INFLUENCE OF ESTRADIOL ON IN VITRO MATURATION OF PORCINE OOCYTES

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ABSTRACT

In vitro production of embryos allows efficient management of herd genetics, reduction of disease impact, and if used in combination with other reproductive technologies it could aid in preserving the threatened genetic diversity of swine. *In vitro* maturation (IVM) is identified as a deficient step in porcine *in vitro* production (IVP) of embryo systems, which decreases the overall success of IVP. There are problems encountered in each step of IVP; chromosomal abnormalities and decreased cell numbers in blastocysts during *in vitro* culturing (IVC), and low monospermic fertilization rates during *in vitro* fertilization (IVF) may be a result of insufficient IVM. As an addition to maturation media, porcine follicular fluid (pFF) can affect IVM. Estrogen can be found in high concentrations in pFF; possibly contributing to the effects seen when pFF is added to IVM. The objective of this thesis was to investigate the effects of estrogen supplementation during IVM on IVP of porcine embryos.

The first objective was to evaluate the *in vitro* maturation rates of porcine oocytes in two maturation media: protein-free and 10% pFF supplemented. Nuclear maturation of oocytes was evaluated using Lamin/Dapi staining of oocytes matured in protein-free and 10% pFF maturation media to ensure the efficiency of the protein-free media. Protein-free and 10% pFF media mature oocytes at similar rates (91% and 89% respectively).

The transcripts within the oocyte can be altered based on the *in vitro* maturation environment, so the second objective was to observe the expression of four chosen maternal effect genes: Basonuclin 1 (*BNC1*), Nucleoplasmin 2 (*NPM2*), Zygote arrest 1 (*ZAR1*), and Tripartite-motif protein 24 (*TRIM24*), using oocytes matured in 50 ng/ml, 100 ng/ml, or 1000 ng/ml of estradiol 17-β (E_2), 10% pFF, or protein-free maturation media. Expression of maternal effect genes, was shown by the ΔCt (cycle threshold) values, obtained from the difference between the Ct values of the normalizing gene (*GAPDH*) and the genes of interest evaluated through QRT-PCR. Values of ΔCt were analyzed in place of fold change to avoid data manipulation. The ΔCt expression of *TRIM24* in 0 ng/ml E_2 maturation medium and the 10% pFF maturation medium were significantly different ($p<0.05$) from the non-matured control, the other maternal determinant genes did not differ in their expression under any treatment.

We hypothesized that estradiol's effects on IVM would be evident when analyzing cleavage and blastocyst formation rates. Cleavage and blastocyst formation rates were examined following *in vitro* fertilization of oocytes matured in 100 ng/ml E_2, 10% pFF, or a protein-free maturation medium to investigate the effect of estradiol on IVP embryos. Cleavage rates for the E_2 (n= 252; 60.2%) or 10% pFF (n= 256; 55.7%) additions to the maturation media did not differ ($p>0.05$) when compared to the protein-free maturation media (n=264; 54.9%). Both 10% pFF and E_2 groups had significantly higher blastocyst formation rates ($p\leq0.05$) than the protein-free maturation media (n=264; 3.5%), although no statistical difference was observed between the blastocyst formation rates of the 10% pFF (n=256; 12.4%) and E2 (n=252; 14.6%) groups.

As a final study, the global gene expression of oocytes matured in a control protein-free media and the protein-free media supplemented with 100 ng/ml E_2 or 10% pFF was investigated using microarray analysis. Genes were not differentially expressed among the matured groups with the outlined threshold values of $-2 \geq \log2(\text{fold change}) \geq 2$, and adjusted p-value ≤0.05. A total of 16 differentially expressed genes between the non-matured and all matured groups exceeded this threshold. Of these genes, 6 are novel transcribed regions with evidence of being an embryonic EST, and 1 is a novel protein-coding gene. The other genes are FBJ murine osteosarcoma viral oncogene homolog (*FOS*), Vimentin (*VIM*), Capthesin C (*CTSC*), Selenium binding protein 1 (*SELENBP1*), Poly(A) binding protein cytoplasmic 1 (*PABPC1*), Tissue factor pathway inhibitor 2 (*TFPI2*), Cysteine-rich, angiogenic inducer 61 (*CYR61*), Acyl-CoA synthetase long-chain family member 6 (*ACSL6*), and Phospholipase A2 group VII (*PLA2G7*).

