following sperm penetration in vitro (Yoshida et al. 1993; Yoshida et al. 1992a). Therefore, supplementation of IVM medium with cysteine or use of a cysteine-rich medium can result in better quality oocytes.

Cysteamine can reduce cystine to cysteine, promoting cysteine uptake and therefore enhancing GSH synthesis (Issels et al. 1988). It can maintain the redox status in oocytes and a high ratio of GSH/GSSG. Cysteamine can also directly protect cells against ionizing radiation, and is known as a scavenger of OH $\cdot$ (Zheng et al. 1988).

Addition of cysteamine to the culture medium improves the maturation and

development of oocytes in several species (Abeydeera et al. 1998a; de Matos et al. 1995; de Matos et al. 2003; Gasparrini et al. 2000; Kobayashi et al. 2006; Zhou et al. 2008). Significant amounts of cysteamine was also detected in follicular fluid (Guyader-Joly et al. 1998).

Pyruvate is important in energy metabolism as well as in redox potential. Pyruvate acts as an extracellular antioxidant (O'Donnell-Tormey et al. 1987). It can be decarboxylated in the presence of H_2O_2 to produce acetate, CO_2 and H_2O . Pyruvate prevents peroxide-induced injury of in vitro cultured bovine embryos (Morales et al. 1999).

Taurine and hypotaurine are synthesized and secreted by oviduct epithelial cells, and large amounts of taurine and hypotaurine are present in oviductal fluid (Guerin and Menezo 1995). Hypotaurine can neutralize $OH^\cdot$ and may have important antioxidant functions for gametes and embryos in the in vivo environment. The by-product of hypotaurine after free radical scavenging is taurine. Taurine can neutralize cytotoxic aldehydes to minimize the detrimental effects of ROS (Ogasawara et al. 1993).

Supplementing taurine or hypotaurine in culture medium has beneficial effects on embryo development (Barnett and Bavister 1992; Dumoulin et al. 1992; Fujitani et al. 1997; Guyader-Joly et al. 1998). Recent studies also demonstrate that enriching the culture medium with taurine can improve in vitro oocyte meiotic maturation and embryo development, possibly through the role of the free radical scavenging effect (Kim et al. 2011; Manjunatha et al. 2009)

Gene transcriptional regulation in the oocyte and early embryo

The transcripts and proteins stored in the cytoplasm of the oocyte are fundamentally important for oocyte maturation and development of early embryos (Meirelles et al. 2004; Piccioni et al. 2005). During oogenesis, oocytes accumulate a larger than necessary amount of transcripts for oocyte maturation and early embryo development (Sasaki et al. 1999). Transcription and storage of most transcripts occurs during the early stages of oocyte growth, but becomes silent as the germinal vesicle undergoes breakdown during meiosis (Brevini-Gandolfi et al. 1999). Epigenetic modifications including DNA methylation and modifications of histones regulate gene transcription without a change in basic structure of DNA. Many studies confirmed that epigenetic modifications play a key role in development (Reik 2007; Rutherford and Henikoff 2003). During oogenesis, oocyte nucleus exhibits a unique chromatin configuration that is subject to dynamic modifications. This process of ‘epigenetic maturation’ is critical for oocyte to complete meiosis and achieve developmental competence (De La Fuente 2006). In human and mouse oocytes, the changes of large scale chromatin structure during meiotic maturation are associated with the decreased metabolic status of the oocyte genome (Bouniol-Baly et al. 1999; Miyara et al. 2003). This transcriptional silencing process is mediated by epigenetic mechanisms. After transcriptional silencing, oocytes rely on stored transcripts to resume meiosis and finish the first cleavage divisions after fertilization (Hodgman et al. 2001; StebbinsBoaz et al. 1996). Thus synthesis and storage of enough maternal transcripts

before the onset of transcriptional silencing are essential for the oocyte to acquire developmental competence (De La Fuente and Eppig 2001).

When transcription is silent, gene expression is controlled at post-transcriptional level, especially by cytoplasmic polyadenylation. In general, polyadenylation elongates the poly (A) tail of transcripts, and is associated with translational activation, whereas deadenylation delete the poly (A) tails and is associated with translational silencing (Piccioni et al., 2005). Deletion of this poly (A) tails is an early step of transcript degradation (Couttet et al. 1997). Studies in bovine oocytes and embryos reveal that polyadenylation of various transcripts involved in the beginning of oocyte meiotic resumption to the first embryo cleavage. When comparing better quality embryos to embryos with low developmental potential, they observed several differences in polyadenylation patterns. In addition, abnormal polyadenylation may cause lower embryonic development potential (Brevini et al. 2002).

Regulation of oocyte meiosis resumption

Mammalian oocytes are arrested at the prophase diplotene stage of meiosis by an inhibitory signal from the surrounding somatic cells in the ovarian follicle before or after birth, and they remain arrested at this stage until resumption of meiosis following stimulation of FSH and LH after puberty. The resumption of meiosis is characterized by germinal vesicle breakdown, followed by completion of Meiosis I and another arrest in metaphase II stage when oocytes are ready for ovulation and subsequent fertilization (Saskova et al. 2008; Schindler and Schultz 2009).

Ovarian follicles consist of theca cells, granulosa cells and several layers of cumulus cells. The follicular environment plays an important role in maintaining oocyte meiotic arrest as removal of the oocytes from these follicles results in gonadotropin independent meiotic resumption in vitro (Edwards 1965; Pincus and Enzmann 1935). The follicular cells also control the oocyte meiotic resumption through an intrinsic mechanism that requires an integration of endocrine, paracrine and autocrine signaling pathway. This section will summarize the current progress in understanding how oocyte meiotic resumption is regulated in the ovarian follicles. A novel in vitro maturation system based on the new insights of meiotic resumption regulation in vivo will also be discussed.

Regulation of oocyte meiotic resumption in granulosa cells and oocytes

It is well known that the preovulatory LH surge induces oocyte maturation. Cyclic AMP (cAMP) is an important mediator of LH induced meiotic resumption. The high level of cAMP in oocyte blocks spontaneous meiotic resumption by suppressing maturation promoting factor (MPF) activity via activation of protein kinase A (PKA) (Wang et al. 2006). Oocyte cAMP can be synthesized by constitutively activation of G-protein-coupled receptors (GPR3) in the oocyte (Mehlmann et al. 2002). It can be transported to oocyte from the granulosa cells through gap junctions (Anderson and Albertini 1976). The oocyte cAMP can be also influenced by the activity of an enzyme, type 3 phosphodiesterase (PDE3), which degrades cAMP in oocyte (Tsafiriri

et al. 1996). The activity of PDE can be inhibited by cyclic GMP (cGMP), another important factor that can be also supplied by granulosa cells.

Signal transduction in follicular somatic cells plays an essential role in regulating oocyte meiosis in response to LH surge. LH, by acting through its receptor, stimulates biosynthesis of EGF-like factors, and this action is mediated by activation of cAMP/PKA (Liang et al. 2007) and protein kinase C (PKC) pathway (Sun et al. 2008). The EGF-like factors, via EGF receptor, activate the ERK signaling pathway, specifically ERK1/2, (Fan et al. 2009) and get involved in the rapid amplification and transduction of the LH signal from the somatic cells to the oocyte (Reizel et al. 2010). The activation of ERK signaling pathway leads to several downstream cascades. Although it has not been fully understood yet, the ERK signaling pathway may involve in phosphorylation of connexin 43, leading to a decrease in gap junctions between the somatic cells and subsequently leads to the decrease of cAMP and cGMP levels in oocytes (Sela-Abramovich et al. 2005). It may also mediate oocyte meiosis resumption through other downstream factors, such as C/EBP β (Fan et al. 2009).

Crosstalk between oocytes and somatic cells

The communication between oocytes and the companion somatic cells are bi-directional. Gonadotropins, via activation of EGFR-ERK1/2 signaling pathway in granulosa cells, induce meiotic resumption of oocytes. On the other hand, the function of granulosa cells is also regulated by oocyte-derived paracrine factors, such as

growth differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP15), and fibroblast growth factor 8B (FGF8B) (Su et al. 2009). GDF9 and BMP15 are two closely related members of the transforming growth factors β (TGF β) superfamily that are exclusively expressed in oocytes (Dube et al. 1998; McGrath et al. 1995). They play essential roles in regulating granulosa cell development and function. Double mutant *Bmp15*^{-/-}*Gdf9*^{+/-} females showed dramatic reduction of fertility (Yan et al. 2001). Following studies revealed that BMP15 and GDF9 cooperate before the LH surge to promote normal differentiation of cumulus cells (Su et al. 2004). BMP15 also participates in the regulation of cumulus cell development and function by promoting metabolism in cumulus cells before the LH surge (Su et al. 2008) and induces cumulus expansion right before ovulation (Yoshino et al. 2006).

GDF9 and BMP15 act on granulosa cells and cumulus cells through their receptors to activate Sma- and Mad- related (SMAD) intracellular signal transducers (SMAD2/3) (Kaivo-oja et al. 2006). The crosstalk between oocyte and somatic cells mainly relies on the EGFR-ERK1/2 and GDF9/BMP15-SMAD2/3 signaling pathways. ERK1/2 activation through EGFR enables the phosphorylation of SMAD3 in its linker region, and leads to the SMAD2/3 mediated gene transcription (Sasseville et al. 2010). In the other hand, oocyte can promote EGFR expression via GDF9/BMP15-SMAD2/3 signaling pathway (Su et al. 2010). The cell to cell interactions during oocyte and follicular development are driven by this oocyte-somatic cell regulatory loop, which plays a fundamental role in oocyte maturation, including oocyte meiotic resumption.

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CHAPTER 2

EFFECT OF AMMONIUM DURING IN VITRO MATURATION ON PORCINE OOCYTE NUCLEAR MATURATION AND SUBSEQUENT EMBRYONIC DEVELOPMENT*

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Abstract

The effects of ammonium in a chemically defined maturation medium on porcine oocyte nuclear maturation and subsequent embryonic development after in vitro fertilization (IVF) and parthenogenetic activation (PA) were examined. Cumulus-oocyte complexes were matured in Purdue Porcine Medium (PPM) supplemented with 0 mM, 0.02 mM, 0.2 mM, 2 mM, or 20 mM ammonium chloride, or TCM199 with 10% porcine follicle fluid (TCM+pFF; positive control) at 38.7 °C in 7% CO₂ in air for 40-44 hr. No significant difference ($P>0.05$) in nuclear maturation was found between oocytes matured in TCM+pFF or PPM with 0 mM, 0.02 mM and 0.2 mM ammonium chloride. However, nuclear maturation was significantly decreased ($P<0.05$) in oocytes matured in PPM with 2 mM or 20 mM ammonium. After IVF, oocytes matured in PPM with 20 mM ammonium resulted in embryos with significantly lower ($P<0.05$) embryonic cleavage and blastocyst development than all other treatment groups. After PA, oocytes matured in PPM with 20 mM ammonium resulted in embryos with lower ($P<0.05$) embryonic cleavage compared to TCM+pFF.

However, PA embryos derived from oocytes matured in PPM with both 2 mM and 20 mM ammonium had lower ($P<0.05$) blastocyst development compared with TCM+pFF. These results demonstrate the detrimental effect of ammonium during *in vitro* oocyte maturation on nuclear progression to metaphase II. Additionally, the presence of ammonium during *in vitro* maturation negatively influences subsequent embryonic development, although PA embryos appear to be more sensitive to the negative effects of ammonium during oocyte maturation than do IVF embryos.

Introduction

Oocyte maturation is a critical step in embryo *in vitro* production (IVP) systems because it influences oocyte quality, which affects embryonic development, fetal development, and even the health of the offspring (Eppig and O'Brien, 1998).

However, oocytes matured *in vitro* have lower developmental competence compared with oocytes matured *in vivo*, indicating that *in vitro* maturation (IVM) systems are suboptimal. Therefore, optimization of current maturation medium is necessary to increase the efficiency of IVM systems.

Amino acids are included in the majority of media for both oocyte maturation and embryo culture, as they serve a variety of physiological functions, including; protein and nucleotide synthesis (Epstein and Smith, 1973; Alexiou and Leese, 1992), provision of energy sources (Gardner, 1998), protection against osmotic shock (Lane, 2001) and oxidative stress (Lindenbaum, 1973), and pH regulation (Edwards et al., 1998). Unfortunately, spontaneous degradation and catabolism of amino acids,

especially glutamine, can result in ammonium production (Gardner and Lane, 1993), which is very toxic for living cells in vivo (Prior and Visek, 1972) and in vitro (Visek et al., 1972). The detrimental effects of ammonium on ruminant (McEvoy et al., 1997; Sinclair et al., 1998) and rodent (Gardner and Lane, 1993) preimplantation embryos have been well documented. The presence of ammonium in culture medium results in reduced intracellular pH, depressed oxidative phosphorylation (Lane et al., 2002), reduced blastocyst cell number (Gardner and Lane, 1993), increased levels of cellular apoptosis, perturbed levels of *Slc2a3* expression and glucose uptake (Zander et al., 2006), altered fetal development and growth rates (McEvoy et al., 1997; Sinclair et al., 1998) and fetal exencephaly (Lane and Gardner, 1994).

Ammonium may also influence the development of preimplantation embryos in vivo, especially in ruminant animals. Excessive degradation of protein/urea in the rumen may result in large amounts of ammonium released into the circulation (Papadopoulos et al., 2001), increasing the ammonium concentrations in the fluids of the reproductive tract, thereby causing embryo toxicity and reduced conception rates (Papadopoulos et al., 2001; Ferguson et al., 1988; Staples et al., 1993). Ammonium concentrations in follicular fluid are influenced by dietary protein intake in the bovine (Hammon et al., 1997), and are negatively associated with follicle size.

Despite many reports on the deleterious effects of ammonium on embryo development, few reports exist examining the effects of ammonium during oocyte maturation. Bae and Foote (1975) reported ammonium production during IVM of

rabbit oocytes, but no inhibitory effect on oocyte maturation was found. Hammon et al. (2000a) investigated the effect of ammonium during IVM of bovine oocytes on subsequent embryo development, and found that exposure to 356 μM ammonium did not adversely influence embryonic development. In another experiment, Hammon et al. (1999) increased the concentration of ammonium in maturation medium to 1400 μM , which is much higher than the ammonium concentrations in physiological conditions, and still found no significant influence on bovine oocyte nuclear maturation or subsequent embryo development. These results suggest that in the bovine, oocytes can resist high concentration ammonium toxicity.

Porcine oocytes have a unique metabolic profile compared to other species (Krisher et al., 2007). Thus, it is possible that porcine oocytes have different sensitivities to ammonium. The aim of this study was to evaluate the effects of ammonium on oocyte nuclear maturation and subsequent embryo development after parthenogenetic activation and in vitro fertilization.

Materials and methods

All chemicals used in this study were purchased from Sigma Chemicals (St. Louis, MO, USA) unless specified otherwise.

Collection of oocytes and in vitro maturation

Ovaries of pre-pubertal gilts were collected at a local slaughterhouse and transferred to the laboratory in 30-35 °C 0.9% (w/v) NaCl. Oocytes were aspirated from follicles

(3-8 mm in diameter) with an 18-gauge needle attached to a vacuum pump. Oocytes with several layers of unexpanded cumulus cells and evenly dark cytoplasm were selected and rinsed in Hepes-buffered synthetic oviductal fluid (SOF-Hepes) containing 0.1% (w/v) BSA, 1.0 mM glutamine, and both essential (1X) and non-essential (1X) amino acids for MEM Eagle (MP Biomedicals, Costa Mesa, CA, USA). Oocytes were then washed twice in maturation medium and cultured in humidified air with 7% CO₂ at 39.7 °C for 40-44 h in 500 µl maturation medium in a four-well plate (50-60 oocytes/well; Nunclon, Nalge Nunc Intl, Rochester, NY, USA). Maturation medium used in this experiment was modified Purdue Porcine Medium (PPM, experimental treatments) (Stroble et al., 2002; Herrick et al., 2003; Herrick et al., 2006) containing 2 mM glucose, 6 mM lactate, 0.5 mM cysteamine, 50 ng/ml EGF, 0.01 units/ml LH, 0.01 units/ml FSH and supplemented with 1% recombunin (Vitrolife, Kungsbacka, Sweden) and 0.2% fetuin, or TCM199 with 10% porcine follicle fluid containing 5 ng/ml EGF, 0.01 units/ml LH and 0.01 units/ml FSH (TCM+pFF; positive control). For experimental treatments, different concentrations of ammonium chloride were added to PPM; 0 mM, 0.02 mM, 0.2 mM, 2 mM, and 20 mM.

Assessment of Meiotic Status

After maturation, oocytes were denuded of cumulus cells by vortexing in SOF-Hepes with 0.01% (w/v; 80-160 unit/ml) hyaluronidase for 3 min. Groups of 10-20 oocytes

were mounted, fixed in chloroform : acetic acid : ethanol (1:3:6, v:v:v) for 72 h, stained with 1% (w:v) orcein in acetic acid, and examined for meiotic stage using a microscope at 400 × magnification. The oocytes developed to stages of telophase and metaphase II were regarded as in vitro matured oocytes and the percentage of oocytes maturation were recorded from each group.

In vitro fertilization and embryo culture

For fertilization, oocytes were washed twice in modified Tris-buffered medium (mTBM) (Abeydeera and Day, 1997) supplemented with 2 mM caffeine, 0.2% (w/v) fraction V BSA and cultured in 50 µl drops of mTBM under 10 ml mineral oil (20 oocytes/drop; Falcon 1007; Becton Dickinson Labware, Franklin Lakes, NJ, USA). 500 µl of fresh, extended (extender was purchased from Swine Genetic International, Ltd. Cambridge, IA, USA), chilled semen was placed onto a gradient of 45%:90% Percoll and centrifuged for 20 min at 700 g. The supernatant was removed and the remaining sperm pellet was washed in 5.0 ml D-PBS twice by centrifuging for 5 min at 1000 g. Sperm were then counted and diluted (1×10^6 sperm/ml) in mTBM and added (50 µl) to fertilization drops containing oocytes for a final sperm concentration of 5×10^5 sperm/ml. Gametes were coincubated for 5 h in 5% CO₂ in humidified air. Following coincubation, zygotes were washed 3 times and cultured in 50 µl NCSU-23 medium (10 zygotes/drop; Falcon 1007) containing 0.4% crystallized BSA (Serologicals Proteins Inc., Kankakee, IL, USA) under 10 ml mineral oil in 5% CO₂,

10% O₂, 85% N₂ for 6 days, when embryonic cleavage and blastocyst development were determined. The blastocysts were stained with 0.01 mg/ml Hoechst 33342 overnight. Total blastocyst cell number was counted under fluorescence at × 400 magnification.

Parthenogenetic activation of oocytes and PA embryo culture

For PA, oocytes were placed between the electrodes of an electroporation chamber filled with activation medium (0.3 M Mannitol, 0.5 mM Hepes, 0.1 mM MgSO₄, 0.1 mM CaCl₂, 0.01 mg/ml BSA) and exposed to a DC electrical pulse of 120 v/mm, 80 μs delivered by BTX Electro Cell Manipulator (Harvard Apparatus, Holliston, MA). After activation treatment, PA embryos were washed 3 times and cultured in Porcine Zygote Medium-3 (PZM-3)(Yoshioka et al., 2002), containing 2 mM N-6 dimethylaminopurine (6DMAP) for 6 h, then washed and cultured in PZM-3 supplemented with 4 mg/ml BSA for 6 days in conditions as described for IVF embryos. Embryonic cleavage and blastocyst development, as well as blastocyst cell number, of PA embryos were determined as described above.

Experimental design and statistical analysis

Three experiments, assessing meiotic status, IVF and PA embryo development, were completed separately. Assessment of meiotic status was replicated six times. In each replicate, 20-40 oocytes were used in each group. A total of 1123 oocytes were used

in this experiment. For oocyte IVF, all treatment groups were replicated four times, except PPM with 20 mM ammonium, which was replicated three times. In each replicate, 50 oocytes were used in each group; a total of 1110 oocytes were used in this experiment. For oocyte PA, TCM+pFF, PPM with 0.2 mM, 2 mM and 20 mM ammonium groups were replicated four times, while PPM with 0 mM and 0.02 mM ammonium groups were replicated three times. In each replicate, 50 oocytes were used in each group; a total of 1107 oocytes were used in this experiment.

Percentages of maturation were analyzed using two-way ANOVA, with maturation medium treatment included in the model as a fixed factor and replicate as a random factor. Cleavage, blastocyst development and blastocyst cell number were analyzed using GLM ANOVA. Percentage data (maturation percentage, embryonic cleavage and blastocyst development) were arcsin transformed. Differences were determined by Bonferroni multiple comparison test. Significance was calculated as $P < 0.05$. Data are reported as mean $\pm$ SEM.

Results

Effects of ammonium on nuclear maturation of porcine IVM oocytes

There was no significant difference in percentages of maturation between TCM+pFF, PPM with 0 mM, 0.02 mM (and 0.2 mM ammonium. However, Percentages of oocytes maturation were significantly decreased when ammonium concentration reached 2 mM and 20 mM during IVM (Table 2.1).