In conclusion, successful nuclear maturation of oocytes derived of prepubertal gilt abattoir derived ovaries can be achieved without pFF or hormone supplementation. The expression of maternal determinant genes is not affected in a dose dependant manner, and removal of E_2 or supplementation of pFF during maturation may alter the expression of *TRIM24* from the non-matured control; where no other maternal effect gene changes through maturation. Estradiol has a similar effect as pFF during *in vitro* maturation of porcine oocytes as seen by cleavage and blastocyst formation rates. And media does not affect the global gene expression of porcine oocytes, though there is a temporal control of gene expression through maturation.

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LIST OF ABBREVIATIONS

ΔCt: Delta cycle threshold

µg: Microgram

µl: Microliter

ACSL6: Acyl-CoA synthetase long-chain family member 6

aRNA: Amplified ribonucleic acid

ATP: Adenosine triphosphate

BAC: Bacterial artificial chromosome

BCB: Brilliant cresyl blue

BNC1: Basonuclin 1

bp: Base pairs

BSA: Bovine Serum Albumin

Ca^{2+}: Calcium

CDCA71: Cell division cycle associated 7 like

cDNA: Complementary Deoxyribonucleic acid

COC: Cumulus oocyte complex

COW: Climbing over the wall

Ct: Cycle threshold

CTSC: Capthesin C

Cy3: Cyanine 3

Cy5: Cyanine 5

CYP17: Cytochrome P450 17α-hydroxylase 17,20 lyase

CYR61: Cysteine-rich, angiogenic inducer 61

DNA: Deoxyribonucleic acid

DPBS: Dulbecco’s phosphate buffered saline

E_2: Estradiol 17-β

EGF: Epidermal growth factor

EPAB: Embryonic poly(A) binding protein

ER: Endoplasmic reticulum

EST: Expressed sequence tags

FF: Follicular fluid

FF-MAS: Follicular fluid meiosis-activating sterol

FOS: FBJ murine osteosarcoma viral oncogene homolog

FSH: Follicle stimulating hormone

g: Gram

G6PD: Glucose-6-phosphate dehydrogenase

GAPDH: Gyceraldehyde-3-phosphate dehydrogenase

GDF9: Growth differentiation factor 9

GPMNB*:* Glycoprotein transmembrane nmb

GSH: Glutathione

GV: Germinal Vesicle

GVBD: Germinal Vesicle Breakdown

h: Hour

i.u.:International unit

IP3: Inositol triphophate

IP3R: Inositol triphophate receptor

IVC: *In vitro* culturing

IVF: *In vitro* fertilization

IVFm-199: *In vitro* fertilization medium 199

IVM: *In vitro* maturation

IVP: *In vitro* production

LH: Luteinizing hormone

M: Molar

mg: Milligram

MI: Metaphase I

MII: Metaphase II

min: Minute

MISS: Microscale integrated sperm sorter

mL: Millilitre

MPN: Male pronucleus

mRNA: Messenger ribonucleic acid

mTBM: Modified tris-buffered medium

mWM: Modified Whitten's medium

mZP3: Mouse zona protein 3 gene

n: Number

NCSU-23: North Carolina State University medium 23

NCSU-37: North Carolina State University medium 37

ng: Nanogram

NPM2: Nucleplasmin 2

PABPC1: Poly(A) binding protein cytoplasmic 1

PBS: Phosphate buffered saline

PAF: Platelet activating factor

PCR: polymerase chain reaction

pFF: Porcine follicular fluid

pg: Picograms

PGMtac4: Porcine gamete medium tac 4

PKC: Protein kinase C

PLA2G7: Phospholipase A2 group VII

POM: Porcine oocyte maturation medium

PZM-5: Porcine zygote medium 5

Q-PCR: Quantitative, real-time polymerase chain reaction

RNA: Ribonucleic acid

rRNA: Ribosomal ribonucleic acid

SELENBP1: Selenium binding protein 1

SEM: Standard error of the mean

sp56: Sperm protein 56

Ta: Annealing temperature

TALP: Tyrode's medium

TCM-199: Tissue culture medium-199

TFPI2: Tissue factor pathway inhibitor 2

TRIM24: Tripartite motif containing 24

VIM: Vimentin

WOW: Well of the well

ZAR1: Zygote Arrest 1

ZP3: Zona protein 3

CHAPTER 1: GENERAL INTRODUCTION