Effects of ammonium during IVM on subsequent embryonic development of IVF and PA porcine embryos

After IVF, no significant difference in the percentage of cleaved embryos or blastocyst formation was found between groups, except oocytes matured in PPM with 20 mM ammonium, which had significantly decreased embryonic cleavage and blastocyst development compared with all other groups (Table 2.2). After PA, oocytes matured in TCM+pFF had a higher ($P<0.05$) percentage of cleaved embryos compared to those in all PPM groups. In PPM groups, a difference in percentage of cleaved embryos was only observed between PPM with 0.2 mM and 20 mM ammonium ($P<0.05$) (Table 2.3). PA embryos derived from oocytes matured in TCM+pFF had a significantly higher percentage of blastocyst development compared with those in PPM with 0.2 mM, 2 mM and 20 mM ammonium. There was no difference in blastocyst development between TCM+pFF and PPM with 0 mM or 0.02 mM ammonium groups. The proportion of PA embryos developing into blastocysts was not different within PPM treatment groups (Table 2.3). No significant difference in blastocyst cell number was found between any treatment group after either IVF or PA (Tables 2.2, 2.3).

Discussion

Supplementation of a defined maturation medium with either 2 mM or 20 mM ammonium decreased nuclear maturation of porcine oocytes. Recently, Tareq et al.

(2007) reported that porcine oocyte maturation was adversely affected when $\geq 300 \mu\text{M}$ exogenous ammonium was added to maturation medium. Since Tareq et al (2007) did not find inhibitory effects of $150 \mu\text{M}$ ammonium and we did not find inhibitory effects of $200 \mu\text{M}$ (0.2 mM) ammonium, it would appear that the threshold ammonium exposure for porcine oocytes that results in toxicity lies between 200 and $300 \mu\text{M}$. Interestingly, porcine follicular fluid contains concentrations of ammonium from $350 \mu\text{M}$ in small follicles (diameter $\leq 2 \text{ mm}$) to $170 \mu\text{M}$ in large follicles (diameter: $5\text{-}6 \text{ mm}$) (Tareq et al., 2005). These results indicate that the apparent threshold concentration of ammonium toxicity for porcine oocytes in vitro ($200\text{-}300 \mu\text{M}$) is greater than what they would be exposed to in a large follicle during nuclear maturation ($170 \mu\text{M}$). In contrast, in vitro exposure of bovine oocytes to ammonium concentrations up to $1400 \mu\text{M}$ for 24 h IVM does not adversely influence oocyte nuclear maturation, even though concentrations of ammonium in bovine follicular fluid range from $366 \pm 51 \mu\text{M}$ in small follicles (diameter $\leq 1 \text{ mm}$) to $33 \pm 10 \mu\text{M}$ in large follicles (diameter: $10\text{-}15 \text{ mm}$) (Hammon et al., 2000a). These results suggest that during in vitro maturation, bovine oocytes are better able to tolerate supraphysiological concentrations of ammonium compared to porcine oocytes. This difference could be attributed to a number of factors, including an extended time of exposure for porcine oocytes (44 h vs 24 h maturation period), or differences in metabolic ammonium production by the cumulus cells. If porcine cumulus cells metabolize more amino acids than bovine cumulus cells, and cumulus-oocyte

complexes are incubated for an extended amount of time, the total amount of ammonium present in the medium after 44 h could be much greater than the amount of exogenous ammonium added at the initiation of culture.

During IVM, cumulus cells are considered to be of vital importance to the success of oocyte maturation (Tanghe et al., 2002). In vitro growth and metabolism of cumulus cells is altered by concentrations of ammonium similar to those measured in follicular fluid, and the ability of these cumulus cells to support in vitro maturation of oocytes was impaired (Rooke et al., 2004). Therefore, decreased oocyte maturation caused by ammonium may not be due solely to direct effects on the oocyte, but also indirect effects on the nursing cumulus cells. In the present study, decreased cumulus cell expansion at the conclusion of IVM was observed in COCs matured in PPM with high (2 mM or 20 mM) concentrations of ammonium (data not shown). These are the same ammonium concentrations that resulted in decreased nuclear maturation.

Alternatively, cumulus cells may reduce the amount of ammonium the oocyte is directly exposed to by detoxifying ammonium in the medium or preventing transport to the oocyte via gap junctions. Previous studies on mouse embryos revealed that 18.8-300 μ M ammonium in culture medium significantly reduces blastocyst cell number, inhibits inner cell mass development and increases cellular apoptosis in the resulting blastocysts (Lane and Gardner, 2003). The deleterious effects of ammonium are more obvious during the cleavage stages in mouse embryos (Zander et al., 2006). In bovine, exposure of embryos to 29-356 μ M ammonium during IVC increased the

percentage of degenerate embryos and decreased blastocyst development (Hammon et al., 2000b), although exposure of bovine oocytes to the same concentrations of ammonium did not influence blastocyst formation (Hammon et al., 2000a). These results indicate that sensitivity to ammonium is dependent on the developmental stage of the oocyte or embryo, and may be related to the presence of cumulus cells.

We used TCM+pFF maturation medium as a positive control in our experiments, as it is the most commonly used medium in porcine in vitro embryo production systems.

We used PPM as background medium for experimental treatment since PPM is a semi-defined medium, and we replaced glutamine with alanyl-glutamine in PPM to minimize ammonium generation. In this study, only oocytes matured in PPM with 20 mM ammonium resulted in decreased embryonic development after IVF compared TCM+pFF and PPM with 0 ammonium controls. In contrast, oocytes matured in PPM with 0.2 mM, 2 mM or 20 mM ammonium prior to PA exhibited decreased development to the blastocyst stage compared with TCM+pFF control ($P < 0.05$).

However these three groups did not exhibit reduced blastocyst development if compared to PPM with 0 mM ammonium control. Compared to reported results in bovine oocytes, porcine oocytes are more sensitive to ammonium concentration, as determined by blastocyst formation. Porcine oocytes showed decreased blastocyst development when matured with 0.2 mM ammonium. In bovine, oocytes 356 μ M ammonium in maturation medium did not adversely influence the blastocyst formation. PZM-3 was used as culture medium for the PA embryos in this experiment,

as PZM-3 supports better development of porcine PA embryos compared to NCSU-23 (Im et al., 2004). In our laboratory, we observe better embryonic development of IVF oocytes when using NCSU-23 as the culture medium. Although development cannot be directly compared between PA and IVF embryos due to this difference in culture media, PA embryos appear to be more susceptible to damage caused by ammonium during IVM compared to IVF embryos.

Exposure of bovine oocytes to low or moderate concentrations of ammonium (29 μM - 88 μM) during IVF increases blastocyst, expanding blastocyst, and hatching blastocyst development (Hammon et al., 2000b). Ovine embryos exposed to elevated utero-oviductal ammonium concentrations up to day 3 postinsemination were more advanced in development and had increased metabolic activities (McEvoy et al., 1997). These authors hypothesized that a microenvironment containing elevated concentrations of ammonium may contribute to reprogramming of the embryo during the earliest stages of development. In the current study, oocytes were not exposed to ammonium during IVF. However, it is possible that ammonium was accumulated in oocytes during IVM via Na^+/K^+ - ATPase or Na^+/K^+ 2Cl^- -cotransporter (Martinelle and H äggstr öm, 1993). Ammonium built up in oocytes may then have a beneficial effect on nuclear reprogramming after IVF, resulting in relatively higher (although not significantly different) blastocyst development from oocytes matured in PPM with 2 mM ammonium. However, PA embryos, which use same maternal nuclear reprogramming strategy as that in normal embryo development (Gao et al., 2007), did

not display increased blastocyst development after treatment with 2 mM ammonium.

Thus, it is possible that small concentrations of ammonium have beneficial effects only on paternal DNA reprogramming.

Different sensitivities to ammonium of oocytes from different species may also be attributed to differing metabolic preferences. Mechanisms of the inhibitory effects of ammonium are still unclear. Gardner and Lane (1993) proposed that ammonium may promote the conversion of α -ketoglutarate to glutamate, resulting in a reduction in flux through the tricarboxylic acid cycle and reduced ATP production. Schneider et al. (1996) suggested two possible mechanisms; transportation of ammonium across membranes requires $\text{Na}^+ / \text{K}^+ - \text{ATPase}$, or, interaction of ammonium with enzymes, participating in a series of futile cycles which detoxify ammonium and result in consumption of ATP. Furthermore, it is known that pyruvate may be used as an ammonium sink by transamination to alanine in early embryos (Orsi and Leese, 2004). Glutamate can also dispose of ammonium by transfer into glutamine in blastocysts, but only in the absence of pyruvate available for transamination to alanine (Orsi and Leese, 2004). These findings suggest that ammonium may be closely associated with the energy metabolism of oocytes. Pig oocytes have distinctive energy metabolic patterns, featuring glucose utilization as their primary energy substrate rather than pyruvate as in mouse and bovine oocytes (Krisher et al., 2007). Glutamine is the most volatile amino acid. It easily breaks down and generates ammonium in culture medium (Lane and Gardner, 2003). Ammonium produced by

medium containing 1 mM glutamine incubated at 37°C for 24h is enough to inhibit embryo development (Lane and Gardner, 2003; Orsi and Leese, 2004; Virant-Klun et al., 2006). One way to reduce toxic ammonium build up is by substituting glutamine with a more stable dipeptide, alanyl-glutamine. Substitution of alanyl-glutamine for glutamine in mouse embryo culture medium results in significantly lower ammonium levels (Lane and Gardner, 2003). This is also an effective way to optimize porcine oocyte IVM systems. A previous study in our laboratory demonstrated that replacing glutamine with alanyl-glutamine in PPM medium can increase porcine oocyte meiotic maturation (Yuan and Krisher, 2007). Elsewhere, glycyl-glutamine has been used to replace glutamine in culture medium and is advantageous to mouse embryonic development (Biggers et al., 2004; Summers et al., 2005). Tareq et al. (2007) confirmed the effects of alanyl-glutamine and glycyl-glutamine in reducing the accumulation of ammonium in porcine IVM and IVF medium, and increasing the rate of nuclear maturation and mono-spermic fertilization.

In conclusion, the results of this study indicate that the presence of ammonium in maturation medium at concentrations of 2 mM or higher compromised nuclear maturation of oocytes. The effects of oocyte exposure to ammonium on embryonic development appear to be less severe for IVF embryos in that embryonic development was not affected until ammonium concentrations reached 20 mM during IVM. In contrast, ammonium concentrations ≥ 0.2 mM negatively affected PA embryo development. Further studies on the relationship of ammonium exposure and oocyte

metabolic pathways are necessary to elucidate the mechanisms whereby ammonium affects both nuclear and cytoplasmic maturation. Replacement of glutamine with alanyl-glutamine or glycyl-glutamine during porcine oocyte IVM should decrease ammonium buildup and protect oocytes from ammonium induced damage that affects both completion of meiosis and subsequent embryonic development.

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Tables

Table 2.1. Effect of ammonium during in vitro maturation (IVM) on nuclear maturation of porcine oocytes.

Medium*	No. oocytes	Percentage (mean±SEM) of oocytes nuclear maturation (%)
TCM+pFF	190	78.4±3.0 ^a
0 mM	162	68.5±4.7 ^a
0.02 mM	195	65.6±3.4 ^a
0.2 mM	186	64.5±3.5 ^a
2.0 mM	204	43.1±3.5 ^b
20.0 mM	186	23.1±3.1 ^c

^{a,b,c} Different superscript letters within a column indicate significantly different values.

* Oocytes were matured in different maturation medium: TCM199 with 10% pFF;

PPM with 0 mM, 0.02 mM, 0.2 mM, 2 mM or 20 mM ammonium.

Table 2.2. Embryonic development of IVF oocytes after exposure to ammonium during IVM.

Medium*	No. oocytes	Cleaved (%)**	Blastocyst (%)**	Blastocyst/Cleaved (%)	Blastocyst cell number
TCM+pFF	200	52.0±3.2 ^a	11.3±2.4 ^a	20.6±4.0 ^a	50.2±5.0
0 mM	196	51.0±3.6 ^a	16.4±3.4 ^a	30.9±5.9 ^a	52.8±4.2
0.02 mM	198	44.0±4.6 ^a	16.8±3.6 ^a	34.1±5.1 ^a	43.7±3.9
0.2 mM	180	42.2±4.2 ^a	10.7±2.6 ^a	28.5±7.8 ^a	48.6±6.1
2.0 mM	189	45.1±5.0 ^a	24.3±4.5 ^a	50.9±0.1 ^a	39.6±2.6
20.0 mM	147	17.8±3.9 ^b	0.8±0.8 ^b	2.3±2.3 ^b	25

^{a,b} Different superscript letters within a column indicate significantly different values.

* Oocytes were matured in different maturation medium: TCM199 with 10% pFF;

PPM with 0 mM, 0.02 mM, 0.2 mM, 2 mM or 20 mM ammonium.

** Percentage of cleaved embryos and blastocysts are calculated from the total number of oocytes.

Table 2.3. Embryonic development of PA oocytes after exposure to ammonium during IVM.

Medium*	No. oocytes	Cleaved (%)**	Blastocyst (%)**	Blastocyst/Cleaved (%)	Blastocyst cell number
TCM+pFF	196	71.9±4.8 ^a	11.8±2.3 ^a	16.0±13.4 ^a	29.6±2.4
0 mM	150	54.4±4.4 ^{bc}	7.5±2.2 ^{ab}	15.0±16.6 ^{ab}	32.4±4.3
0.02 mM	140	49.3±4.0 ^{bc}	5.5 ±1.9 ^{ab}	12.1±16.4 ^a	26.8±2.4
0.2 mM	211	50.1±4.1 ^b	3.0±1.2 ^b	5.1±8.6 ^b	23.7±2.3
2.0 mM	206	40.7±4.1 ^{bc}	1.9±1.1 ^b	4.4±10.2 ^b	23.2±1.3
20.0 mM	204	25.2±3.0 ^c	0.9±0.6 ^b	3.9±10.4 ^{ab}	24

^{a,b} Different superscript letters within a column indicate significantly different values.

* Oocytes were matured in different maturation medium: TCM199 with 10% pFF;

PPM with 0mM, 0.02mM, 0.2mM, 2mM or 20 mM ammonium.

** Percentage of cleaved embryos and blastocysts are calculated from the total number of oocytes.

CHAPTER 3

IDENTIFICATION OF DEVELOPMENTAL COMPETENCE RELATED GENES IN MATURE PORCINE OOCYTES

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Abstract

Oocyte competence is a key factor limiting female fertility. However, the underlying molecular mechanisms that contribute to oocyte competence remain unclear. The objective of this study was to elucidate specific genes whose function contributes to oocyte competence. We observed that 6 of 20 target genes examined were differentially expressed between adult (more competent) and prepubertal (less competent) porcine in vitro matured (IVM) oocytes. These genes were cholesterol synthesis related gene HMG-CoA reductase (*HMGCR*), fatty acid oxidation genes acyl-CoA synthetase long-chain family member 3 (*ACSL3*) and long-chain acyl-CoA dehydrogenase (*ACADL*), glycolytic genes fructose 1,6 bisphosphate aldolase (*ALDOA*) and lactate dehydrogenase C (*LDHC*), and tumor necrosis factor- α (*TNF*). These 6 genes, as well as 3 other genes (porcine endogenous retrovirus (*PERV*), transcribed loci 10 (*TL10*), serine/arginine-rich splicing factor 1 (*SRSF1*)), were further analyzed by comparing transcript abundance in IVM and in vivo matured (VVM) prepubertal and adult

porcine oocytes. Among these 9 target genes, five were differentially expressed between IVM and VVM prepubertal oocytes, while eight genes were differentially expressed between IVM and VVM adult oocytes. None was differentially expressed between VVM prepubertal and adult oocytes. A functional study of *TNF* demonstrated that depletion of endogenous TNF decreased oocyte competence and *TNFAIP6* expression in cumulus cells, while *TNF* in IVM medium regulated *TNFAIP6* expression in cumulus cells. Differential expression of the genes identified in this study suggests that these genes may be functionally relevant to oocyte competence.

Introduction

Oocyte quality, or developmental competence, affects embryonic development, fetal growth, and even health of the offspring (Eppig and O'Brien 1998). Improving and predicting oocyte quality in vitro is critical for successful application of assisted reproductive technologies to both reproductive medicine and animal agriculture. Pigs are important to biomedical research, because they are physiologically more similar to humans than the mouse (Lunney 2007). Thus, a better understanding of porcine oocyte competence could improve in vitro oocyte maturation not only in pigs but also in other mammalian species, including humans, resulting in better quality oocytes and a significant positive effect on human medicine, agricultural animal production and biomedical applications.

During maturation, the oocyte must undergo a series of cellular events, such as reorganization of cytoplasmic organelles, synthesis and storage of transcripts and

proteins, and changes in cellular metabolism (Krisher 2004). These cellular events, collectively referred to as cytoplasmic maturation, are required for successful subsequent embryonic development (Eppig 1996). During oogenesis, oocytes accumulate a larger than necessary amount of transcripts that are important for oocyte maturation and early embryo development (Sasaki et al. 1999). Transcription and storage of most transcripts occurs during the early stages of oocyte growth. Transcription is then generally silenced as the germinal vesicle breaks down during resumption of meiosis (Brevini-Gandolfi et al. 1999). After transcriptional silencing, oocytes rely on stored transcripts to both continue meiosis and complete the initial cleavage divisions after fertilization, until the maternal zygotic transition (Hodgman et al. 2001; Stebbins-Boaz et al. 1996). Thus synthesis and storage of maternal transcripts prior to the onset of transcriptional silencing are essential for the oocyte to acquire developmental competence (De La Fuente and Eppig 2001). During oocyte meiotic maturation, degradation of transcripts also occurs; almost half of the existing transcripts undergo some degree of deadenylation, and about 30% of the total mRNA is degraded (Paynton et al. 1988). This degradation of mRNA during the transition from the germinal vesicle (GV) to metaphase II (MII) stage of meiosis is a selective process based on gene function and stage of oocyte development (Su et al. 2007). Thus, either aberrant degradation or maintenance of transcripts during oocyte maturation could also be deleterious to oocyte quality.

Oocyte competence is progressively achieved as females approach puberty. The

difference in developmental competence between oocytes derived from prepubertal and adult animals has been confirmed in bovine (Khatir et al. 1996; Revel et al. 1995) and ovine (O'Brien et al. 1996) species. In pigs, oocytes derived from prepubertal animals have decreased meiotic maturation, increased polyspermy and compromised embryonic development in vitro (Marchal et al. 2001). Nuclear transfer studies in cattle demonstrate that using enucleated prepubertal oocytes as the recipient cytoplasm result in decreased blastocyst formation compared with enucleated adult oocytes (Mermillod et al. 1998; Salamone et al. 2001). By comparing the relative abundance of transcripts between GV stage ovine oocytes derived from prepubertal and adult animals, seven developmentally important genes ($\text{Na}^+\text{K}^+\text{ATPase}$, p34^{cdc2} , glucose-transporter I, activin, zona occludens protein 2, poli(A)polymerase, E-cadherin) related to developmental competence were identified (Leoni et al. 2007). These results indicate that the decreased developmental competence of prepubertal derived oocytes is likely associated with incomplete cytoplasmic maturation, and may be specifically reflected in altered transcript abundance.

Elucidation of an aberrant transcript profile in prepubertal oocytes could lead to identification of important cellular mechanisms involved in oocyte competence. Previous microarray analysis in our laboratory detected a total of 999 genes that were differentially expressed in prepubertal versus adult MII stage oocytes (Paczkowski and Krisher, unpublished results). Further studies of target genes suggested by the microarray, as well as additional genes in these target pathways, may enhance our

understanding of molecular mechanisms important to oocyte quality. The objective of this experiment using pig as the model was to elucidate specific genes whose function have shown to influence oocyte competence in other species. Expression of 20 selected target genes were compared between adult (more competent) and prepubertal (less competent) oocytes by quantitative real time PCR (qPCR), which revealed a select subset of differentially expressed mRNAs related to a narrow range of cellular functions. This selective set of differentially expressed mRNAs was further validated in a second model system using VVM (more competent) and IVM (less competent) oocytes. Furthermore, we performed a functional study to validate the relationship of one selected gene, *TNF*, on oocyte competence.

Results

Experiment 1. Comparative gene expression following IVM of oocytes derived from prepubertal and adult animals.

Transcript abundance of 20 candidate genes was compared between oocytes derived from adult and prepubertal animals by qPCR. These genes are related to apoptosis (*MAPK8*, *TNF*, *CAPS8* and *CASP3*), cholesterol synthesis (*HMGCR*, *SREBF1*), fatty acid oxidation (*ACSL3*, *ACADL*), pentose phosphate pathway (*G6PD*, *PGD*), glycolysis (*HK1*, *ALDOA*, *PGK1*, *ENO1*, *PKM2*, *LDHC*, *PDHA1*), the citric acid cycle (*IDH3B*, *ATP5G1*), and calcium homeostasis (*ATP2B1*). Among these genes, *TNF*, *ACSL3*, *ACADL*, *ALDOA* and *LDHC* showed a significantly higher expression in

oocytes from prepubertal animals. Only *HMGCR* was highly ($P<0.05$) expressed in adult derived oocytes (Figure 3.1).

Experiment 2. Comparative gene expression in IVM and VVM oocytes derived from prepubertal and adult animals.

Using gene expression profiles from our prepubertal-adult model in Experiment 1, as well as a few candidate genes suggested by previous microarray and qPCR studies (Paczkowski and Krisher, unpublished results), we selected a panel of 9 differentially expressed genes that are associated with oocyte competence. These genes are porcine endogenous retrovirus (*PERV*), transcribed loci 10 (*TL10*), serine/arginine-rich splicing factor 1 (*SRSF1*), tumor necrosis factor- α (*TNF*), HMG-CoA reductase (*HMGCR*), acyl-CoA synthetase long-chain family member 3 (*ACSL3*), long-chain acyl-CoA dehydrogenase (*ACADL*), fructose 1,6 bisphosphate aldolase (*ALDOA*) and lactate dehydrogenase C (*LDHC*). Expression patterns of these 9 competence-related genes were then compared in IVM and VVM oocytes obtained from both prepubertal and adult animals. The comparison between IVM prepubertal and adult MII oocytes validated 55.6% (5/9) of genes that were determined to be differentially expressed by previous qPCR analyses in this model of oocyte competence. The comparison between IVM and VVM prepubertal oocytes determined that 55.6% (5/9) of our target genes were differentially expressed, and the comparison between IVM and VVM adult oocytes revealed that 88.9% (8/9) of the target genes were differentially expressed,

between more and less competent oocytes. None of the genes examined were differentially expressed between VVM prepubertal and adult oocytes (Table 3.1).

Experiment 3. Validation of the relationship of TNF to oocyte competence.

Experiment 3.1. The effect of TNF during IVM on oocyte nuclear maturation, developmental competence and TNFAIP6 expression in cumulus cells.

There was no significant difference in the percentage of mature oocytes between those matured in the presence of 0 ng/ml, 0.1 ng/ml or 1 ng/ml TNF (Table 3.2). After IVF, no significant difference in the percentage of cleaved embryos or blastocyst development, as well as blastocyst cell number, was found between treatment groups (Table 3.2). The level of *TNFAIP6* mRNA in cumulus cells increased dramatically from zero to 24 hours post maturation (hpm), and then declined at 44 hpm, in all TNF treatments ($P<0.05$). Expression of *TNFAIP6* was significantly increased after treatment with both 0.1 ng/ml and 1 ng/ml TNF compared with control (0 ng/ml) at 24 hpm, although there was no significant difference between 0.1 ng/ml and 1 ng/ml TNF treatment at this time (Figure 3.2). At 44 hpm, *TNFAIP6* mRNA was significantly increased in 0.1 ng/ml TNF compared with control and 1 ng/ml treatment, but there was no significant difference in *TNFAIP6* expression between 1 ng/ml and control at this time (Figure 3.2).

Experiment 3.2. The effect of anti-TNF antibody during IVM on oocyte nuclear maturation, developmental competence and TNFAIP6 expression in cumulus cells.

The percentage of mature oocytes was significantly decreased after addition of 100 µg/ml anti-TNF during maturation (Table 3.3). In addition, percentages of cleaved embryos and blastocyst development were also significantly decreased, although blastocyst cell number was not different (Table 3.3). Embryonic development was also examined after IVM with nonspecific immunoglobulin (IgG) or anti-TNF antibody; no significant difference in the percentage of cleaved embryos was observed between either treatment group and control, although treatment with anti-TNF resulted in reduced embryonic cleavage compared to treatment with IgG. Blastocyst formation, both as a percentage of total oocytes and cleaved embryos, was significantly decreased following anti-TNF treatment during IVM when compared with control, whereas no differences were observed between controls and IgG treatment (Table 3.4). Although treatment with IgG significantly decreased expression of *TNFAIP6* in cumulus cells when compared with control at 24 hpm, a further significant reduction in *TNFAIP6* expression was observed following treatment with anti-TNF. At 44 hpm *TNFAIP6* mRNA was not significantly different between any treatment group (Figure 3.3).

Discussion

In this study, we tested 20 candidate genes that are related to oocyte competence in prepubertal-adult model. Based on results, we identified 6 differentially expressed genes (*TNF*, *HMGCR*, *ACSL3*, *ACADL*, *ALDOA*, *LDHC*) and a 3 more genes (*PERV*,

TL10, *SRSF1*) based on a previous study (Paczkowski and Krisher, unpublished results). We further tested expression of this panel of genes in both in vitro and in vivo matured oocytes derived from both adult and prepubertal females, to validate their importance in a second model system of oocyte competence. In addition, this model allowed us to evaluate how the expression of these genes is altered by the challenges of the in vitro environment. To our knowledge, this is the first time that two well-established models have been used in the same study to determine the relationship between gene expression and oocyte competence in pigs.

The 5 differentially expressed genes (*TL10*, *SRSF1*, *TNF*, *ACSL3* and *LDHC*) identified in both experiments were all upregulated in less competent oocytes in the in vitro prepubertal versus adult model. Interestingly, in adult oocytes, in the in vitro-in vivo matured model where 8 out of 9 genes were differentially expressed, all genes were downregulated in less competent oocytes. We hypothesize that this discrepancy between up and down regulation in less competent oocytes between prepubertal and adult animals is due to differences in the maturity of cellular mechanisms. During the final phase of oocyte development, global transcriptional activity decreases, allowing the oocytes to progress through meiosis (Brevini-Gandolfi et al. 1999). This transcriptional silencing process is mediated by epigenetic mechanisms, including DNA methylation (Curradi et al. 2002). The genome-wide methylation status is significantly lower in IVM prepubertal oocytes than in IVM adult oocytes at the GV stage, indicating that regulation of gene expression mediated by DNA methylation is

not fully established in prepubertal oocytes (Ptak et al. 2006). This could result in prepubertal oocytes being more transcriptionally active prior to re-initiation of meiosis, as well as inappropriately continuing some minor level of expression even during meiotic maturation. Even more significantly, transcripts may not be appropriately degraded during meiotic maturation, due to the immature cellular mechanisms in IVM prepubertal oocytes. Thus, cellular mechanisms that control transcription and transcript degradation may not be functioning correctly in the immature oocyte, resulting in the observed upregulation of genes in IVM oocytes compared to adult. It is important to realize that upregulation of gene expression in prepubertal oocytes does not necessarily imply improved oocyte quality.

In adult oocytes, the genome methylation pattern is well-established and the transcriptional control system mature. The downregulation of target genes in less competent IVM adult oocytes may indicate either insufficient transcript storage before GVBD in oocytes collected from abattoir derived ovarian follicles not yet near ovulation or excessive transcript degradation after GVBD due to the suboptimal in vitro culture environment.

In the prepubertal in vivo-in vitro matured model, in vitro culture environment may cause both upregulation of genes due to immature cellular transcription control mechanisms, or downregulation of genes due to the suboptimal environment.

Therefore, genes were regulated in different directions in these oocytes. Regulation of specific genes may depend on how extensive the immature gene regulation

mechanisms have been altered by the in vitro culture environment in the prepubertal oocyte. We did not compare gene expression patterns between IVM prepubertal oocytes and VVM adult oocytes, as both female age and maturation environment are both present in this comparison. Thus, it is difficult to draw meaningful conclusions from this comparison as both female age and maturation environment are contributing factors, making it impossible to determine which factor is responsible for the effects observed.

An important point to note is that the differences we observed in gene expression patterns between prepubertal and adult oocytes were only observed after maturation in the in vitro environment, not in vivo, indicating that the prepubertal oocyte is less able to handle the challenges of the in vitro environment, and that these stresses may then negatively alter gene expression and or transcript degradation when compared to adult oocytes.

Of our panel of 9 oocyte competence genes, 5 were further validated in the second experiment by again comparing gene expression levels between IVM prepubertal and adult oocytes. The discrepancies observed in qPCR results between experiments 1 and 2 may be attributed to the independent pools of cDNA utilized for qPCR analysis in these two independent experiments. A previous study of cumulus cells demonstrated that using qPCR analysis to validate microarray data could reach 60% (15 out of 25 target genes) validation rate in the original samples; however, only 8 of the 15 validated genes could be confirmed using independent cumulus cell samples (van

Montfoort et al. 2008). Gene expression is variable between pools of cells, particularly when oocytes are pooled in large number and across several females. However, using independent pools of cDNA for qPCR analysis may actually increase the reliability of any consistently identified differences in transcript abundance between two treatment groups.

HMGCR is the rate limiting enzyme of the cholesterol biosynthetic pathway (Brown and Goldstein 1990). Cholesterol-enriched lipid rafts are present in membranes of mouse oocytes and pre-implantation embryos, and treating zygotes with a cholesterol-depleting drug prevents embryonic development (Comiskey and Warner 2007).

Exposure to follicular fluid meiosis-activating sterol, an intermediate of cholesterol biosynthesis, during IVM can increase the quality of porcine oocytes (Faerge et al. 2006). These findings suggest that cholesterol is important in oocytes and embryos for supporting pre-implantation development. These results support previous findings that cholesterol synthesis is important to oocyte developmental potential (Faerge et al. 2006).

ACSL3 and *ACADL* are two important enzymes in the lipid β -oxidation pathway, while *LDHC* and *ALDOA* are two enzymes related to glycolysis. A recent study in mice demonstrated that lipid β -oxidation is essential for oocyte developmental competence and early embryo development (Dunning et al. 2010). Porcine oocytes, compared with other mammalian species, are characterized by a high lipid content (McEvoy et al. 2000), stored mainly as lipid droplets in the cytoplasm that are co-localized with

mitochondria. Exposure to inhibitors of lipid β -oxidation during oocyte maturation results in developmental failure post IVF (Sturmey et al. 2006). Elevated glucose metabolism via glycolysis in oocytes has been correlated to improved developmental competence in cattle, cats and pigs (Herrick et al. 2006; Krisher and Bavister 1999; Spindler et al. 2000). Recently, our laboratory also demonstrated aberrant protein abundance of *ALDOA* and lactate dehydrogenase A (*LDH-A*) in prepubertal COCs, suggesting that these two genes may play a role in the mechanisms resulting in developmental competence (Paczkowski and Krisher 2010). These results substantiate previous work suggesting that fatty acid and glucose metabolism play an important role in oocyte competence (Krisher 2004; Sturmey et al. 2009).

TL10 represents a transcribed locus with unknown gene identification and functional annotation. *SRSF1* is an essential sequence specific splicing factor involved in pre-mRNA splicing (Kim et al. 2009). In addition, *SRSF1* also mediates post-splicing activities, such as mRNA nuclear export and translation (Michlewski et al. 2008). We currently have no information about how these genes function in the context of oocyte quality.

In the current study, *TNF* was upregulated in less competent oocytes in the in vitro prepubertal-adult model as well as the prepubertal in vivo-in vitro model. This suggests that high levels of TNF may be detrimental to oocyte competence. Exposure of porcine oocytes to high concentrations of TNF (5 ng/ml) in vitro decreased the percentage of meiotic maturation, whereas exposure to 10 or 100 ng/ml TNF resulted in a significant

increase in the frequency of defective spindles or abnormal microfilament distribution (Ma et al. 2010). Despite the negative effects of TNF on oocytes in vitro, several in vivo studies suggested an important role of TNF during follicle development and oocyte growth (Johnson et al. 1999; Onagbesan et al. 2000). Our results demonstrate that some level of TNF is required for oocyte competence, as anti-TNF antibody specifically decreased embryonic development. Together, our findings suggest that TNF is an important component of oocyte maturation, but that excessive concentrations may be detrimental.

Cumulus expansion is mediated by the production of an extracellular matrix comprised of a hyaluronan backbone, and is stabilized by various hyaluronan-binding proteins, including TNFAIP6 (Fulop et al. 1997). In our study, *TNFAIP6* expression in cumulus cells increased dramatically at 24 hpm and was responsive to TNF treatment. This pattern of *TNFAIP6* expression agrees with that in porcine preovulatory follicles reported by another group. Interestingly, the pattern of *TNFAIP6* expression in cumulus cells during IVM is also similar to the pattern of in vivo TNF secretion by oocytes (Johnson et al. 1999). As suggested by several studies, cumulus cell TNFAIP6 expression may potentially be a marker of oocyte competence (Assidi et al. 2010; Assidi et al. 2008; Tesfaye et al. 2009). Our functional study of TNF revealed a positive correlation between the concentration of TNF in IVM medium and *TNFAIP6* mRNA expression in cumulus cells. Depletion of endogenous TNF decreased TNFAIP6 mRNA expression levels in cumulus cells and resulted in decreased meiotic

maturation and embryonic development. These results suggest that oocyte derived TNF regulates the expression of *TNFAIP6* in cumulus cells in vitro, and thus may indeed be reflective of oocyte quality. We hypothesize that a minimal concentration of endogenous oocyte TNF, reflected by a critical transcript level, may be important in regulating appropriate *TNFAIP6* expression and function in cumulus cells. In this study, however, high levels of *TNFAIP6* in cumulus cells, induced by adding exogenous TNF, did not result in increased oocyte competence. This suggests that a minimal level of *TNFAIP6* expression in cumulus cells is required for oocyte competence, but that further increasing this level may not be beneficial. Thus, increased concentrations of TNF in poor quality oocytes, although detrimental, may be reflected by increased *TNFAIP6* levels in cumulus cells, suggesting that higher *TNFAIP6* expression is not necessarily better for oocyte quality.

The present study provides a molecular sketch of genes and metabolic pathways, including the cholesterol synthesis, fatty acid oxidation and glycolysis, which may be important regulators of oocyte quality in the pig. For one of these genes, TNF, we demonstrate functional importance. Additional functional studies of the remaining target genes in our panel may further elucidate the contribution of these genes and the pathways they participate in to oocyte quality. Ultimately, this information may result not only in a better understanding of oocyte competence and how better to support optimal competence during in vitro maturation, but also in the identification of biomarkers that could be used to predict oocyte quality.

Materials and Methods

Materials

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

Animals

All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee, and have been judged to minimize discomfort, distress, injury and pain.

In vitro maturation

Ovaries of cycling adult sows and prepubertal gilts were collected from two local abattoirs (Momence Packing Co. and Indiana Packers Corp., respectively), and transferred to the laboratory at 30 – 34 °C in 0.9% (w/v) NaCl. Prepubertal ovaries were confirmed by the absence of developed corpora lutea. Oocytes were aspirated from 3-8 mm follicles using an 18-gauge needle. Oocytes with several layers of unexpanded cumulus cells and evenly dark cytoplasm were selected and rinsed in Hepes-buffered synthetic oviductal fluid supplemented with 0.1% BSA (SOF-Hepes (Good and Frishette 1966)). Selected oocytes were matured in vitro in Purdue Porcine Medium modified for maturation (PPMmat) (Herrick et al. 2006; Herrick et al. 2003; Stroble et al. 2002) containing 2 mM glucose, 6 mM lactate, 0.2 mM pyruvate, 0.5 mM

cysteamine, 1% recombinant human albumin (recombunin, G-MM, Vitrolife, Kungsbäcka, Sweden), 0.2% fetuin, 50 ng/ml EGF, 0.01 units/ml each LH and FSH (Sioux Biochemicals, Sioux City, IA) for 42 to 44 h in 7% CO₂ in humidified air at 38.7 °C.

Assessment of Meiotic Status

After maturation, oocytes were denuded by vortexing for 3 minutes in SOF-Hepes with 0.01% (w/v; 80-160 unit/ml) hyaluronidase. Denuded oocytes were mounted, fixed in chloroform:acetic acid:ethanol (1:3:6, v/v/v) for 72 h, stained with 1% (w/v) orcein in acetic acid, and examined for meiotic stage at 400 × magnification. Oocytes reaching telophase and metaphase II were considered mature.

In vitro fertilization (IVF) and embryo culture (IVC)

After maturation, oocytes were denuded as described above, washed twice in modified Tris-buffered medium (mTBM) (Abeydeera and Day 1997) supplemented with 2 mM caffeine and 0.2% (w/v) fraction V BSA and placed in 50 µl drops of mTBM under 10 ml embryo tested, washed mineral oil (20 oocytes/drop). Semen from the same boar was used throughout the experiments. Sperm preparation was performed by placing 500 µl of chilled, extended (1:5 dilution, Androhep EnduraGuard, Minitube of America Inc., Verona, WI, USA) semen, onto a gradient of 45% :90% Percoll (GE Healthcare Life Sciences, Uppsala, Sweden) and centrifuged for 20 min at 700 x g. The

supernatant was removed and the remaining sperm pellet washed twice with 5.0 ml D-PBS by centrifuging for 5 min at 1000 x g. Sperm were then counted and diluted (1×10^6 sperm/ml) in mTBM (Abeydeera and Day 1997) and added (50 μ l) to fertilization drops containing oocytes for a final sperm concentration of 5×10^5 sperm/ml. Gametes were co-incubated for 5 h in 6% CO₂ in humidified air. Following co-incubation, putative zygotes were washed 3 times and cultured in 50 μ l NCSU-23 medium (Long et al. 1999; Machaty et al. 1998; Petters and Wells 1993) (10 zygotes/drop) containing 0.4% crystallized BSA (MP Biomedicals, Solon, OH, USA) under 10 ml mineral oil in 6% CO₂, 10% O₂, balance N₂ for 6 days, when embryonic cleavage and blastocyst development were determined. Blastocysts were stained with 0.01 mg/ml Hoechst 33342 to ascertain total blastocyst cell number under fluorescence at 400 x magnification.

In vivo matured ovulated oocyte collection

Adult and prepubertal pigs were synchronized then superstimulated prior to surgical oocyte retrieval. Superovulation maximizes the number of oocytes retrieved per female so that costs can be minimized. However, superovulation does influence oocyte quality (Baart et al. 2009; Foote and Ellington 1988). Therefore the oocytes used in this study, although in vivo matured, are somewhat different than naturally ovulated oocytes. It is possible that expression of our target genes may differ between superovulated and naturally ovulated in vivo matured oocytes. In spite of the physiologic differences

reported in superovulated oocytes, such oocytes are still routinely used to produce normal offspring in both human medicine and agricultural animal production systems. Compared with IVM, superovulation has lesser effects on oocyte quality, and thus has been considered a more competent oocyte in this study. The animals' estrous cycles were synchronized by feeding 15 mg/day of an oral progestin, altrenogest (Matrix; Intervet America Inc. Millsboro, DE, USA) for 14-18 days. The day following the final day of matrix feeding, females received 1000 IU of pregnant mares' serum gonadotropin (Calbiochem, La Jolla, CA, USA) followed in 72 h by 1000 IU of human chorionic gonadotropin (hCG, Calbiochem). Surgical oocyte retrieval was performed 40 h post hCG injection. Pigs were restrained with a limp rope snare and injected with an initial intra-muscular injection of 3 ml anesthetic cocktail consisting of 5.9 mg/kg Ketamine, 1.47 mg/kg Telazol, 2.9 mg/kg Xylazine and 0.09 mg/kg atropine, followed by 7 ml intra-venous injection of the same solution via ear vein canula. Endotracheal tubes were inserted into the nares for connection to the anesthesia apparatus, supplying oxygen (2 L/min) and 5% halothane. A midline, lower abdominal incision was made through the body wall, the oviduct and part of the uterus exteriorized, and ovulated oocytes recovered via retrograde flush of the oviducts using approximately 20 ml warm SOF-Hepes. If necessary, oocytes were denuded via vortexing for 3 min in SOF-Hepes with 0.01% (w/v; 80-160 unit/ml) hyaluronidase.