Swine constitutes 40% of the worlds meat, making it the most important meat source globally [1]. However there is a pending threat to the genetic diversity of this important food source, with 151 breeds of pigs already classed as extinct and 132 more breeds at risk [2]. In 2007 the Global Plan of Action for Animal Genetic Resources was adopted by all countries attending the International Conference on Animal Genetic Resources for Food and Agriculture; Canada was one on these countries [3]. As a result producers and researchers now face the challenge of reducing the loss as well as preserving genetic diversity for all livestock, including swine. Advances in reproductive technologies provide researchers and producers with the means of accomplishing the set task. *In vitro* production of embryos is one available technique which allows efficient management of herd genetics, reduction of disease impact, and when used in conjunction with other reproductive technologies will aid in preserving swine genetic diversity.

In vitro production of embryos (IVP) is the creation of embryos outside of the female tract. It involves three separate and interdependent steps: *In vitro* maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* culturing (IVC) of the embryos. Unfortunately IVP in swine is not as successful as in other livestock species [4]. There are problems encountered in each step of IVP: chromosomal abnormalities and decreased cell numbers in blastocysts during IVC [5], low monospermic fertilization rates in IVF [6], and incomplete maturation from IVM [7,8]. Many of the problems in porcine IVP are associated with insufficient IVM; as IVM is the first step in IVP it is capable of influencing the success of all subsequent steps.

Oocyte competence is described as the quality of the oocyte and its resulting potential to develop into an embryo and the subsequent live offspring. The competence of an oocyte is influenced by many different factors during IVM; including the source of the oocyte [9], the environment in which it is matured [10,11], and the medium it is matured in [6,12-15]. Different supplements within the IVM medium have proved effective at improving IVM rates and the subsequent IVP steps [16-20]. Porcine FF is one of the most common supplements to maturation media [12-14], factors with pFF improve nuclear maturation, cytoplasmic maturation, fertilization rates and embryo development [21]. There are an abundance of metabolites,

cytokines, growth factors and steroid hormones within pFF [22]. There is a wide range of steroid hormone concentrations in porcine follicles [23], and estradiol can be present in low and high concentrations depending on the age of the donor animal, the size of follicle and the stage of the estrus cycle [23,24]. Supplementation of maturation medium with estrogen has proven controversial, there are reports of improved maturation with E_2 introduced in the first half of IVM in NCSU-23 maturation medium which contains pFF [25], and some of decreased maturation with large amounts of E_2 introduced into modified Whitten's Medium which also contains pFF [26]. The success of IVM can determine the success of the IVP system, and the effects of E_2 during IVM should be defined.

When comparing bovine *in vitro* and *in vivo* matured oocytes, differentially expressed genes were observed [27,28]. As the *in vitro* environment is known to affect the gene expression of oocytes, the maturation environment may also influence the expression of gene transcripts within the oocyte which have been correlated with oocyte competence [29,30]. Prior to the resumption of meiosis during IVM, oocytes undergo a growth phase. During this time they accumulate important maternal RNA transcripts; when the oocyte resumes meiosis during IVM, transcription is not observed and the stored RNA and transcripts are used for maturation, fertilization and early embryogenesis [31]. This maternally derived RNA is the only known regulator of early embryogenesis before the embryonic genome is activated [32]. Maternal effect genes are responsible for storing some of transcripts and defects in these genes can lead to failure of embryogenesis [33]. The alteration of gene transcripts during IVM may then affect the quality of oocytes and the resulting success of IVP, as the stored transcripts are the only source of proteins during this phase.