Quantitative PCR

Oocytes

Metaphase II stage oocytes were identified by extrusion of the first polar body before being frozen at -80 °C with lysis buffer (Dynabeads mRNA DIRECT Kit, Invitrogen, Carlsbad, CA). Three separate pools of 20 oocytes each were collected for each treatment for qPCR analysis. Poly A⁺ RNA was extracted from the samples using the Dynabeads mRNA DIRECT Kit (Invitrogen). Half of the mRNA from each sample was used to assess genomic DNA contamination by reverse transcriptase (RT) and mock RT reactions. RNA samples (3 µl) were incubated with 1 µl dNTP (10 mM), 2 µl random hexamer primers (dN6; 100 ng/µl), and 4 µl water for 5 min at 65 °C. Random primed RNA samples were divided for RT and mock RT reactions. For RT reactions, 50% of random primed RNA (5 µl) was incubated with 2 µl 5x transcription buffer, 1 µl dithiothreitol (0.1 mM), 0.75 µl Moloney Murine Leukemia Virus (MMLV; 200 U/µl), 0.5 µl RNase Out (40 U/µl), and 0.75 µl water. Mock RT reactions were performed using the remaining 50% of random primed RNA as described above; however, MMLV and RNase Out were excluded from the samples. Reverse transcription was performed at 25 °C for 10 min, 37 °C for 50 min, and 70 °C for 10 min. cDNA samples were amplified for *GAPDH* with 0.5 µl dNTP (10 mM), 0.5 µl forward (20 µM) and 0.5 µl reverse (20 µM) primers, 2.5 µl 10x transcription buffer, 0.25 µl HotMaster Taq (5 U/µl) polymerase (Eppendorf, Hamburg, Germany), and 15.75 µl water. Samples were incubated at 94 °C for 1 min and 40 seconds, 40-cycles

of 94 °C for 20 seconds, 58 °C for 10 seconds, and 65 °C for 30 seconds, followed by 65 °C for 2 min. PCR products were separated by gel electrophoresis and stained with ethidium bromide. The presence of a gel band in mock PCR samples indicated genomic DNA contamination. All samples displayed a positive RT reaction and did not contain genomic DNA. For the remaining half of the mRNA, single strand and double strand cDNA were generated using the RT template switching SMART technology (Wang et al. 2000; Zhu et al. 2001) and DNA samples were purified using the Machery-Nagel (Easton, PA) PCR Clean-up Gel Extraction Kit. Linear amplification was performed using the MEGAscript High Yield Transcription Kit (Ambion, Austin, TX), followed by DNase treatment. Amplified mRNA samples were purified from template cDNA using the Qiagen (Valencia, CA) RNeasy Mini Kit and single strand cDNA were generated by random primed cDNA synthesis. Primer design and qPCR were performed as previously described (Fleming-Waddell et al. 2007). Current gene annotations were obtained from the Genbank and primers were designed for qPCR using Primer3 (Rozen and Skaletsky 2000). Accession number, primer sequence, and product length of target genes are presented in Table 3.5. Information for primers for *GAPDH*, *PERV*, *SRSF1*, *TL10* (Paczkowski and Krisher, unpublished results) and *TNFAIP6* (Shimada et al. 2004) were previously reported. For the remaining genes, primers were tested on pools of 20 porcine oocytes and primer specificity was determined by melting curve analysis and gel electrophoresis. PCR products were cloned into pCR 2.1 TOPO vectors and transformed into One Shot TOP10 chemically

competent *E. coli*. Plasmids were sequenced to confirm the identity of the transcript and quantified using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Target genes were analyzed using QuantiFast SYBR Green PCR reagents (Qiagen) on a MasterCycler Realplex² (Eppendorf North America, Inc; Westbury, NY, USA). A standard curve was generated from serial dilutions of EcoRI digested plasmids (10^7 to 10^1 molecules) and the efficiency of the primers was calculated.

Cumulus cells

After removal from oocytes, cumulus cells were transferred to a 1.5 ml tube and centrifuged for 1 min at 700 x g. The supernatant was removed and the cumulus cells were frozen at -80 °C for qPCR analysis. Total RNA was extracted from pooled samples of cumulus cells using the RNeasy kit (Qiagen) as recommended by the manufacturer, followed by DNase treatment and eluted in 30 µl water. After extraction, the total RNA concentration of cumulus cell samples was determined by using the Quant-iT RiboGreen RNA Assay Kit (Invitrogen). Since the number of cumulus cells varied between pooled samples, the lowest RNA concentration in all samples was used as the standard for normalization and an equal amount of RNA was used for all reverse transcription reactions. Reverse transcription was performed with SuperScript III (Invitrogen) and random hexamer primers (dN6; 100 ng/µl). Reactions were performed at 42 °C for 15 min, 50 °C for 50 min and at 70 °C for 15 min for enzyme inactivation. Primer design and qPCR were performed as described above.

Experimental design

Experiment 1. The objective of this experiment was to compare transcript abundance of 20 candidate genes between prepubertal and adult MII oocytes derived following IVM(prepubertal-adult model) to identify a ‘panel’ of candidate genes that are reflective of oocyte competence. Each treatment was replicated three times, with 20 oocytes per pool in each replicate.

Experiment 2. The objective of this experiment was to further examine a select subset of genes from Experiment 1, which were differentially expressed between IVM and VVM MII oocytes collected from both prepubertal and adult females (2x2 factorial; in vitro-in vivo matured model). In vivo matured ovulated MII oocytes were collected from 6 prepubertal and 6 adult females. Transcript abundance of the selected genes was compared between these 4 treatment groups. In each treatment, oocytes were pooled in 3 separate replicates with 20 oocytes per replicate.

Experiment 3. The objective of this experiment was to validate the functional relationship of *TNF* to oocyte competence in adult oocytes, suggested by our gene expression analysis results. In experiment 3.1, the effect of TNF supplementation during IVM on a) oocyte nuclear maturation and subsequent embryo development and b) *TNFAIP6* mRNA expression in cumulus cells was examined. Adult oocytes were matured in PPMmat with TNF (0, 0.1 and 1 ng/ml). In each replicate, 10 oocytes were used to determine meiotic status after IVM, and 40-50 oocytes from the same pool were used for IVF/C to assess embryonic development. This experiment was replicated

6 times. In addition, three separate pools of cumulus cells from 10 COCs were independently sampled from each treatment at 0 h, 24 h and 44 hpm to analyze *TNFAIP6* expression. In experiment 3.2, the effect of anti-TNF antibody during in vitro oocyte maturation on a) subsequent embryonic development and b) *TNFAIP6* mRNA expression in cumulus cells was examined. Adult oocytes were matured in PPMmat or PPMmat with 100 µg/ml anti-TNF antibody. In each replicate, 10 oocytes were used to determine meiotic status after IVM, and 40-50 oocytes from the same pool were used for IVF/C to assess embryonic development. This experiment was replicated 4 times. To determine the specificity of antibody effects, oocytes were matured in PPMmat without antibody, and PPMmat with 100 µg/ml of either IgG or anti-TNF antibody. Cumulus cells from 10 COCs were sampled from each treatment at 0 h, 24 h and 44 hpm for qPCR analysis. The remaining oocytes in each pool were used for IVF/C to assess subsequent embryonic developmental. This experiment was replicated 3 times.

Statistical analysis

For oocyte qPCR results, the mRNA abundance of target genes was normalized to an internal control, *GAPDH*, for sample to sample comparisons. For cumulus cell qPCR results, in which the RNA could be quantified, the expression level of the target gene at 0 hpm was set at 1, and data from other time points were calculated accordingly. Relative expression ratios were obtained by the comparative threshold cycle method (Livak and Schmittgen 2001). Data were analyzed using the relative expression

software tool, REST 2009 version 2.0.13 (Pfaffl et al. 2002). Percentages of maturation, embryonic cleavage, and blastocyst development, as well as blastocyst total cell number were analyzed using two-way ANOVA, with maturation treatment included in the model as a fixed factor and replicate as a random factor. Percentage data (maturation success, embryonic cleavage and blastocyst development) were arcsin transformed. Treatment differences were determined by Bonferroni or Fisher's LSD (Experiment 4) multiple comparison test when treatment was significant. Significance was determined as $P < 0.05$. Data are reported as mean $\pm$ SEM.

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Tables and Figures

Table 3.1. Summary of gene symbol, accession number, primer sequences and amplicon product length for the target genes examined.

Gene Symbol	Accession Number	Forward Primer Sequence	Reverse Primer Sequence	Product Length
<i>GAPDH</i>	AF017079	acatcaagaaggtggtgaag	attgtcgtaccaggaaatgag	151
<i>PERV</i>	AF038599	tggtttgagggatggttca	acgggctttggactgttgt	190
<i>SRSF1</i>	NM_001038007	tctgcatgtcctctgtgtga	tctaagtctcccaactgcaa	211
<i>TL10</i>	CN162638	aggagcttgatcccatgt	ccctttcccagcttcataga	230
<i>MAPK8</i>	AK230496	atgaagcatcctgctcacct	cctcgataccctcaaaact	176
<i>TNF</i>	NM_001031779	cccattcaggctcaacaat	gcctggactacatcccat	185
<i>CASP3</i>	AJ583707	tgctttgtggaatcaagcac	cccaactgaccaaagtcaa	233
<i>CASP8</i>	AF069648.1	tgttgcgagagcaatttagc	ttggacaaacacctccatga	153
<i>HMCGR</i>	BP436947	gtgaaaaggagcgtgagctt	tgaatgtttcagtcgccaac	106
<i>SREBF1</i>	NM_214157	cactttctgacccgcttctt	gcttgctccaagagggttc	236
<i>ACSL3</i>	NM_001143698	ctggtgacagatgccttcaa	aggaatggagttgcctcac	184
<i>ACADL</i>	NM_213897	cttatgtggatgcccgagtt	tagtggtccctcccttcca	183
<i>G6PD</i>	XM_583628	ccagaatctcatggtgctga	gatgacacaggcgatgttgt	199
<i>PGD</i>	CN159092	aactgtcccacacactgg	tcttcaccgactgtcca	172
<i>HK1</i>	BX915001	aaggcagaagccacctaag	gaaacggacgccactaaact	166
<i>ALDOA</i>	CA780251	gctgagtgcagagaagtgtg	gccagggtattccaaggag	233
<i>PGK1</i>	AY677198.1	cttgactgtggtcctgaga	gcacagcaggtagcagtgtc	204
<i>ENO1</i>	CK462922	cacacatggctccagacact	tccctgtgatgtctcactgc	154
<i>PKM2</i>	CN166623	cttcctcatcccttgcttg	tagtacctgtgccgtgatgc	224
<i>LDHC</i>	U95378.1	cctctgggctattggactg	cctcctcctcagcattcaag	198
<i>PDHA1</i>	X52990.1	ggattgctctggcctgtaag	gctctctccacagacgttcc	181
<i>IDH3B</i>	DQ507860	tgcatggaccagagaagag	ccaatgacagcctcagtga	220
<i>ATP5G1</i>	CF367963	ttgtgtgatggtcaggaagc	caacctcctcttcgtcctgt	158
<i>ATP2B1</i>	NM_214352.1	cttgcttcggaaaccttacg	ccgtgcatttatttcgttga	241
<i>TNFAIP6</i>	M31165	tcataactccatattggcttgaac	tcttcgtactcatttgggaagcc	391

Table 3.2. Comparative expression level of competence-related genes in in vivo or in vitro matured oocytes derived from prepubertal or adult pigs.*

Gene Symbol	In vitro prepubertal vs. adult		Prepubertal in vitro vs. in vivo		Adult in vitro vs. in vivo		In vivo prepubertal vs. adult	
<i>PERV</i>	0.821	nsd	0.384	nsd	0.244	down	0.522	nsd
<i>TL10</i>	4.035	up	1.786	up	0.209	down	0.473	nsd
<i>SRSF1</i>	3.259	up	1.357	nsd	0.166	down	0.398	nsd
<i>TNF</i>	4.899	up	1.87	up	0.272	down	0.713	nsd
<i>HMCGR</i>	0.827	nsd	0.461	down	0.439	nsd	0.766	nsd
<i>ACSL3</i>	1.963	up	1.287	nsd	0.432	down	0.659	nsd
<i>ACADL</i>	1.23	nsd	0.54	down	0.222	down	0.506	nsd
<i>ALDOA</i>	1.55	nsd	1.38	nsd	0.428	down	0.48	nsd
<i>LDHC</i>	2.176	up	1.374	up	0.299	down	0.473	nsd

* In each column, gene expression was compared between two groups, stated in the column heading. The numbers in each column showed the relative gene expression level of the first group normalized to that of the second group. ‘up’ means that the gene was upregulated in the first group compared to the second group of the comparison, and likewise ‘down’ means the gene was down regulated in the first group compared to the second group; ‘nsd’ not significantly different.

Table 3.3. Effects of TNF during in vitro maturation on meiotic maturation and subsequent embryonic development of porcine oocytes following IVF/IVC*.

TNF concentration	Percentage of oocytes reaching to MII (n)	Embryonic cleavage (n)	Blastocyst development (n)	Blastocyst/cleaved §
0 ng/ml	66.1 ± 6.1% (62)	57.8 ± 2.9% (288)	23.0 ± 2.7% (288)	39.4 ± 4.0%
0.1 ng/ml	62.5 ± 6.5% (58)	64.2 ± 3.0% (249)	25.8 ± 2.8% (249)	39.7 ± 3.9%
1 ng/ml	50.0 ± 7.3% (48)	63.2 ± 2.7% (279)	26.1 ± 3.7% (279)	38.2 ± 4.8%

* Data are reported as mean ± SEM. No significant differences ($P > 0.05$) were found in percentage of oocytes reaching to MII and embryonic development in all treatment groups.

§ Percentage of blastocysts is calculated from the cleaved embryos.

Table 3.4. Effects of anti-TNF during in vitro maturation on oocyte meiotic maturation and subsequent embryonic development following IVF/IVC.

Anti-TNF concentration	Meiotic maturation (n)	Cleaved embryos (n)	Blastocyst development (n)	Blastocyst/cleaved §
0 µg/ml	56.6 ± 6.9% ^a (53)	53.8 ± 2.7% ^a (291)	17.7 ± 2.0% ^a (291)	33.4 ± 3.7% ^a
100 µg/ml	31.4 ± 8.0% ^b (35)	39.2 ± 4.2% ^b (119)	9.9 ± 2.6% ^b (119)	24.2 ± 6.9% ^b

^{ab} Different superscripts within a column denote a significant difference, $P < 0.05$. Data are reported as mean ± SEM.

§ Percentage of blastocysts is calculated from the cleaved embryos.

Table 3.5. Effects of anti-TNF- α and IgG during porcine oocyte in vitro maturation on subsequent embryonic development following IVF/IVC.

Treatment	Cleaved embryos (n)	Blastocyst development (n)	Blastocyst/cleaved
Control	49.7 $\pm$ 3.3% ^{ab} (115)	11.1 $\pm$ 1.7% ^a (115)	23.7 $\pm$ 3.9% ^a
100 μ g/ml IgG	64.0 $\pm$ 5.6% ^a (81)	8.6 $\pm$ 1.8% ^{ab} (81)	14.3 $\pm$ 3.1% ^{ab}
100 μ g/ml anti TNF	47.0 $\pm$ 3.9% ^b (91)	3.0 $\pm$ 1.5% ^b (91)	5.3 $\pm$ 2.7% ^b

^{ab} Different superscripts within a column denote significant difference, $P < 0.05$. Data are reported as mean $\pm$ SEM.

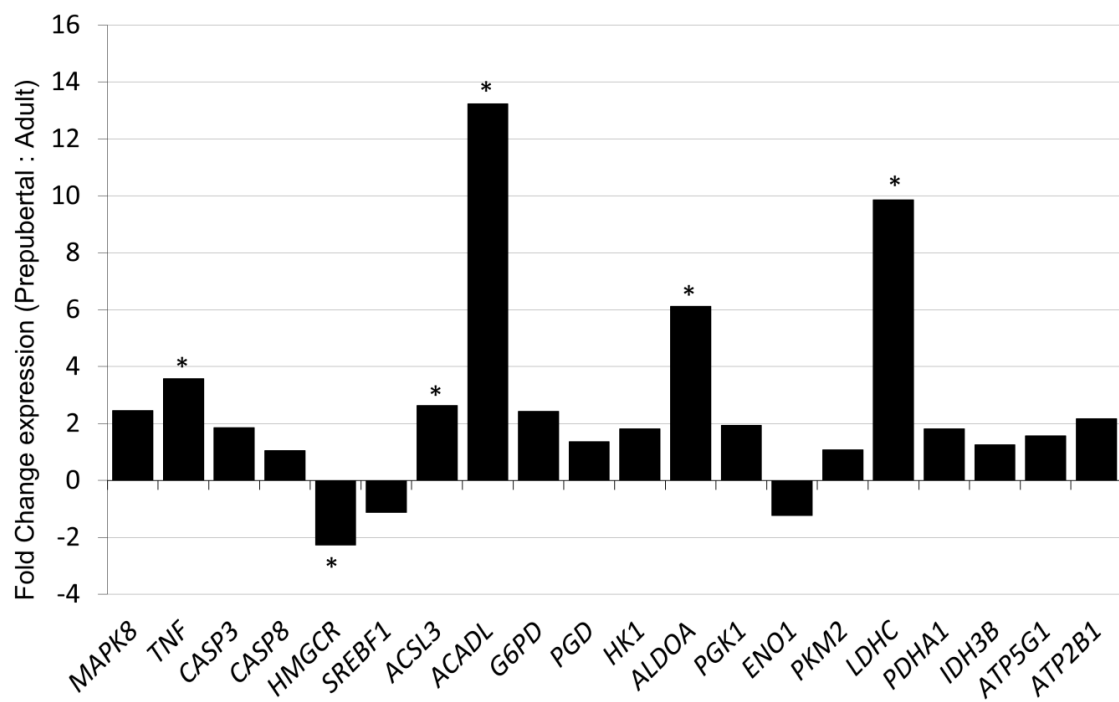


Figure 3.1. Expression of 20 target genes in oocytes derived from prepubertal compared to adult pigs, determined by qPCR analyses and relative to *GAPDH*. Bars above the X axis represent genes that were upregulated in prepubertal oocytes; bars below X axis represent genes that were upregulated in adult oocytes. * represents a significant difference in gene expression between prepubertal and adult oocytes ($P < 0.05$).

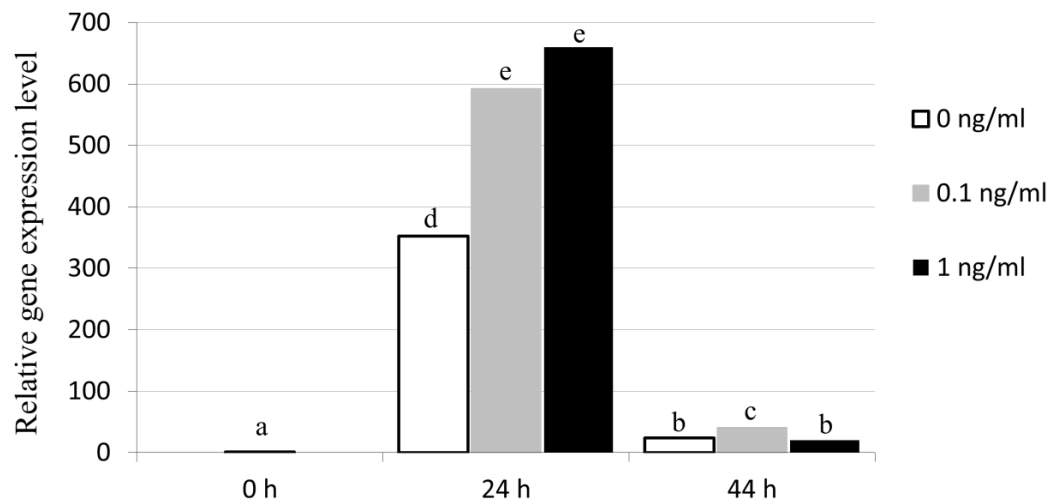


Figure 3.2. Relative expression of *TNFAIP6* in porcine cumulus cells treated with different concentrations of TNF (0 ng/ml, 0.1 ng/ml, 1 ng/ml) during in vitro maturation, as determined by qPCR analysis. Data were normalized to *TNFAIP6* expression at 0 h post maturation. Columns with different superscript letters differ significantly between time points and TNF treatments ($P < 0.05$).

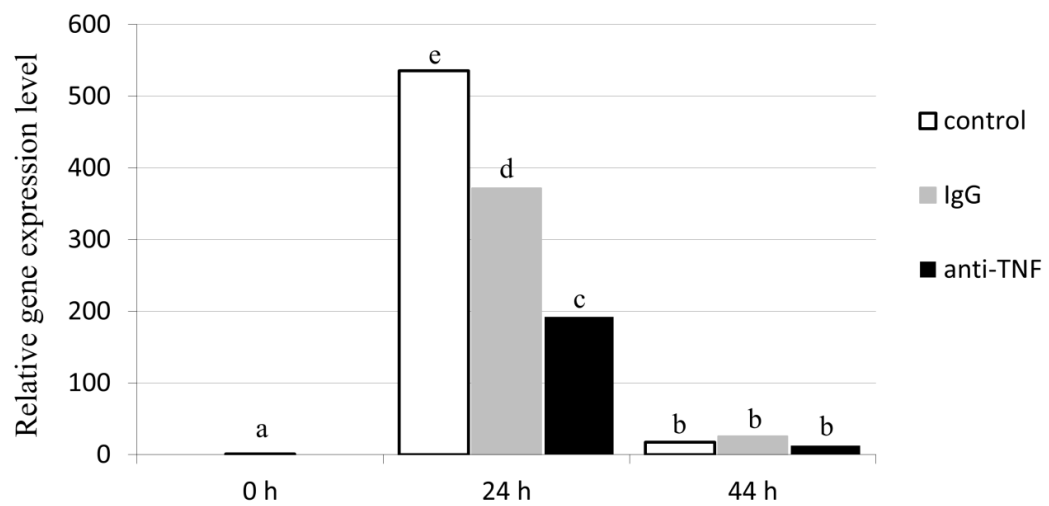


Figure 3.3. Relative expression of *TNFAIP6* in porcine cumulus cells treated with 100 $\mu\text{g/ml}$ of either IgG or anti-TNF during in vitro maturation, as determined by qPCR analysis. Data were normalized against the *TNFAIP6* expression level at 0 h post maturation. Bars with different superscripts differ significantly between time points and TNF treatments ($P < 0.05$).