In vitro production of embryos is an appealing biotechnology for the conservation of swine genetic diversity. *In vitro* maturation is seen to influence the success of IVP, and the supplements within IVM medium contribute to this effect. Porcine follicular fluid (pFF) is commonly used as a supplement during IVM, and as a result high amounts of estrogen can be introduced into maturation medium. Estrogen has controversial effects on IVM success in pigs, and may be a contributor to the difficulties in porcine IVP. Unsuccessful IVP can sometimes be attributed to alterations in certain genes within the oocyte. As maturation media is seen to affect

some gene transcripts during IVM, estrogen may have further effects on gene transcripts within the oocyte which determines the oocytes potential to develop into an embryo.

The objectives of this study were to examine the effects of estradiol on porcine IVM through maturation rates, cleavage and blastocyst formation rates, the changes in maternal effect gene expression, and the global gene expression using microarray. *In vitro* production of embryos may be improved with further understanding of estrogen's influence during IVM; thus making IVP a feasible biotechnology to aid in the conservation of swine genetic diversity.

CHAPTER 2

I. LITERATURE REVIEW

2.1. *Purpose of In Vitro Production of Embryos*

The advances in reproductive technologies have given many options to producers and researchers. The use of *in vitro* technologies shows much promise as it can be used to manage herd genetics more selectively, reduce the impact of disease on both herd and breed genetics, and conserve genetic diversity when combined with other reproductive technologies. The commercialization of *in vitro* production (IVP) of embryos has risen in other livestock species such as cattle; research is improving IVP in swine to advance in that same direction.

Reproductive technologies, like IVP of pig embryos, can help producers to efficiently manage their herds and allow more control of the animals' genetics. Certain traits such as the feed efficiency, growth rate, and certain carcass characteristics allow a producer to reduce the cost of production [34]. Using IVP embryos could improve the accuracy and intensity of selection for these traits by using multiple embryos from a select donor in various recipients. As the female role in reproduction is a limiting factor due to the length of pregnancy, the production and use of more females through IVP and associated technologies such as embryo sexing, would increase overall swine production.

With the constant threat of disease, IVP embryos reduce the risk of contamination from another herd through the processes involved in breeding. If there is an outbreak of disease, the genetic material of the herd can be preserved through the creation of IVP embryos. Other technologies, such as cryopreservation of IVP embryos, permit the genetic material to be stored for long-term use; this would protect genetic diversity. Piglets have been produced using sex-sorted semen and IVP, with a similar rate of success (based on litter size and cleavage rate of embryos) as with using unsorted semen [35]. The use of IVP in combination with other

technologies such as cryopreservation of embryos and sex-sorted sperm for sex specific embryos increases the possible uses of this technology. IVP using frozen sex-sorted boar semen would allow more efficient production of sex specific embryos [36] which are favourable as it will routinely produce more females.

Cryopreservation of IVP embryos has been achieved, however the efficiency of the present protocols must be improved before it can become a tool for producers [37-39]. Embryos produced *in vitro* and then frozen would be more cost effective when transporting genetic material, both nationally and internationally [40]. The costs introduced through transportation and maintenance of a whole animal, the increased risk of disease, and the problems associated with acclimatization of the animal would be reduced through the use of IVP embryos. By refining the processes involved in IVP these techniques may become effective reproductive tools for the pork production industry.

The use of IVP embryos has risen dramatically in the cattle industry; however this technology remains to be a small percentage of the hundreds of millions of cows which can be used [41]. It has been reported that 265,000 IVP embryos were transferred worldwide in 2005 [42]. In 2007, 245,257 IVP bovine embryos were reported [43]. There were few reports on the number of porcine IVP embryos, and 64,147 IVP embryos of the reported 68,156 transferable embryos collected (both *in vivo* and *in vitro*) were produced in Korea [42]. There is speculation that these numbers are higher but are not reported, and the limited use of the IVP technologies is not due to the efficiency of the technologies itself [41]. In the swine industry, embryo transfer is actively being introduced with 30,000 embryos transferred in 2005 [42]. Many of these were experimental, but with improvements in IVP this technique could benefit the swine industry as it has the cattle industry. Currently porcine IVP of embryos remains inferior to other livestock species [4]; improvement is needed to increase the efficiency of this technology.