CHAPTER 4

DISRUPTED REDOX HOMEOSTASIS AND ABERRANT REDOX GENE EXPRESSION PATTERNS IN PREPUBERTAL PORCINE OOCYTES CONTRIBUTE TO DECREASED DEVELOPMENTAL COMPETENCE

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Abstract

There is a growing body of evidence suggesting that redox homeostasis in oocytes is closely related to developmental competence; however, the underlying mechanisms are unclear. The objective of this study was to identify specific redox related genes whose function contributes to oocyte competence, and characterize the role of redox homeostasis in oocyte competence. We determined that the redox genes *GLRX2*, *PDIA4*, *PDIA6* and *TRXR1* were differentially expressed between adult (more competent) and prepubertal (less competent) porcine in vitro matured (IVM) oocytes. These genes were further validated by comparing transcript abundance in IVM and in vivo matured (VVM) prepubertal and adult porcine oocytes. Differential expression of these genes suggested that they may be functionally relevant to oocyte competence. By maturing oocytes in different redox environments, we found that a balanced redox environment is critical for oocyte development. Elevated glutathione in prepubertal oocytes suggested the disrupted redox homeostasis exists in these cells. By further comparing *GLRX2*, *PDIA4*, *PDIA6* and *TRXR1* expression in oocytes matured under different redox environments, we found aberrant expression patterns in prepubertal oocytes but not in adult oocytes when extra antioxidant was supplemented to the maturation medium. These results suggested that prepubertal oocytes are less competent in regulating redox balance compared to adult oocytes. The aberrant

expression patterns of *GLRX2*, *PDIA4*, *PDIA6* and *TRXR1* may contribute to the decreased developmental competence in prepubertal porcine oocytes.

Introduction

Oocyte IVM holds great promise as a tool for providing more viable oocytes for embryo production in agriculture and enhancing infertility treatment in human medicine. However, in many species, oocytes matured in vitro are less competent to support subsequent embryonic development compared with oocytes matured in vivo (Combelles et al. 2002; Dieleman et al. 2002; Farin et al. 2001; Holm et al. 2002). This suggests that current maturation conditions are not effective in supporting the correct development of oocyte competence. The establishment of oocyte competence during maturation is highly complex, requiring the completion of both nuclear and cytoplasmic maturation. Many IVM oocytes can successfully complete meiosis but fail to complete embryonic development due to incomplete cytoplasmic maturation (Krisher 2004). During oogenesis, oocytes accumulate a larger than necessary amount of transcripts for oocyte maturation and early embryo development (Sasaki et al. 1999). The transcripts and proteins stored in the cytoplasm of the oocyte are fundamentally important for the oocyte maturation and development of early embryos (Meirelles et al. 2004; Piccioni et al. 2005). Therefore, inappropriate transcript accumulation may reflect compromised competence in oocytes after IVM. ROS such as hydrogen peroxide (H_2O_2), hydroxyl radicals ($\cdot OH$), superoxide anions (O_2^-) and nitric oxide (NO) are produced during normal cellular metabolism as well as accumulated indirectly from the surrounding environment. ROS are highly reactive and are known to cause widespread damage to proteins, nucleic acids, lipids, and other biomolecules (Storz and Imlay 1999). Cells have intrinsic antioxidant mechanisms to counteract this problem. A series of antioxidant enzymes and molecules, including

catalases (Chelikani et al. 2004), superoxide dismutases (Johnson and Giulivi 2005), and peroxidases (Brigelius-Flohe 1999) are expressed in cells that act to neutralize ROS. In addition, there are small thiol molecules that act to maintain redox balance of cells. Two major thiol-redox systems (thioredoxin and glutaredoxin) maintain the reducing capacity of the cytosol and prevent unwanted disulfide bond formation in proteins (Prinz et al. 1997). The thioredoxin system consists of thioredoxin reductase (*TRXR1*) and thioredoxin (*TRx*). The glutaredoxin system is mainly composed of glutathione reductase (*GSR*), Glutathione (GSH), and three glutaredoxins (*GLRX1-3*) (Prinz et al. 1997). Glutathione is the major redox buffer in the cytoplasm that is responsible for reducing disulfide bonds in cytosolic proteins, providing cells with a reduced environment and protecting against oxidative stress (Aslund and Beckwith 1999; Meister and Tate 1976). When ROS concentrations rise above a certain level, the antioxidant mechanisms are not sufficient to prevent oxidative damage, and increased ROS can directly oxidize protein thiols and rapidly form disulfide bonds (Claiborne et al. 1999), putting the cell into a state that is defined as oxidative stress (Sies 1991). During IVM, the oocytes are exposed to higher oxygen concentrations than in the in vivo environment. Thus, oxidative stress is a unique challenge to oocytes matured in vitro. To counteract elevated oxidative stress in the in vitro environment, several small thiol compounds can be added to the maturation medium to stimulate GSH synthesis, such as cysteine and cystine, cysteamine, or β -mercaptoethanol. Supplementation of these small thiols during oocyte IVM may stimulate GSH synthesis and thus improve embryo development to the blastocyst stage (de Matos and Furnus 2000; de Matos et al. 2002).

Maternal age has a significant influence on oocyte developmental competence, as oocyte competence is progressively obtained during females undergo puberty and the

first several estrus cycles (Gandolfi et al. 1998; Khatir et al. 1998). Therefore, it is an excellent model to study oocyte developmental competence. In pigs, oocytes derived from prepubertal gilts showed decreased meiotic maturation, increased polyspermy and compromised embryonic development (Marchal et al. 2001). It is possible that redox regulation pathways may not yet be fully developed in prepubertal oocytes, partially contributing to the decreased developmental competence observed. We hypothesize that by comparing the redox related gene expression between prepubertal and adult derived oocytes, important redox regulation mechanisms in oocytes can be identified, and effective strategies can be developed to alter these critical pathways to improve oocyte competence.

Materials and Methods

Materials

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

Animals

All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee, and have been judged to minimize discomfort, distress, injury and pain.

Oocytes In vitro maturation

Porcine ovaries were collected from prepubertal gilts or adult sows at two local abattoirs (Indiana Packers Corp. and Momence Packing Co., respectively), and transported to the laboratory in 0.9% (w/v) NaCl at 30 - 34 °C. Prepubertal ovaries

were confirmed by the absence of developed corpora lutea. Cumulus-oocyte complexes (COCs) were vacuum aspirated from 3-8 mm follicles using an 18-gauge needle. Oocytes with several layers of unexpanded cumulus cells and evenly dark cytoplasm were selected and rinsed in Hepes-buffered synthetic oviductal fluid supplemented with 0.1% BSA (SOF-Hepes (Good and Frishette 1966)). Selected COCs were matured in vitro in modified Purdue Porcine Medium (PPMmat) (Herrick et al. 2006; Herrick et al. 2003; Stroble et al. 2002) containing 2 mM glucose, 6 mM lactate, 0.2 mM pyruvate, 0.34 mM cysteamine, 1% recombinant human albumin (recombumin, G-MM, Vitrolife, Kungsbacka, Sweden), 0.2% fetuin, 50 ng/ml EGF, 0.01 units/ml each LH and FSH (Sioux Biochemicals, Sioux City, IA) for 42 to 44 h at 38.7 °C in 7% CO₂ in humidified air.

Identification of meiotic maturation

After maturation, COCs were vortexed for 3 min in SOF-Hepes with 0.01% (w/v; 80-160 unit/ml) hyaluronidase and washed three times in SOF-Hepes. Denuded oocytes were mounted, fixed in chloroform:acetic acid:ethanol (1:3:6, v/v/v) for 72 h, stained with 1% (w/v) orcein in acetic acid. Oocytes were examined for meiotic stage at 400 × magnification. Oocytes reaching telophase and MII were considered mature.

In vitro fertilization (IVF) and embryo culture (IVC)

After maturation, oocytes were removed from the surrounding cumulus cells as described above, washed three times in modified Tris-buffered medium (mTBM) (Abeydeera and Day 1997) supplemented with 2 mM caffeine, 0.2% (w/v) fraction V BSA, and 1 × PSA (10,000 U penicillin, 10,000 µg streptomycin, 25 µg amphotercin;

Invitrogen, Carlsbad, CA, USA). Oocytes were cultured in 50 μ l drops of mTBM under 10 ml embryo tested, washed mineral oil (20 oocytes/drop). Fresh ejaculated semen from a single boar was collected weekly. Chilled and extended (1:5 dilution, Androhep EnduraGuard, Minitube of America Inc., Verona, WI, USA) semen was warmed at 37 °C for 20min, then 500 μ l warmed semen was placed onto a gradient of 45% :90% Percoll (GE Healthcare Life Sciences, Uppsala, Sweden) and centrifuged for 20 min at 700 $\times$ g. The supernatant was removed and the remaining sperm pellet washed twice with 5 ml Dulbecco's phosphate buffered saline (D-PBS; Invitrogen) by centrifuging for 5 min at 1000 $\times$ g. Sperm were then counted and diluted (1×10^6 sperm/ml) in mTBM and added (50 μ l) to fertilization drops containing oocytes for a final sperm concentration of 5×10^5 sperm/ml in 100 μ l. Gametes were co-incubated for 5 h at 38.7 °C in 6% CO₂ in humidified air. After co-incubation, presumptive zygotes were washed 3 times and cultured in 50 μ l NCSU-23 medium (10 zygotes/drop) containing 0.4% crystallized BSA (MP Biomedicals, Solon, OH, USA) under 10 ml mineral oil in 6% CO₂, 10% O₂, balance N₂ for 6 days, when embryonic cleavage and blastocyst development were determined. Blastocysts were stained with 0.01 mg/ml Hoechst 33342 overnight to determine total blastocyst cell number under fluorescence at 400 $\times$ magnification.

In vivo matured oocyte collection

Animals were synchronized then superstimulated prior to surgical oocyte retrieval. The animals' estrous cycles were synchronized by feeding 15 mg/day of an oral progestin, altrenogest (Matrix; Intervet America Inc. Millsboro, DE, USA) for 14-18 days. The day following the final day of matrix feeding, females received 1000 IU of PMSG (Calbiochem, La Jolla, CA, USA) followed in 72 h by 1000 IU of hCG (Calbiochem).

Surgical oocyte retrieval was performed 40 h post hCG injection. Pigs were restrained with a limp rope snare and injected with an initial intra-muscular injection of 3 ml anesthetic cocktail consisting of 5.9 mg/kg Ketamine, 1.47 mg/kg Telazol, 2.9 mg/kg Xylazine and 0.09 mg/kg atropine, followed by 7 ml intra-venous injection of the same solution via ear vein canula. Endotracheal tubes were inserted into the nares for connection to the anesthesia apparatus, supplying oxygen (2 L/min) and 5% halothane. A midline, lower abdominal incision was made through the body wall, the oviduct and part of the uterus exteriorized, and oocytes recovered via retrograde flush of the oviducts using approximately 20 ml warm SOF-Hepes. If necessary, oocytes were denuded via vortexing for 3 min in SOF-Hepes with 0.01% (w/v) hyaluronidase.

Oocyte quantitative PCR

For experiment 1 and 2, MII oocytes were identified by extrusion of the first polar body before being frozen at -80 °C with lysis buffer (Dynabeads mRNA DIRECT Kit, Invitrogen). Three separate pools of 20 oocytes each were collected for each treatment for qPCR analysis. Poly A+ RNA was extracted from the samples using the Dynabeads mRNA DIRECT Kit (Invitrogen). Genomic DNA contamination was assessed by reverse transcriptase (RT) and mock RT reactions using half of the mRNA from each sample. RNA samples (3 µl) were incubated with 1 µl dNTP (10 mM), 2 µl random hexamer primers (dN6; 100 ng/µl), and 4 µl water for 5 min at 65 °C. Random primed RNA samples were divided for RT and mock RT reactions. RT reactions were performed by using Moloney Murine Leukemia Virus (MMLV; 200 U/µl) with 40 U/µl RNase Out. Mock RT reactions were performed using the remaining 50% of random primed RNA; however, MMLV and RNase Out were excluded from the samples. cDNA samples were amplified for *GAPDH* by using HotMaster Taq (HMT; 5 U/µl)

polymerase (Eppendorf, Hamburg, Germany). PCR products were separated by gel electrophoresis and stained with ethidium bromide. The presence of a gel band in mock PCR samples indicated genomic DNA contamination. All samples displayed a positive RT reaction and did not contain genomic DNA. For the remaining half of the mRNA, single strand and double strand cDNA were generated using the RT template switching SMART technology (Wang et al. 2000; Zhu et al. 2001) and DNA samples were purified using the Machery-Nagel (Easton, PA) PCR Clean-up Gel Extraction Kit. Linear amplification was performed using the MEGAscript High Yield Transcription Kit (Ambion, Austin, TX), followed by DNase treatment. Amplified mRNA samples were purified from template cDNA using the Qiagen (Valencia, CA) RNeasy Mini Kit and single strand cDNA were generated by random primed cDNA synthesis. Primer design and qPCR were performed as previously described (Fleming-Waddell et al. 2007). Current gene annotations were obtained from the Genbank and primers were designed for qPCR using Primer3 (Rozen and Skaletsky 2000). Accession number, primer sequence, and product length of target genes are presented in Table 4.1. Information about primers for *GAPDH* was previously reported (Paczkowski and Krisher, in press). For the remaining genes, primers were tested on pools of 20 porcine oocytes and primer specificity was determined by melting curve analysis and gel electrophoresis. PCR products were cloned into pCR 2.1 TOPO vectors and transformed into One Shot TOP10 chemically competent *E. coli*. Plasmids were sequenced to confirm the identity of the transcript and quantified using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Target genes were analyzed using QuantiFast SYBR Green PCR reagents (Qiagen) on a MasterCycler Realplex² (Eppendorf North America, Inc; Westbury, NY, USA). A standard curve was generated from serial dilutions of EcoRI digested plasmids (10^7 to

10¹ molecules) and the efficiency of the primers was calculated.

For experiment 7, MII oocytes identified by extrusion of first polar body were frozen at -80 °C with lysis buffer (Arcturus PicoPure RNA Isolation Kit, Applied Biosystems, Foster City, CA, USA). Three separate pools of 20 oocytes each were collected for each treatment for qPCR analysis. Total RNA was extracted by using the Picopure Kit (Applied Biosystems) as described in the manufacturer's protocol with an additional DNase treatment to prevent genomic DNA contamination. cDNA synthesis was performed by RT reaction (Sensiscript RT Kit, Qiagen, Valencia, CA, USA) with random hexamer primers. Target genes were analyzed using QuantiFast SYBR Green PCR reagents (Qiagen) as described above.

Assay of GSH content

In experiment 3, total GSH content was measured by the dithiobisnitrobenzoic acid (DTNB)-glutathione disulphide reductase recycling assays (Baker et al. 1990; Herrick et al. 2006). After IVM, MII oocytes identified by extrusion of the first polar body were frozen at -80 °C in groups of 30 with 0.4 M NaPO₄ buffer containing 10 mM EDTA. When the assay was performed, 45µl water was added in each sample tube including cell-free media blank tube, and the samples were loaded to a 96 well plate. GSH standards (50µl water with 0.4-200pmol GSH) were also prepared and added to individual wells. DTNB (1mM, 5ml), NADPH (1mM, 5ml) and glutathione reductase (200U/ml, 0.1 ml) will be added in assay buffer (200mM NaPO₄, 1mM EDTA, pH 7.5) to make reaction mixture. 100µl of this reaction mixture was added in each well to initiate the reaction. Absorbance was measured five times by spectrophotometer at 30s intervals at 412nm.

In experiment 5, total GSH content was measured by using the DetectX Glutathione

Fluorescent Detection Kit (Arbor Assays, Ann Arbor, MI, USA). In brief, oocytes from each treatment were collected at 0, 24 and 44 hour post maturation (hpm). Oocytes were denuded and frozen at -80 °C in groups of 30 with 5% (w/v) aqueous 5-sulfo-salicylic acid dehydrate solution. Then the oocytes were lysed and deproteinized by vigorous vortexing and freeze/thaw cycles. The oocytes were incubated at 4 °C for 10 min followed by centrifugation for 10 min at 14,000 rpm and 4 °C. The centrifuged supernatants were diluted 1:5 with assay buffer and used for total GSH content assay as described in manufacturer's protocol.

Detection of ROS concentrations in oocytes

The ROS concentration in oocytes was measured using the Image-iT LIVE Green ROS Detection Kit (Invitrogen). In brief, oocytes from each treatment were collected at 0, 24 and 44 hpm. Oocytes were denuded and washed 3 times in D-PBS supplemented with 0.1% BSA. Oocytes were then incubated with 25 µM 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H₂DCFDA) in D-PBS with 0.1% BSA for 30 min. Subsequently, oocytes were washed with D-PBS with 0.1% BSA three times and were placed on a glass slide with D-PBS and covered with cover slip. The digital photographs were recorded 5 sec after excitation of oocytes at 480 nm and emission at 510 nm under a fluorescent microscope. The intensity of florescence in each oocyte were measured by ImageJ 1.43u software (Abràmoff et al. 2004), which indicated the ROS concentration in each oocyte.

Experimental design

Experiment 1. The transcript abundance of 13 redox genes was compared between prepubertal and adult derived IVM MII oocytes to identify the redox genes that are

reflective of oocyte competence. Each treatment was replicated three times, with 20 oocytes per pool in each replicate.

Experiment 2. To further validate a selected subset of redox genes from Experiment 1, transcript abundance of the selected genes were compared between IVM and VVM MII oocytes collected from both prepubertal and adult females (2×2 factorial design). In vivo matured MII oocytes were collected from 6 prepubertal and 6 adult females. In each treatment, oocytes were pooled in 3 separate replicates with 20 oocytes per replicate.

Experiment 3. Total GSH content was compared between IVM prepubertal and adult oocytes. In each treatment, oocytes were pooled in 4 separate replicates with 30 oocytes per replicate.

Experiment 4. Adult oocytes were matured in different redox environments, and nuclear maturation and subsequent developmental competence were examined. The basic maturation medium was PPMmat supplemented with 1mM hypoxanthine. ROS were generated by the hypoxanthine-xanthine oxidase (XOD) system at three different concentrations: XOD0 (0 mU), XOD1 (1 mU) and XOD10 (10 mU). In each XOD treatment, two different concentrations of cysteine (Cys) were added as an antioxidant: Cys1 (0.57mM) and Cys2 (1.14mM). This resulted in six experimental treatments in a 3×2 factorial design; XOD0-Cys1 was considered the control. A total of 3 replicates with 442 oocytes (20-30 oocytes/treatment/replicate) were performed to determine nuclear maturation. A total of 4 replicates with 1219 oocytes (50 oocytes/treatment/replicate) were performed to assess embryonic development after IVF/C.

Experiment 5. Adult oocytes matured in treatment group XOD0-Cys1, XOD0-Cys2, XOD10-Cys1 and XOD10-Cys2 were randomly sampled from each treatment at 0, 24 and 44 hpm to assess total GSH content. A total of 4 replicates with 1080 oocytes (20-30 oocytes/treatment/replicate) were performed.

Experiment 6. Adult oocytes matured in treatment groups XOD0-Cys1, XOD0-Cys2, XOD10-Cys1 and XOD10-Cys2 were randomly sampled from each treatment at 0, 24 and 44 hpm to examine ROS concentration. A total of 3 replicates with 619 oocytes (20-30 oocytes/treatment/replicate) were performed in this experiment.

Experiment 7. Adult and prepubertal oocytes were matured in different redox environments: XOD0-Cys1, XOD0-Cys2, XOD10-Cys1 and XOD10-Cys2. After IVF, MII adult and prepubertal oocytes were collected and transcript abundance of our selected subset of redox genes from experiment 1 were compared. In each treatment, oocytes were pooled in 3 separate replicates with 20 oocytes per replicate.

Statistical analysis

For qPCR results in experiment 1 and 2, the mRNA abundance of a target gene was normalized to an internal control, *GAPDH*, for sample to sample comparisons. In experiment 7, the qPCR results were similarly normalized to *18s*. Relative expression ratios were obtained by the comparative threshold cycle method (Livak and Schmittgen 2001). Data were analyzed using the relative expression software tool, REST 2009 version 2.0.13 (Pfaffl et al. 2002). GSH content (experiments 3 and 5) and ROS concentration (experiment 6) values were analyzed using one-way ANOVA, with treatment included in the model as a fixed factor. Differences were determined by Bonferroni (all-pairwise) multiple comparison test when treatment was significant. In experiment 4, percentages of maturation, embryonic cleavage, and blastocyst

development, as well as blastocyst total cell number were analyzed using two-way ANOVA, with maturation treatment included in the model as a fixed factor and replicate as a random factor. Percentage data (maturation success, embryonic cleavage and blastocyst development) were arcsin transformed. Differences were determined by Fisher's LSD multiple comparison test when treatment was significant. Significance was determined as $P < 0.05$. Data are reported as mean $\pm$ SEM.

Results

Experiment 1. Comparative gene expression in IVM oocytes derived from prepubertal and adult animals.

Transcript abundance of 13 redox genes was compared between IVM oocytes derived from adult and prepubertal animals by qPCR. Analysis of gene expression showed that glutaredoxin 2 (*GLRX2*), protein disulfide isomerase 4 (*PDIA4*), protein disulfide isomerase 6 (*PDIA6*), and thioredoxin reductase 1 (*TRXR1*) were more highly expressed in prepubertal derived oocytes ($P < 0.05$). No difference was found for the remaining analyzed transcripts (*GSR*, *SRx*, *AP1B1*, *GPXI*, *GSS*, *GSTO1*, *QSOX1*, *GLRX1*) (Figure 4.1). Transcripts from all genes examined, with the exception of *SRx*, were more abundant in prepubertal derived oocytes, even if not differentially expressed.

Experiment 2. Comparative gene expression in IVM and VVM oocytes derived from prepubertal and adult animals.

Based on results from Experiment 1, *GLRX2*, *PDIA 4*, *PDIA6* and *TRXR1* were considered to be associated with oocyte competence, and the expression patterns of these genes were then compared in IVM and VVM oocytes obtained from both

prepubertal and adult animals. The comparison between IVM prepubertal and adult MII oocytes confirmed that *GLRX2*, *PDIA 4* and *TRXR1* were upregulated in IVM prepubertal oocytes, whereas *PDIA6* was not differentially expressed in this experiment. When expression patterns of these 4 genes were compared between IVM and VVM prepubertal oocytes, *GLRX2*, *PDIA 4* and *TRXR1* were upregulated, and *PDIA6* was downregulated in IVM prepubertal oocytes. The comparison between IVM and VVM adult oocytes revealed that *GLRX2*, *PDIA 4* and *PDIA6* were downregulated in IVM adult oocytes, and *TRXR1* was not differentially expressed between IVM and VVM adult oocytes. When comparing the gene expression patterns between VVM prepubertal and adult oocytes, *PDIA 4*, *PDIA6* and *TRXR1* were not differentially expressed. Only *GLRX2* was downregulated in VVM prepubertal oocytes (Table 4.2).

Experiment 3. Comparison of total GSH content in IVM oocytes derived from prepubertal and adult animals.

Total GSH content was significantly higher in prepubertal oocytes compared to adult oocytes after IVM (7.0 ± 0.6 pmol/oocyte and 5.8 ± 0.4 pmol/oocyte, respectively).

Experiment 4. Effects of different redox environments on adult derived oocyte nuclear maturation and developmental competence.

There was no significant difference in the percentage of meiotic maturation between oocytes treated with 1mU/ml XOD or without XOD. However, maturation was significantly decreased when oocytes were treated with 10mU/ml XOD (Table 4.3). Supplementation with additional cysteine could not rescue maturation. Embryonic cleavage of oocytes treated with 10mU/ml XOD was also significantly decreased compared to control, and also could not be rescued by additional cysteine (Table 4.3).

Blastocyst development was significantly decreased when oocytes were treated with extra cysteine alone (XOD0-Cys2) or with 10mU/ml XOD without extra cysteine (XOD10-Cys1) when compared to control (Table 4.3). The deleterious effect of 10mU/ml XOD could be somewhat negated by adding extra antioxidant capacity to the environment (XOD10-Cys2); this treatment was not significantly different than control or XOD10-1X (Table 4.3). There were no differences in blastocyst total cell number between any treatments ($P>0.05$) (Table 4.3).

Experiment 5. Effects of different redox environments on the total GSH content of adult oocyte during IVM.

Total GSH content in mature control oocytes significantly increased during IVM (2.5 ± 0.3 pmol/oocyte at 0 hpm, 7.8 ± 2.0 pmol/oocyte at 24 hpm, and 11.7 ± 0.4 at 44 hpm; Figure 4.2). Extra cysteine alone (XOD0-Cys2) did not influence total oocyte GSH content at either 24 hpm or 44 hpm when compared with control (7.6 ± 0.2 pmol/oocyte and 10.5 ± 0.4 pmol/oocyte, respectively) ($P>0.05$). Oocytes treated with 10mU/ml XOD (XOD10-Cys1) had significantly lower total GSH content at both 24 hpm and 44 hpm compared to control (5.6 ± 0.7 pmol/oocyte at 24 hpm and 7.0 ± 1.3 pmol/oocyte at 44 hpm). Extra cysteine could not rescue the decreased total GSH content in oocytes treated with 10mU/ml XOD (XOD10-Cys2) at either 24 hpm or 44 hpm (4.8 ± 1.0 pmol/oocyte and 6.5 ± 0.8 pmol/oocyte, respectively).

Experiment 6. Effects of different redox environments on the ROS concentration of adult oocytes during IVM.

ROS concentration in control (XOD0-CYS1) oocytes matured in control group was significantly decreased during IVM (87.3 ± 5.2 unit at 0 hpm, 52.3 ± 4.1 unit at 24

hpm, and 26.8 ± 2.1 unit at 44 hpm; Figure 4.3). Oocytes treated with extra cysteine alone (XOD0-Cys2) had significantly decreased ROS concentrations at both 24 hpm and 44 hpm when compared with control (41.0 ± 3.2 unit and 52.3 ± 4.1 unit, respectively at 24 hpm, and 18.26 ± 1.38 unit and 26.8 ± 2.1 unit, respectively, at 44 hpm). However, ROS concentration in oocytes treated with 10mU/ml XOD (XOD10-Cys1) was not significantly different from the control at either 24 hpm or 44 hpm (51.3 ± 5.0 unit and 29.5 ± 2.9 unit, respectively). Extra cysteine did not decrease the ROS concentration in oocytes treated with 10mU/ml XOD (XOD10-Cys2) at both 24 hpm and 44 hpm (51.2 ± 3.5 unit and 30.7 ± 2.9 unit, respectively) (Figure 4.3).

Experiment 7. Effects of different redox environments on redox gene expression patterns in prepubertal and adult MII oocytes.

All redox related genes tested were downregulated in prepubertal oocytes matured with extra cysteine alone (XOD0-Cys2) when compared to control. However, none of the redox genes were differentially expressed in adult oocytes matured with extra cysteine alone when compared to control (XOD0-CYS1; Figure 4.4A). When oocytes were matured with 10mU/ml XOD without extra cysteine (XOD10-Cys1), none of the redox genes were differentially expressed in prepubertal oocytes and only GLRX2 was downregulated in adult oocytes when compared to control (Figure 4.4B). When treated with 10mU/ml XOD with extra cysteine (XOD10-Cys2), all redox genes except *PDIA6* were downregulated in prepubertal oocytes when compared to control. However, none of the redox genes were differentially expressed in adult oocytes (Figure 4.4C).

Discussion

Differential expression of 4 redox related genes between IVM oocytes derived from adult (good quality) and prepubertal (poor quality) animals suggested that these genes are related to oocyte competence. We further tested expression of these genes in both in vitro and in vivo matured oocytes derived from both adult and prepubertal animals, to validate the importance of these four genes to oocyte competence. Three out of four genes were further validated in the second model by again comparing gene expression levels between IVM prepubertal and adult oocytes.

The discrepancy in PDIA6 expression between experiment 1 and 2 may due to the independent pools of cDNA used for each experiment. When comparing gene expression levels between in vitro and in vivo matured oocytes, all four genes were differentially expressed in prepubertal oocytes, and three genes were differentially expressed in adult oocytes. Overall, 7 out of 8 gene comparisons were validated in the in vitro-in vivo matured model, indicating the importance of these four genes to oocyte competence. In comparisons between different types of oocytes, the same genes were both up- and down-regulated regulated in less competent oocytes. We hypothesize that this discrepancy between up and down regulation in less competent oocytes between different models of oocyte competence is due to differences in the maturity of cellular mechanisms between prepubertal and adult oocytes, as well as the ability of these two types of oocytes to handle in vitro environmental stressors (Yuan et al., in press). When comparing gene expression between prepubertal and adult oocytes after in vivo maturation, only *GLRX2* was differentially expressed, indicating that the dysregulation of redox genes in prepubertal oocytes found in the first experiment are primarily due to the suboptimal in vitro environment. Therefore, prepubertal oocytes may be less able to regulate redox balance in the in vitro environment, resulting in altered gene

expression and reduced quality when compared to adult oocytes.

Among the four redox genes associated with oocyte competence in this study, *GLRX2* belongs to the oxidoreductase family that participates in a variety of cellular redox reactions. *GLRX2* could prevent H₂O₂-induced cell apoptosis by protecting complex I activity in the mitochondria (Wu et al. 2010). *PDIA4* and *PDIA6* are members of protein disulfide isomerase family A, which catalyze the formation and breakage of disulfide bonds between cysteine residues within proteins as they fold (Wilkinson and Gilbert 2004). *TRXR1* is the only enzyme known to catalyze the reduction of thioredoxin and is a central component in the thioredoxin system (Mustacich and Powis 2000). The upregulation of these redox genes in oocytes derived from prepubertal females may be indicative of a decreased ability of the prepubertal oocytes to regulate the redox balance. We found that total GSH content was significantly higher in prepubertal oocytes compared to adult oocytes. It is likely that prepubertal oocytes may overreact to the oxidative stress of the in vitro environment, resulting in production of excessive amounts of GSH in an attempt to counteract oxidative stress. Supplementation of cysteamine during IVM of adult mouse oocytes improved embryo development, although addition of cysteamine during IVM of prepubertal oocytes had no effect on embryo development (de Matos et al. 2003). Cysteamine is a low molecular-weight thiol compound that can reduce cystine to cysteine and enhance oocyte glutathione synthesis (Bannai 1984; Issels et al. 1988). Based on our results, prepubertal oocytes may have already produced excessive GSH during IVM, such that supplementation of cysteamine may not enhance embryo development.

To assess how oxidative stress influences oocyte maturation and subsequent embryonic development, we matured adult oocytes in different levels of oxidative stress with or without additional cysteine. Cysteine is the precursor of GSH (de Matos and Furnus

2000), which has been confirmed to be the main non-enzymatic defense system against oxidative stress in oocytes and embryos (de Matos and Furnus 2000; Gardiner and Reed 1994; Takahashi et al. 1993; Yoshida 1993). In bovine oocytes, supplementation with cysteine, but not N-acetyl-L-cysteine, catalase or superoxide dismutase, during IVM improved embryonic development (Ali et al. 2003). Supplementation of cysteine at 1.14 mM and 2.28 mM during pig oocyte IVM has a significant beneficial effect on blastocyst development (Jeong and Yang 2001). In our experiment, addition of 1.14 mM cysteine was detrimental to blastocyst formation when compared to control in which 0.57 mM cysteine was added. This discrepancy may be explained by differences in the maturation systems used in the two studies. In the previous study, NCSU23 with 10% porcine follicular fluid without addition of cysteamine and pyruvate was utilized. Cysteine is highly unstable and easily oxidized to cystine (Bannai 1984; Sagara et al. 1993). We add 0.34 mM cysteamine and 0.2 mM pyruvate in our completely defined maturation medium. Cysteamine can reduce cystine to cysteine, stabilize cysteine and promote cysteine uptake into cells (Issels et al. 1988). Pyruvate can be decarboxylated by H_2O_2 to produce acetate, carbon dioxide and H_2O . It plays a role in protecting cells against oxidative stress as an extracellular antioxidant (O'Donnell-Tormey et al. 1987). Therefore, addition of 1.14mM cysteine in our maturation medium may create an environment that is too reduced and inhibits oocyte developmental competence. This detrimental effect of extra cysteine could be abrogated by adding a mild oxidative stress in the form of 1mU/ml XOD to the maturation medium. It is likely that the free radicals generated by supplementing 1 mU/ml XOD neutralize the excessive reducing agents and balance the redox environment in the maturation medium. When supplemented with 10 mU/XOD, oocyte nuclear maturation and blastocyst formation were both decreased, suggesting that this intensive oxidative stress has a deleterious

effect on oocyte competence. This effect could be somewhat negated by adding extra cysteine to the environment. Overall, these results demonstrate the importance of keeping the redox environment balanced during oocyte maturation. Excessive oxidative or reducing environments are both detrimental to oocyte developmental competence.

During IVM, total GSH content significantly increased, reaching the highest level at the MII stage. This finding is consistent with previous reports in mouse (Calvin et al. 1986), hamster (Zuelke et al. 2003) and pig (Yoshida 1993; Yoshida et al. 1993), indicating that GSH synthesis is active throughout maturation. Intracellular GSH deposition protects oocytes against oxidative stress during the maturation period. GSH also facilitates male pronuclear formation after sperm penetration (Yoshida et al. 1993). In addition, GSH synthesized during maturation may serve as a reservoir of antioxidant protection for embryo development until the blastocyst stage (Zuelke et al. 2003). In this study, extra cysteine supplementation did not increase the GSH levels in oocytes, a finding supported by previous work (Abeydeera et al. 1999). It is possible that only a certain amount of small thiol compounds, such as cysteine, can be utilized by oocytes to stimulate GSH synthesis. When treated with 10 mU/XOD, total GSH content in oocytes was significantly decreased, indicating that the oocytes were undergoing oxidative stress and utilizing available GSH pools. Supplementation of additional cysteine in the maturation medium did not increase total GSH content in oxidatively stressed oocytes, possibly because oocytes could not utilize the extra cysteine to stimulate GSH synthesis. Additional cysteine may also react with ROS in the medium directly and partially neutralize the oxidative environment. Thus, as we observed, addition of extra cysteine alleviated oxidative stress in oocytes and partially rescued blastocyst development.

Intracellular ROS concentration in oocytes significantly decreases during IVM. High levels of ROS in oocytes immediately post collection may be due in part to elevated oxidative stress during removal from the follicular environment. During IVM, the continuous production of GSH in oocytes appears to neutralize ROS. Thus, increasing intracellular GSH levels during IVM may contribute to decreasing ROS concentrations in oocytes. In bovine oocytes, increasing demand for oxygen consumption throughout IVM (Sutton et al. 2003) may be driven by mitochondrial oxidative phosphorylation, the major source of ATP synthesis (Dumollard et al. 2004). Contrary to our observation of continuously decreasing ROS concentrations through IVM, in bovine oocytes ROS levels were decreased over the first 12 h and increased in the last 10 h of maturation (Morado et al. 2009). Several differences between the two studies, such as species, maturation medium, and ROS detection methods, may contribute to this discrepancy.

ROS concentration was significantly decreased when extra cysteine was added to the maturation medium. Extra cysteine may create a reduced environment and inhibit intracellular ROS production during IVM. The role of ROS in oocyte maturation is still controversial. It is well known that excessive ROS is detrimental to oocyte and embryo development (Hashimoto et al. 2000; Yang et al. 1998). Several studies demonstrated negative correlations between follicular fluid ROS levels and successful IVF and pregnancy in humans (Bedaiwy et al. 2005; Pasqualotto et al. 2004). However, ROS also plays an important role in regulating proper cellular functions (Agarwal et al. 2004; Rhee 2006). It regulates various reproductive processes, including maintaining the proper function of sperm (Gagnon et al. 1991) as well as being involved in sperm-oocyte interaction (deLamirande et al. 1997). A transient increase of intracellular ROS level may signal meiotic resumption from diplotene arrest in mammalian oocytes

(Pandey et al. 2010). In addition, ROS is indispensable for cumulus cell expansion, and ovarian production of ROS is an essential preovulatory signaling event (Shkolnik et al. 2011). Decreased ROS in oocytes matured with extra cysteine alone may contribute to the compromised blastocyst formation after IVF, suggesting that crucial ROS signaling pathways during IVM may be disrupted by the reduced environment, negatively impacting oocyte competence. However, we did not observe an increase in ROS concentration in the oocytes under oxidative stress, although oocyte competence was still compromised as reflected by reduced blastocyst development.

We found increased expression of *GLRX2*, *PDIA4*, *PDIA6* and *TRXR1*, and significantly increased GSH level in IVM prepubertal oocytes when compared to adult oocytes. These results suggest that prepubertal oocytes may be less able to regulate redox balance in the in vitro environment. Additional cysteine in maturation medium, with or without added oxidative stress, resulted in significantly decreased redox gene expression in mature prepubertal oocytes, while gene expression in mature adult oocytes was not altered by the different redox environment. Cysteine is the rate-limiting precursor of glutathione synthesis (Beutler 1989), and can thus be used as an antioxidant.. In this study, the increased redox gene expression and GSH level in prepubertal porcine oocytes may cause a too reduced intracellular environment. The disrupted redox homeostasis may interrupt the cell function of utilizing the cysteine in the medium to facilitate the GSH synthesis. Therefore, supplementation of additional cysteine was detrimental and inhibits redox-related gene expression in prepubertal oocytes.

In conclusion, the current study demonstrates that redox balance and the redox related genes *GLRX2*, *PDIA4*, *PDIA6* and *TRXR1*, are functionally relevant to oocyte developmental competence. A balanced redox environment is required for normal

oocyte development. The disrupted redox homeostasis and aberrant redox gene expression patterns in prepubertal porcine oocytes may contribute to the decreased developmental competence. Future work will focus on development of techniques to control these important redox regulating pathways to achieve better oocyte competence following IVM.

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Tables and Figures

Table 4.1. Summary of gene symbol, accession number, primer sequences and amplicon product length for the target genes examined.

Gene Symbol	Accession Number	Forward Primer Sequence	Reverse Primer Sequence	Product Length
<i>GAPDH</i>	AF017079	acatcaagaaggtggtgaag	attgtcgtaccaggaaatgag	151
<i>18S</i>	X01117	cggctaccacatccaaggaa	ctccaatcggatcctcgtaaagg	147
<i>GLRX2</i>	AW656565	gcttgaatacggagccagt	gcaaagttgtaagcgctct	215
<i>GSR</i>	AY368271	tgtggcaatcaggatgtga	tacgtgagccgactgaacac	153
<i>PDIA4</i>	AK236576	aaactgaggccccgttatcaa	gtggcccttgacttcttgc	201
<i>PDIA6</i>	AK238249	acgacctggagaaggatgag	cggatcgagtgtacttctg	150
<i>SRx</i>	BF711868	ctgccaagaaccagaagtc	gctccacgctgttcagt	162
<i>TRXR1</i>	AK236755	catctcccaattcctcgtgt	ctgatagccacggcatgtt	222
<i>AP1B1</i>	BQ604280	taggagggtgctctgttg	atgaagcatcctgtcacct	221
<i>GPX1</i>	NM_214201	gtcgggtgtatgccttctct	ctgatgtccaaactggtgc	212
<i>GSS</i>	AK237297	catccacaagcaagtcctga	ttggcagcttcttggctctt	219
<i>GSTO1</i>	NM_214050	cgcctttgctaatacgcttt	gggccagatgaggtaatacaa	167
<i>QSOX1</i>	BX917843	gatgggaagtcggtgacact	gtacctggccctgatctttg	150
<i>TRx</i>	NM_001137629	ctacaaaccaccagggcatt	gtcgggctacttcattctgg	227
<i>GLRX1</i>	NM_214233	caatggtgtgaagggtctga	cacttaatggcccgaact	159

Table 4.2. Relative expression level of competence-related redox genes in in vivo or in vitro matured oocytes derived from prepubertal or adult pigs.*

Gene Symbol	In vitro prepubertal vs. adult		Prepubertal in vitro vs. in vivo		Adult in vitro vs. in vivo		In vivo prepubertal vs. adult	
GLRX2	4.472	up	1.874	up	0.153	down	0.364	down
PDIA4	3.151	up	1.865	up	0.411	down	0.695	nsd
PDIA6	1.541	nsd	0.405	down	0.174	down	0.66	nsd
TRXR1	2.353	up	3.545	up	1.043	nsd	0.692	nsd

* In each column, gene expression was compared between two groups stated in the column heading. The numbers in each column showed the relative gene expression level of the first group normalized to that of the second group. ‘Up’ means that the gene was upregulated in the first group compared to the second group of the comparison, and likewise ‘down’ means the gene was down regulated in the first group compared to the second group; ‘nsd’ not significantly different.

Table 4.3. Effects of different redox environments on adult oocyte nuclear maturation and developmental competence.

Treatment	Percentage of oocytes reaching MII (n)	Percent embryonic cleavage (n)	Percent blastocyst development (n)	Blastocyst cell number
XOD0-Cys1	80.3 ± 4.8 ^a (71)	59.1 ± 3.5 ^a (209)	27.0 ± 4.0 ^a (209)	57.8 ± 3.9
XOD0-Cys2	68.9 ± 5.4 ^{ab} (74)	48.6 ± 5.2 ^{ab} (209)	14.9 ± 3.4 ^b (209)	56.2 ± 5.7
XOD1-Cys1	77.3 ± 4.9 ^a (75)	59.1 ± 2.9 ^a (204)	22.4 ± 3.9 ^{ab} (204)	70.0 ± 5.4
XOD1-Cys2	80.8 ± 4.6 ^a (73)	57.5 ± 4.1 ^a (194)	27.0 ± 4.6 ^a (194)	65.5 ± 4.2
XOD10-Cys1	48.1 ± 5.7 ^c (79)	36.4 ± 5.6 ^b (195)	12.5 ± 2.8 ^b (195)	65.4 ± 6.4
XOD10-Cys2	58.6 ± 5.9 ^{bc} (70)	38.3 ± 4.5 ^b (208)	18.4 ± 3.9 ^{ab} (208)	54.9 ± 4.2

^{abc} Different superscripts within a column denote a significant difference, $P < 0.05$. Data are reported as mean ± SEM.