2.2.*Conservation of Swine Genetics*

Pork constitutes the world's most important source of meat, as it makes up 40% of the

worlds meat. Beef, although popular in North America, only constitutes 29% of the global meat used [1]. It would seem that swine would not be considered an at risk livestock species due to its popularity. It is not just the pure numbers of pigs which assign the risk status, but also the number within the breeds. There are an estimated 649 pig breeds being raised globally [2]. Also taken into account, 47 breeds are categorized as critical, another 85 have a risk status of endangered, and a total of 151 have already been classed as extinct [2]. Many of these pig breeds are found in Asia, and the importation of more standard breeds such as Duroc, Landrace, and Large White are replacing the indigenous breeds. The loss of the indigenous breeds is associated with the loss of genetic diversity of the species, and this makes swine a species in need of conservation.

The once diverse genetics of swine are being lost mainly due to standardized, economical, and efficient pig production systems. Globally farmers are choosing to raise breeds which will meet the demands of these production system [1]. Pigs do not produce any secondary products such as wool, or milk, and they do not provide power or transport. The only use for swine is for meat, thus selection of pigs is focused on that aspect; breeds which do not provide the most efficient production characteristics are disregarded. The feed efficiency, growth rate, and carcass characteristics are current traits which producers select for as they provide an economic advantage, and other important characteristics can be neglected [34]. As well, some breeds posses more heritability of selected traits. Landrace and Large White pigs both posses heritability of back fat and muscle depth carcass characteristics [44]. While those traits are selected for, other traits such as disease resistance may be overlooked even though these traits are also heritable [45].

As traits are selected the genetic diversity decreases within and between breeds with cross breeding. The level of inbreeding is rising with this artificial selection, and thus the potential for changing or losing allele frequencies is a concern for conservation. The population and incidence of inbreeding per generation is of concern for both Berkshire and Landrace breeds. While Duroc, Hampshire and Yorkshire are currently at an acceptable level, the rate of inbreeding is rising [46]. A report in Canada showed that the rate of inbreeding was not yet as high as in the United States [47]. Even with an underestimated rate of inbreeding, the trend should still be of concern to producers [47].

Producers face a challenge in reducing the loss of genetic diversity while maintaining performance. In 2007 the International Technical Conference on Animal Genetic Resources for Food and Agriculture was held in Interlaken, Switzerland. The countries which were present at the conference, including Canada, adopted the Global Plan of Action for Animal Genetic Resources. This plan promotes the responsible management and conservation of vital genetic resources [3]. As a result researchers face the challenge of preserving the genetic diversity which currently remains. There is a role for reproductive technologies for both producers and researchers to solve the current problems as well as prevent the loss of any more breeds.

2.3. *In Vitro Production of Embryos*

Production of *in vitro* embryos involves three subsequent steps: *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culturing (IVC). These *in vitro* processes are meant to mimic the processes *in vivo*, which result in an embryo. These steps will be discussed in more detail in subsequent sections of this thesis.

2.3.1. IVM

2.3.1.1.Nuclear Maturation

Mammalian oocytes arise from the female primordial germ cells during foetal development. In porcine foetal ovaries, all the gametes are contained within nests before developing into the earliest form of the follicle, the primordial follicle [48]. It has been observed that 60 days post coitus only egg nests are seen within the fetal ovary, but by day 70 the number of primordial follicles equals the number of egg nests [48]. The number of egg nests declines as fetal age increases until none exist, and only follicles can be observed. The primordial follicle

contains a quiescent primary oocyte, which is surrounded by a single layer of flattened somatic cells. These somatic cells are presumed to be the progenitors of the granulosa cells in later follicles [49]. It should be noted that gestation length is only 115 days in swine; therefore the total amount oocytes for its lifespan have developed into primordial follicles before it is born.

The primary germ cells proliferate via mitosis and enter into the first meiotic division, prophase I. Germ cells such as oocytes undergo meiosis to reduce their homologous chromosome pairs into haploid cells. For an oocyte to join with haploid sperm to create a diploid embryo the oocyte must also be haploid. The chromosomes condense and recombine to the diplotene stage of meiosis I where they then disperse and the oocyte, now called primary oocyte is arrested at this stage, surrounded by the somatic precursors to granulosa cells [50]. At the time that oocytes arrest in diplotene stage, folliculogenesis begins as the somatic cells surround the oocyte to form a basal lamina and thus form a follicle [51]. By day 70 post coitum primary follicles can be found within the ovary [48]. At birth more secondary follicles develop and increase to 30% of the present follicles at 90 days post partum, and tertiary follicles appear after day 90 [48]. Very few of the original primordial follicles ever develop into tertiary follicles which can ovulate and provide a competent oocyte [49].