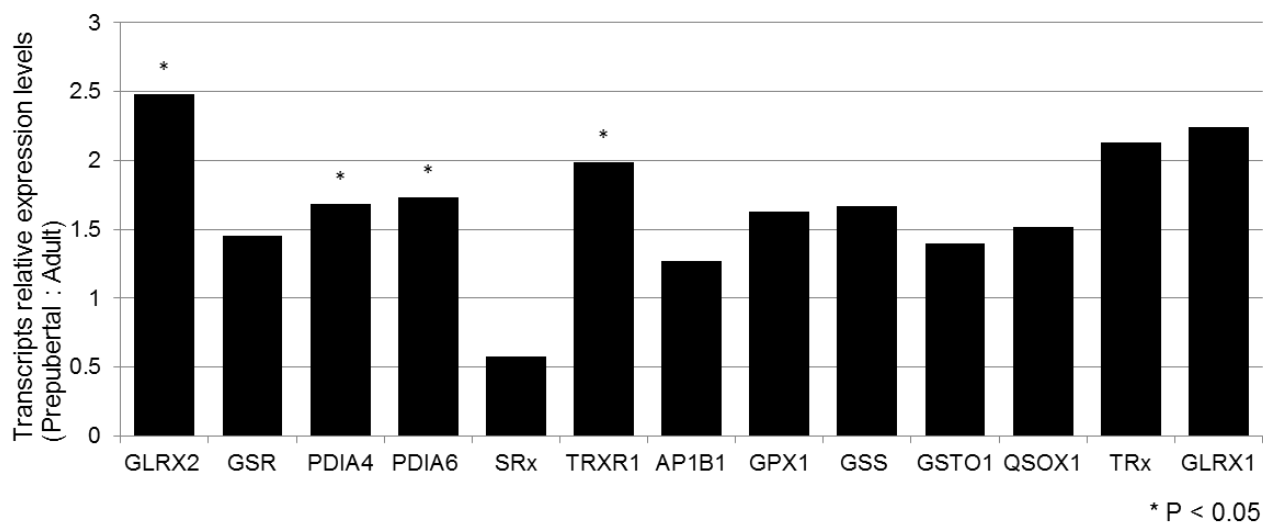


Figure 4.1. Expression of 13 redox genes in oocytes derived from prepubertal compared to adult pigs, determined by qPCR analyses and relative to *GAPDH*. * represents a significant difference in gene expression between prepubertal and adult oocytes ($P < 0.05$).

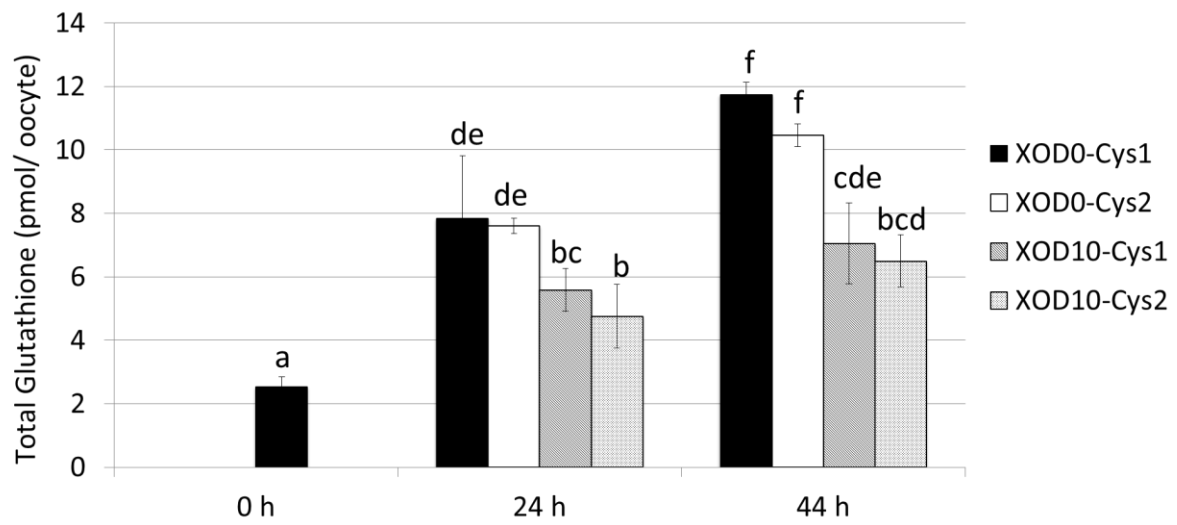


Figure 4.2. Effects of different redox environments on the total GSH content of adult oocyte during IVM. The values are expressed as mean $\pm$ SEM. Values with different superscripts are significantly different ($P < 0.05$).

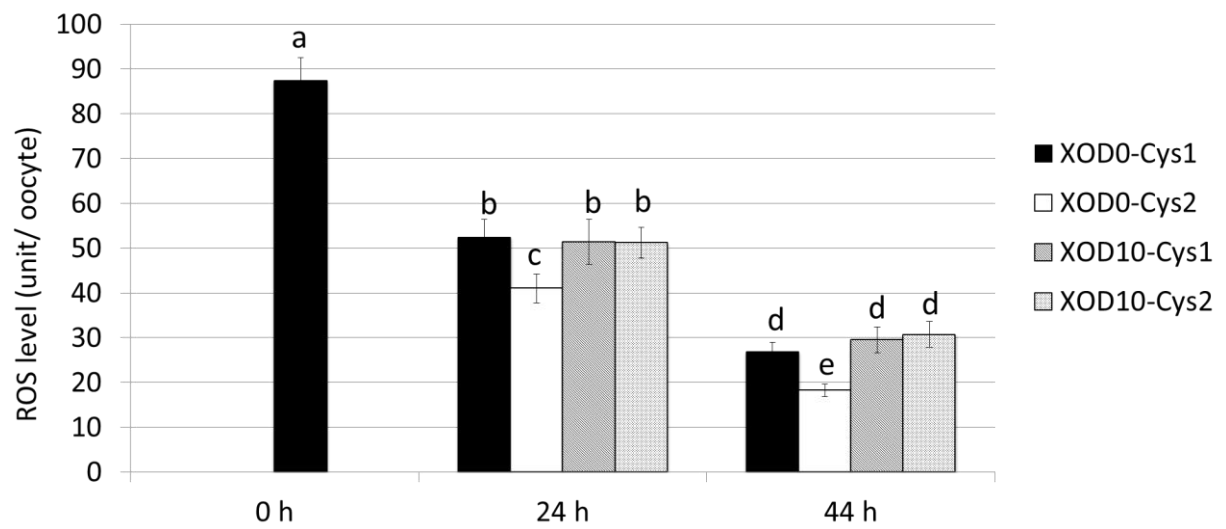


Figure 4.3. Effects of different redox environments on the ROS concentrations of adult oocyte during IVM. The values are expressed as mean $\pm$ SEM. Values with different superscripts are significantly different ($P < 0.05$).

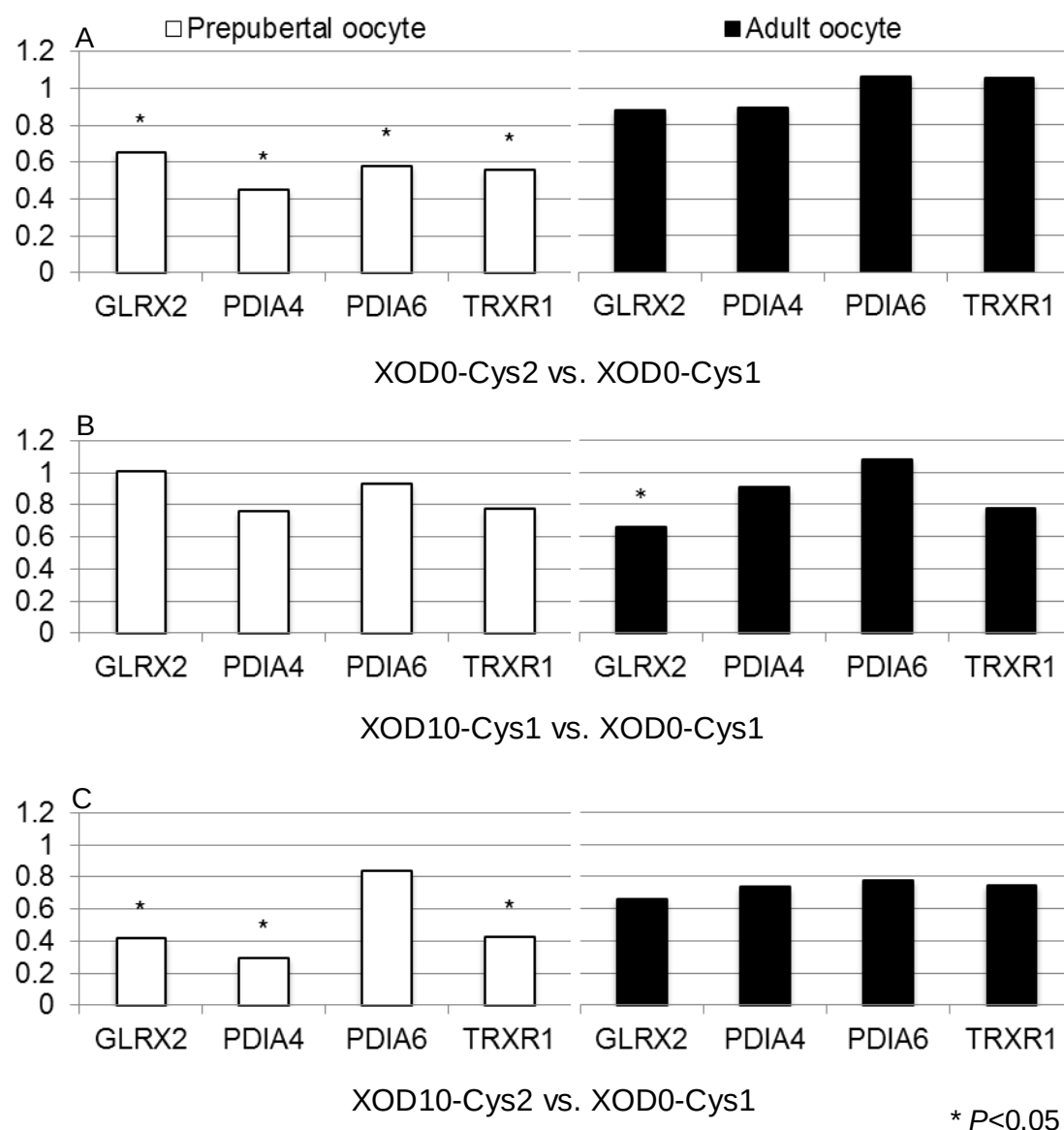


Figure 4.4. Effects of different redox environments on redox gene expression patterns in prepubertal and adult MII oocytes. Expression of redox genes of oocytes in XOD0-Cys2 (A), XOD10-Cys1 (B), or XOD10-Cys2 (C) treatment was compared with oocytes in control group in both prepubertal and adult oocytes. The relative expression level of genes in treatment against control group were determined by qPCR analyses and relative to *18S*. * represents a significant difference in gene expression between treatment and control group ($P < 0.05$).

CHAPTER 5

USE OF A MICROFLUIDIC WELL INSERT TO SUCCESSFULLY MATURE PORCINE OOCYTES AND CULTURE EMBRYOS INDIVIDUALLY IN VITRO

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Abstract

The physical environment used in in vitro embryo production (IVP) has changed little over the years. The application of microfluidics to embryo production provides tools that may allow the development of a more physiological, efficient culture system; meanwhile, tracking of individual oocytes and embryos become feasible using this technology. In this study, our objective was to evaluate the effect of a novel microfluidic system for individual oocyte in vitro maturation (IVM) and embryo in vitro culture (IVC) in pigs. In the first experiment, microfluidic well inserts consisting of multiple microwells with connecting microchannels resulted in equivalent percentages of blastocyst development and blastocyst total cell numbers compared with standard microdrop conditions for embryo IVC. In the second and third experiments, these microfluidic well inserts were further evaluated for individual IVC and IVM in a rocking and motionless environment. The rocking environment during both oocyte IVM and embryo culture had detrimental effects on oocyte and embryo development compared with a motionless environment. Importantly, blastocyst development of oocytes and embryos cultured in the microfluidic wells in the motionless environment was equivalent to that of standard microdrops. In conclusion, this microfluidic well insert system can successfully mature individual oocytes and culture individual embryos without compromising developmental potential.

Introduction

The in vitro production (IVP) of porcine embryos, including in vitro oocyte maturation (IVM), fertilization (IVF) and embryo culture (IVC), is historically difficult. Tremendous efforts have been made to optimize this system to allow more efficient production of viable embryos (Abeydeera and Day 1997; Funahashi and Day 1997; Nagai et al. 1984; Yoshida et al. 1990; Yoshioka et al. 2002). The majority of research has focused on studying the in vivo fluid environment as well as oocyte/embryo metabolism to manipulate the chemical components of culture media used to support in vitro development. Energy substrates and various growth factors have been examined in detail to establish optimized concentrations at different time points during IVP to achieve optimal development of viable embryos. Despite dramatic progress made in developing appropriate media environments for IVP, relatively little work has been accomplished optimizing the physical culture environment.

In the in vivo environment, ovulated oocytes before fertilization and preimplantation embryos are confined in the female reproductive tract in a small volume of oviductal or uterine fluid. However, the in vitro scenario is entirely different. In vitro, oocytes and embryos are cultured in a relatively large volume of culture medium, typically in groups. Culturing embryos in groups in smaller volumes of medium can increase blastocyst formation and blastocyst total cell number (Gardner et al. 1994; Lane and Gardner 1992; Paria and Dey 1990). In the pig and bovine, embryos in closer proximity to each other developed better (Gopichandran and Leese 2006; Somfai et al. 2010; Stokes et al. 2005). This is thought to be due to the production of autocrine/paracrine factors by the embryos themselves that have beneficial effects on their own development as well as adjacent embryos (Gardner et al. 1994;

Gopichandran and Leese 2006). However, it is difficult to increase cell to fluid volume ratios in standard microdrop condition, as small volumes evaporate quickly during dish preparation, causing potential chemical and osmotic toxicities. In addition, too little medium in standard microdrop condition can also be detrimental (Canseco et al. 1992). This may due to the accumulation of harmful by-products, such as ammonia, and insufficient nutrients available for complete embryo development. Therefore, changing the physical environment for embryo culture to take advantage of the autocrine/paracrine effect, while providing enough nutrients and limiting toxic by-products could potentially lead to the development of a novel culture system that better supports in vitro embryo production.

Unlike the in vivo environment, where embryos are moved via ciliated epithelium through a changing environment, the traditional microdrop cultures are static. Use of a dynamic culture environment may benefit embryo development as it may more closely mimic the in vivo situation. Blastocyst formation from 2-cell stage mouse and human embryos and blastocyst cell number were significantly increased when cultured in a tilting system compared with static culture (Matsuura et al. 2010).

Mechanical vibration at 30 or 60 min intervals for 5 s during oocyte maturation improved porcine oocyte developmental competence, although no significant effect was observed when vibration was applied to activated embryos (Mizobe et al. 2010).

Using a controlled fluid flow device, one cell mouse embryos developed to the blastocyst stage more often, with higher blastocyst cell numbers, resulting in increased implantation and pregnancy rates compared with static control (Heo et al. 2010).

Because typical IVP conditions utilize relatively large microdrops with groups of embryos, it is difficult to successfully develop individual or even small groups of

embryos in this system. A single gamete/embryo culture system would benefit human IVF and wildlife conservation, where few oocytes or embryos are often recovered. Such a system would also allow tracking and monitoring of single oocytes and embryos, permitting more specific data collection for experimental purposes. In this study, we assessed a novel microfluidic well insert in both a static and dynamic environment for use in porcine IVM and IVC. We hypothesize that the microfluidic inserts can mature individual oocytes and culture individual embryos successfully, resulting in better developmental potential.

Materials and Methods

Chemicals

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

Oocyte in vitro maturation (IVM)

Ovaries from sows were collected at a local abattoir, and transported to the laboratory in saline solution (0.9% NaCl) at 30-34 °C. Cumulus-oocyte complexes (COCs) were vacuum aspirated from 3-8 mm follicles using an 18-gauge needle. Oocytes with several layers of unexpanded cumulus cells and evenly dark cytoplasm were selected and rinsed in Hepes-buffered synthetic oviductal fluid supplemented with 0.1% BSA (SOF-Hepes (Good and Frishette 1966; Herrick et al. 2006)). Selected COCs were matured in vitro in TCM 199 with 10% porcine follicular fluid, 5 ng/ml EGF, and 0.01 units/ml each LH and FSH (Sioux Biochemicals, Sioux City, IA, USA) for 42 h at 38.7 °C in 7% CO₂ in humidified air in either bulk IVM control condition (50 oocytes/well in Nunc 4-well culture dishes (Nunclon; Nalge Nunc Intl. Rochester, NY,

USA) in 500 µl maturation medium covered with 500 µl mineral oil.) or the microfluidics IVM system.

Assessment of nuclear maturation

After maturation, oocytes were denuded of cumulus cells by vortexing in SOF-Hepes with 0.01% (w/v; 80-160 unit/ml) hyaluronidase for 3 min. Oocytes (approximately 10 per slide) were mounted onto a glass slide and fixed in chloroform:acetic acid:ethanol (1:3:6) for a minimum of 48 h. Oocytes were then stained with 1% (w/v) orcein in acetic acid and examined at 400 × magnification on an inverted phase contrast microscope. Oocytes at telophase and metaphase II (MII) were considered mature.

In vitro fertilization (IVF)

After maturation, denuded oocytes were washed three times in modified Tris-buffered medium (mTBM) (Abeydeera and Day 1997) supplemented with 2 mM caffeine, 0.2% (w/v) fraction V BSA, and 1× PSA (10,000 U penicillin, 10,000 µg streptomycin, 25 µg amphotercin; Invitrogen, Carlsbad, CA, USA) and placed into 50 µl drops of mTBM under 10 ml mineral oil (20 oocytes/drop) in a Falcon 1007 petri dish (Becton Dickinson Labware, Franklin Lakes, NJ, USA). Fresh ejaculated semen from a single boar was collected weekly. Chilled and extended (1:5 dilution, Androhep EnduraGuard, Minitube of America Inc., Verona, WI, USA) semen was warmed at 37 °C for 20min, then 500 µl warmed semen was processed through a 45%-90% gradient of Percoll (GE Healthcare Life Sciences, Uppsala, Sweden) by centrifugation at 700 ×g for 20 min. The supernatant was removed and the sperm pellet washed twice with 5 ml Dulbecco's phosphate buffered saline (D-PBS; Invitrogen) by centrifuging at 1000 ×g for 5 min. Sperm were diluted (1×10^6 sperm/ml) in mTBM and 50 µl of diluted sperm were

added to the fertilization drops containing oocytes for a final sperm concentration of 5×10^5 sperm/ml in 100 μ l. Gametes were co-incubated for 5 h at 38.7 °C in 6% CO₂ in humidified air.

In vitro embryo culture

After IVF, presumptive zygotes were washed three times and cultured in either NCSU-23 medium with 0.4% (w/v) crystalized BSA (MP Biomedicals) or Purdue Porcine Medium (PPM, (Herrick et al. 2006; Herrick et al. 2003; Stroble et al. 2002) culture system in 6% CO₂, 10% O₂, 85% N₂ for 6 days in standard microdrop condition (10 embryos in 50 μ l culture medium under 10 ml mineral oil in Falcon 1007 petri dishes), Nunc wells (25 embryos in 500 μ l culture medium under 500 μ l mineral oil per well), or the microfluidics system. When using PPM as culture medium, presumptive zygotes were cultured in PPM1 for the first 3 days then moved to PPM2 for an additional 3 days. On day 6 of culture, embryocleavage and blastocyst formation were determined and blastocysts were stained with 0.01 mg/ml Hoechst 33342 overnight to determine total blastocyst cell number under fluorescence at 400 $\times$ magnification.

Microfluidic well inserts

All the microfluidic well inserts are fabricated of polydimethylsiloxane (PDMS) using standard photolithography and micromoulding techniques. When culturing embryos or oocytes, inserts are attached to the bottom of a Nunc 4-well culture dish, and covered with 500 μ L maturation medium and 500 μ L mineral oil. The microfluidics well inserts for embryo culture used in experiment 1 were composed of 49 (7 $\times$ 7) microwells with a diameter of 260 μ M each. Each microwell is 1 mm apart. Adjacent microwells are either isolated or connected via a microchannel underneath the surface of the insert

(Figure 5.1a and 5.1b). The microfluidics well inserts for embryo culture used in experiment 2 are composed of 25 (5×5) microwells with a diameter of 260 μM each. Each microwell is 500 μm apart. Adjacent microwells are connected via microchannel underneath the surface of the insert (Figure 5.1c). The microfluidics well inserts for oocyte maturation used in experiment 3 are composed of 49 (7×7) microwells with a diameter of 520 μM each. Each microwell is 1 mm apart. Adjacent microwells are connected via microchannel underneath the surface of the insert.

Experimental design

Experiment 1. The effects of the microfluidic well insert system, as well as the connecting microchannel between microfluidic wells, on embryonic development were examined in two different media environments. After standard IVM/IVF, presumptive zygotes were cultured in either NCSU-23 or PPM medium in 1) standard microdrops, 2) microfluidic wells with or 3) without connecting channels (2×3 factorial design). This experiment was replicated five times. In each replicate, 20-30 embryos were used in each treatment. A total of 896 embryos were used in this experiment.

Experiment 2. The effect of the microfluidic system for embryo culture in both a rocking and motionless environment on embryonic development was examined in this experiment. After standard IVM/IVF, presumptive zygotes were cultured in a rocking or a motionless culture environment in NCSU-23 in 1) standard microdrop conditions, 2) wells, or 3) microfluidic wells with channels (3×2 factorial design). To create a rocking culture environment, the culture dishes were placed on a rocking platform inside the incubator with a speed of 10 rpm during the entire culture period (Figure 5.2). All treatment groups were replicated four times, except wells in a rocking

environment, which was replicated three times. In each replicate, 25-50 embryos were used in each treatment. A total of 802 embryos were used in this experiment.

Experiment 3. The effects of the microfluidic system for oocyte maturation in both a rocking and a motionless environment on oocyte nuclear maturation and developmental competence after standard IVF/IVC were examined in this experiment.

Oocytes were matured in either standard well dishes in groups of 50 or microfluidic wells with channels, in a rocking or motionless culture environment (2×2 factorial design). The rocking culture environment was set up as described in experiment 2.

Assessment of nuclear maturation was replicated six times. In each replicate, 10 oocytes were examined in each treatment. A total of 231 oocytes were used in this experiment to assess meiotic maturation. To assess oocyte developmental competence, all treatment groups were replicated five times. In each replicate, 30-50 oocytes were used in each treatment. A total of 740 oocytes were used in this experiment to assess oocyte developmental competence.

Statistical analysis

In experiment 1 and 3, percentages of maturation, embryonic cleavage, and blastocyst development, as well as blastocyst total cell number were analyzed using two-way ANOVA, with treatment included in the model as a fixed factor and replicate as a random factor. In experiment 2, percentages of embryonic cleavage and blastocyst development, as well as blastocyst total cell number were analyzed using GLM ANOVA, also with treatment included in the model as a fixed factor and replicate as a random factor. Percentage data (maturation success, embryonic cleavage and blastocyst development) were arcsin transformed in all analyses. Differences were

determined by Bonferroni (All-Pairwise) Multiple Comparison Test when treatment was significant. Significance was determined as $P < 0.05$. Data are reported as mean $\pm$ SEM.

Results

Experiment 1. Effects of medium and physical environment, including microchannels between microfluidic wells, during porcine IVC on embryonic development

There was no significant difference in the percentage of cleaved embryos between treatments. There was also no significant difference in blastocyst development between culture systems within the same medium. However, when embryos were cultured in microfluidic wells without connecting microchannels, blastocyst development was significantly lower when PPM was used as the culture medium compared to NCSU-23; the other 2 environments did not differ between media. When cultured in NCSU-23, blastocyst total cell number of embryos cultured in microfluidic wells without microchannels was significantly decreased compared to embryos cultured in microdrops. Blastocyst total cell number was not significantly different between culture systems when PPM medium was used (Table 5.1).

Experiment 2. Effects of physical environment in combination with a rocking or motionless environment during porcine IVC on embryonic development.

No significant difference in the percentage of embryonic cleavage or blastocyst total cell number was found between treatments. There was no significant difference in blastocyst development between different culture systems in a motionless environment. However, in a rocking environment, the percentage of blastocyst

development was significantly decreased when embryos were cultured in microdrops compared with those cultured in wells or microfluidic wells. Embryos cultured in a rocking environment had decreased blastocyst development compared to those cultured in a motionless environment regardless of the culture system employed, although only microdrop culture was significantly different between the two types of motion (Table 5.2).

Experiment 3. Effect of physical environment in combination with a rocking or motionless environment during porcine oocyte IVM on nuclear maturation and subsequent embryonic development.

There were no significant differences in the percentage of nuclear maturation, percentage of embryonic cleavage or blastocyst total cell number between treatments. When oocytes were matured in microfluidic wells, blastocyst development was significantly decreased in a rocking environment compared to a motionless environment. Blastocyst development from oocytes matured in the microfluidic wells was equal to that of oocytes matured in standard control wells in either a motionless or a rocking environment (Table 5.3).

Discussion

The microfluidic well insert culture system provides a unique physical environment for embryo culture, in which the constricted culture space more closely mimics the in vivo situation compared with standard microdrop embryo culture. Using this microfluidics system, we can successfully mature individual oocytes and culture individual embryos with success rates for subsequent blastocyst development equal to that of standard systems, in which individual culture is not possible. This is an

important advance that allows tracking, assessment the development of single oocytes and embryos, a technique particularly important when only limited numbers of oocytes or embryos are available.

We compared two types of porcine embryo culture medium in standard and microwell environments, as we hypothesize that a novel culture medium may be required for microfluidic culture due to the unique microenvironment in which the embryo develops. Embryo culture media currently in use has been developed using the relatively large volume microdrop system, which may not accurately reflect embryonic requirements in vivo and/or in small volumes. We observed a significant difference in blastocyst development between NCSU-23 and PPM only when embryos were cultured in microfluidic wells without connecting microchannels.

Possibly, PPM medium promotes increased secretion of embryotropic factors, such that without microchannels to assist in movement of these factors between embryos development is compromised. This also suggests that in NCSU-23, embryos do not need to rely on secreted factors from neighboring embryos. This interaction between the culture medium and the physical environment certainly suggests that medium for embryo culture should be developed and optimized for specific physical culture environments. Studies to optimize the culture medium for embryo development in this microfluidic system may result in enhanced embryo development greater than that currently achieved in standard conditions or microfluidic conditions with current culture media.

Culturing embryos in groups in smaller volumes of medium can improve blastocyst development and quality (Gardner et al. 1994; Lane and Gardner 1992; Paria and Dey 1990). Autocrine/paracrine factors are suggested to play a major role in this beneficial effect (Gardner et al. 1994; Gopichandran and Leese 2006). However, there is no

direct evidence that these factors actually can and do participate in communication between embryos. Interestingly, group culture benefits embryos of monovular species as well as polyovular, so this effect may or may not be physiologically relevant in vivo. In this study, we observed lower blastocyst total cell numbers, reflecting poorer quality embryos, in embryos cultured in microfluidic wells without microchannels. This may indicate that the connecting microchannels between wells increase embryo viability, although the connecting channels had no effect on blastocyst development. This result also suggests that the microfluidic wells with connecting microchannels possibly allow embryo to embryo communication to occur, increasing embryo quality. Alternatively, the connecting microchannels may facilitate medium circulation between the wells and the culture medium reservoir on the top of the wells, replenishing the medium in the wells to provide additional nutrients and remove detrimental metabolic by-products in a more appropriate manner. Dynamic fluid movement may benefit embryo development by removal of harmful by-products, replenishment of substrates, disruption of environmental gradients, and physical stimulation/activation of signaling pathways (Swain and Smith 2011). However, we demonstrated that a rocking environment with a speed of 10 rpm during culture had a detrimental effect on embryo development. It is important to note that the pattern and the intensity of the movement are critical factors that can influence fluid movement dynamics and thus embryo development. Embryos can sense shear stress, and excessive mechanical stress can damage cells (Xie et al. 2006). The rocking environment applied in this study may have been too intensive, causing shear stress or excessive displacement of beneficial embryo secreted factors. The pattern of fluid movement should also be considered in order to achieve an optimal dynamic culture environment. Matsuura et al. (year) utilized a tilting embryo culture system

with various angles at various speeds to try to mimic similar forces experienced by embryos in vivo. Using this approach, dishes tilted to 20° at 1° per second and held for 1 min yielded an estimated shear force of 0.7 dyn/mm^2 . This type of movement resulted in improved blastocyst development in the mouse (Matsuura et al. 2010). We redesigned the microfluidics inserts with microchannels in the second experiment by decreasing the number of microwells per insert as well as the distance between each microwell, so that it would be more suitable for culturing small numbers of embryos and might increase embryo to embryo communication through the connecting microchannels. The development of individual pig zygotes to the blastocyst stage was optimal when they were cultured 81-160 μm apart, suggesting that this could be the ideal distance that autocrine/paracrine factors can effectively communicate between embryos (Stokes et al. 2005). However, this experiment was done in microdrop conditions, where the autocrine/paracrine factors secreted by the embryos may be easily diluted by the large volume of surrounding culture medium. In our microfluidic culture system, embryo secreted factors may be more concentrated around each embryo within each microwell, due to the restricted, low volume culture environment. This may be a contributing factor as to why single embryos in microwells in this study produced equivalent development results to that of group culture in standard conditions. It is also possible that due to the low volumes present in the microwells and connecting microchannels, gradients of secreted factors from one embryo may be able to reach another other well in effective concentrations. A similar microwell system, called well-of-the-well (WOW), also produces results equal to that of conventional microdrop controls. WOW cultured embryos had lower amounts of apoptosis in both inner cell mass and trophectoderm, and oxygen consumption of WOW derived blastocysts was closer to physiological levels than that

of controls. Ultimately, these WOW culture embryos resulted in significantly higher pregnancy rates (Sugimura et al. 2010). Compared with the microfluidic system used in this study, the WOW system lacks connecting microchannels between wells, making it difficult to optimize the existing WOW system by taking advantage of multiple embryo culture. In addition, the WOW system lacks consistency in size of WOW. Further comparisons of implantation and pregnancy rates after embryo transfer may delineate difference in the viabilities of embryos derived from our microfluidic system versus control.

By increasing the diameter of the microwell, we successfully adapted the microfluidic device to individual oocyte IVM without compromising oocyte competence. A dynamic environment may be beneficial to oocyte IVM, as mechanical vibration of oocytes at 30 or 60 min intervals for 5 s resulted in improved cytoplasmic maturation (Mizobe et al. 2010). However, the rocking environment applied in our study was detrimental, possibly due to movement that was too intensive.

One important application for the microfluidic system is to be able to successfully track and culture one single oocyte through IVP to the blastocyst stage before transfer. In this study, we demonstrated that our current system can successfully mature oocytes and culture embryos individually without compromising quality, as measured by in vitro development. Currently, several different methods have been developed for single embryo culture, such as microdroplets, glass capillaries, WOW, adhesive matrix or filaments of a polyester mesh. Most are not widely used for clinical or experimental purposes except the WOW system (Krisher and Wheeler 2010). Our microfluidic system provides an alternative way to culture individual oocytes/embryos, in relatively small or large numbers. The fabrication technology is well developed (Walters et al. 2004; Wheeler et al. 2007; Zeringue et al. 2001). The

parameters of the microwells can easily be altered to meet the species-specific dimensions of oocytes and embryos. The inserts can be attached to the bottom of any existing culture plastic ware. Oocytes and embryos can be easily loaded into the devices manually. These microfluidic devices provide a currently available, inexpensive and easy to use method for individual oocyte and embryo culture. In conclusion, our study demonstrates that a microfluidic well system with connecting microchannels between wells can successfully mature oocytes and culture embryos individually without compromising oocyte quality or blastocyst formation. Future studies will examine varying physical parameters of the inserts to determine the optimal configuration, as well as adaptation of this microfluidic system for individual oocyte IVF, thus fully integrating each step of individual embryo IVP.

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Tables and Figures

Table 5.1. The effect of medium and physical environment, including a channel connecting the microwells during porcine IVC on embryonic development.

Medium	Environment	No. oocytes	Embryonic cleavage (%)	Blastocyst development (%)*	Blastocyst total cell number
NCSU23	Microdrop	141	60.6 ± 4.1	20.0 ± 5.9 ^{abc}	47.4 ± 3.5 ^a
	Microwell with channel	149	54.5 ± 5.6	23.1 ± 6.3 ^{ab}	39.0 ± 3.7 ^{ab}
	Microwell without channel	151	58.0 ± 2.3	25.7 ± 6.1 ^a	35.4 ± 2.5 ^b
PPM1/2	Microdrop	156	65.9 ± 2.7	14.6 ± 3.4 ^c	36.2 ± 2.7 ^{ab}
	Microwell with channel	149	56.9 ± 7.2	15.5 ± 3.7 ^{bc}	28.4 ± 1.9 ^b
	Microwell without channel	150	50.3 ± 5.2	13.3 ± 3.6 ^c	29.4 ± 2.2 ^b

^{abc} Different superscripts within a column denote a significant difference, $P < 0.05$.

Data are reported as mean ± SEM.

*Calculated as the percent of total presumptive zygotes placed into culture

Table 5.2. The effect of motion and physical environment during porcine IVC on embryonic development.

Type of motion	Environment	No. oocytes	Embryonic cleavage (%)	Blastocyst development (%) [*]	Blastocyst total cell number
Motionless	Microdrop	171	58.0 ± 5.7	29.4 ± 7.1 ^a	54.7 ± 6.0
	Well	106	51.1 ± 9.1	20.68 ± 3.3 ^{ab}	51.1 ± 5.6
	Microwell	150	61.0 ± 7.3	29.0 ± 5.0 ^a	57.8 ± 4.6
Rocking	Microdrop	150	46.0 ± 6.2	2.6 ± 1.1 ^c	40.3 ± 4.7
	Well	75	53.3 ± 8.1	12.0 ± 4.0 ^b	48.6 ± 7.2
	Microwell	150	54.0 ± 4.2	18.0 ± 2.0 ^{ab}	46.0 ± 6.1

^{abc} Different superscripts within a column denote a significant difference, $P < 0.05$.

Data are reported as mean ± SEM.

^{*}calculated as the percent of total presumptive zygotes placed into culture.

Table 5.3. The effect of motion and physical environment during oocyte IVM on nuclear maturation and embryonic development.

Type of motion	Environment	Percentage of nuclear maturation (n)	Percentage of embryonic cleavage (n)	Percentage of blastocyst development*	Blastocyst total cell number
Motionless	Well	77.0 ± 5.4 (61)	63.0 ± 4.1 (193)	33.3 ± 4.6 ^a	61.0 ± 3.5
	Microwell	75.0 ± 5.8 (56)	61.1 ± 3.9 (177)	36.3 ± 3.9 ^a	57.4 ± 3.3
Rocking	Well	78.9 ± 5.4 (57)	63.0 ± 3.5 (187)	24.6 ± 3.8 ^{ab}	50.3 ± 4.0
	Microwell	75.4 ± 5.7 (57)	50.9 ± 3.9 (183)	18.9 ± 3.2 ^b	53.1 ± 4.4

^{abc} Different superscripts within a column denote a significant difference, P < 0.05.

Data are reported as mean ± SEM.

*calculated as the percent of total presumptive zygotes placed into culture.

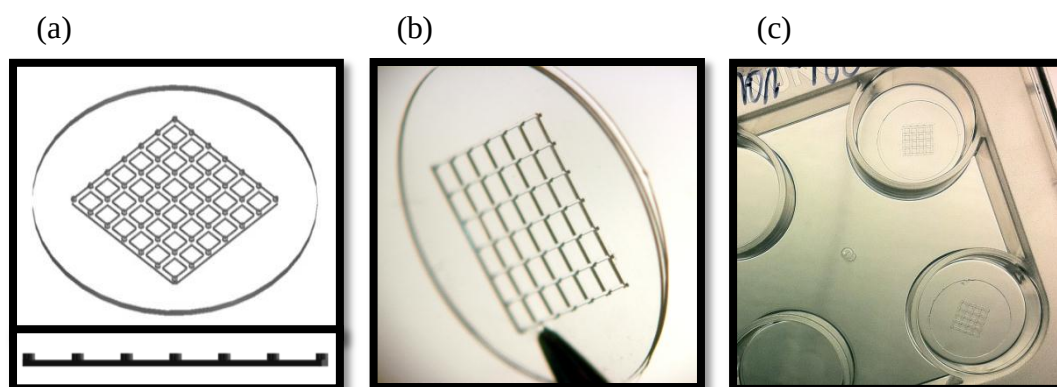


Figure 5.1. The microfluidic well insert.(a) Diagram of a microwell insert with connecting microchannel, from the top and side. Black area in sideview indicates the microwells and connecting microchannels (b) A 7×7 microfluidic well insert. (c) 5×5 maturation microfluidic well inserts attached to the bottom of Nunc 4-well plate.

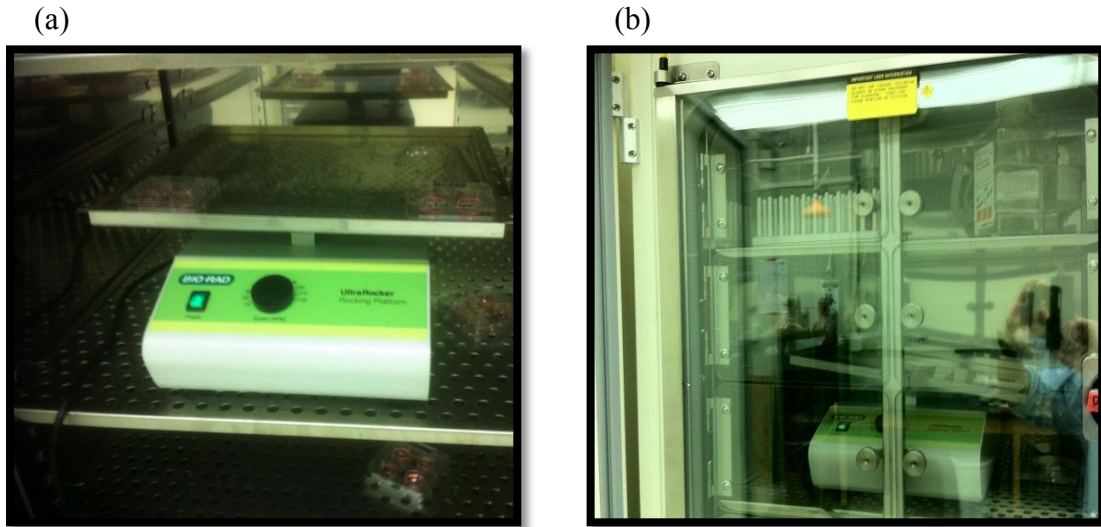


Figure 5.2. The rocking culture environment. (a) Close view of the rocking platform with culture dishes placed on top. (b) Set up of the rocking platform inside the incubator.

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-Teaching assistant for 'Animal Reproduction Experimentation'

Publications

Peer Reviewed Manuscripts

- Yuan, Y., Ida, J., Paczkowski, M., Krisher, R. Identification of developmental competence related genes in mature porcine oocytes. Mol. Reprod. Dev.(In Press)
- Paczkowski, M., Yuan Y., Fleming-Waddell, J., Bidwell C., Spurlock, D., and Krisher, R., Alterations in the transcriptome of porcine oocytes derived from prepubertal and adult females is correlated with developmental potential. J. Anim Sci (In Press.)
- Yuan, Y., Krisher, R. (2010) Effect of ammonium during in vitro maturation on oocyte nuclear maturation and subsequent embryonic development in pigs. Anim. Reprod. Sci. 117:302-307.
- Yuan, Y., Hao ZD., Liu J., Wu, Y., Yang, L., Liu, GS., Tian, J.H., Zhu, S.E., Zeng, S.M. (2008) Heat shock at the germinal vesicle breakdown stage induces apoptosis in surrounding cumulus cells and reduces maturation rates of porcine oocytes in vitro. Theriogenology. 70(2):168-78.

Abstracts

- Yuan, Y., Wheeler, M., Krisher, R. (2011) Use of a microfluidic system to successfully mature porcine oocytes individually in vitro.. Society for the Study of Reproduction annual meeting abstract (Submitted).
- Yuan, Y., Krisher, R. (2011) Effect of reactive oxygen species during in vitro maturation on porcine oocyte nuclear maturation and developmental competence. International Embryo Transfer Society annual meeting (abst.275).
- Yuan, Y., Paczkowski, M., Krisher, R. (2010) Identification of developmental competence related genes in mature porcine oocytes. Society for the Study of Reproduction annual meeting (abst. 390).
- Yuan, Y., Krisher, R. (2009) Glutaredoxin pathway genes are differentially expressed in mature porcine oocytes with varying developmental potentials. Biol. Reprod.81:370 (abst. 370).
- Yuan, Y., Krisher, R. (2008) The presence of ammonium in chemically defined maturation medium inhibits porcine oocyte nuclear maturation and subsequent embryonic development in vitro. Reprod. Fertil. Dev. 20(1):208-209 (abst. 257).
- Yuan, Y., Krisher, R. (2007) Replacing glutamine with alanyl-glutamine in chemically defined maturation medium can increase porcine oocyte nuclear maturation in vitro. Biol. Reprod. Special Issue 224 (abst. 574).

Book Chapters

- Ye Yuan and Rebecca Krisher (In press) In vitro maturation (IVM) of porcine oocytes. In: Methods of Molecular Biology in Germplasm Development: Methods and Protocols by Human Press.

Professional Organizations and Memberships (member since)

- Society for the Study of Reproduction Minority Affairs Committee (2011)
- Society for the Study of reproduction Trainee Affairs Committee (2011)
- Society for the Study of Reproduction (2007)
- International Embryo Transfer Society (2007)