The primary oocytes remain in prophase I arrest until puberty, at which point the oocytes enter a growth phase. As the growth phase nears completion, a variety of events occur within the oocyte; the transcriptome becomes quiescent, the nucleolus restructures, and there is a reorganization of cytoplasmic organelles [52]. The now fully grown oocytes are found within pre-ovulatory tertiary follicles, and in response to luteinizing hormone (LH) the dominant antral follicle continues to grow and the oocytes resume meiosis [53,54]. Oocyte arrested at prophase I can be identified by the intact nuclear envelope, the germinal vesicle (GV). The germinal vesicle breakdown (GVBD) is a clear sign of the resumption of meiosis. After GVBD, a metaphase I (MI) spindle forms and once all chromosome pairs have established stable microtubule-kinetochore interactions, anaphase I of meiosis I begins. Continuing through meiosis, telophase I results in two diploid daughter cells, but one of the daughter cells does not move on through meiosis and instead forms the first polar body [55]. Meiosis resumes once again when the sperm enters the ooplasm, after another cell cycle arrest during metaphase II (MII). The oocyte's haploid chromosomes form female pronuclei as the sperm head decondenses to form male

pronuclei. The resulting daughter cell is released to form the second polar body and meiosis is complete.

Oocyte maturation is completed in phases; first acquiring the capacity to fuse with spermatozoon, then cytoplasmic maturation, which is followed by completing nuclear maturation [56]. Nuclear maturation requires the ovum to reach MII of meiosis. Nuclear maturation can take place in culture without the ovum ever achieving full competence [57]. It is a separate process from cytoplasmic maturation; thus as an oocyte which may be deemed competent based on nuclear maturation may not have the ability to be fertilized or have male pronuclear formation [58].

2.3.1.2.Cytoplasmic Maturation

Cytoplasmic maturation entails reorganization of the oocyte's internal structure, and accumulation of mRNA and proteins [59,60]. Measures of cytoplasmic maturation are intracellular glutathione (GSH) content, cumulus expansion, ability to be fertilized and the subsequent cleavage and blastocysts formation rates [4,61,62]. Oocytes which have attained cytoplasmic maturation, and have gained the ability to resume meiosis [63], can then be successfully fertilized.

Mitochondria are important for oocyte metabolism as they are the cell powerhouses, and the number of mitochondria within the cell relate to the oocytes functional competence. Reduced numbers of mitochondria lead to abnormal distribution of the organelles during early embryogenesis [64]. Mitochondria are moved from their peripheral location in GV oocytes to the inner ooplasm in an even spacial distribution throughout maturation (*in vivo* and *in vitro*) [65,66]. During the maturation period of mammalian oocytes, mitochondria synthesize the necessary adenosine triphosphate (ATP) to synthesize proteins which will support the maturation processes and further embryo development [67]. The reorganization of mitochondria occurs most efficiently in oocytes with high developmental competence [68]. In most mammals, the ATP levels differ between morphologically good and poor oocytes as does the mitochondrial

relocation. Lack of evenly distributed mitochondria and low ATP content within oocyte can implicates poor developmental capability of the oocyte [67]. However, no significant differences in ATP content were found between oocytes matured *in vivo* or *in vitro* in the pig [69]. As the *in vivo* matured oocyte has higher competence than the *in vitro* matured counterpart in the pig, the reorganization of the mitochondria may be adequate in the *in vitro* derived oocyte.

The ribosome is another cytoplasmic organelle that is reorganized throughout maturation. The oocyte of a primordial follicle is transcriptionally inactive and the nucleolus is composed exclusively of the granular portion, thus signalling an absence of ribosome activity [70]. As the oocyte resumes meiosis the activity of protein synthesis gradually increases, about three fold from the GVBD stage to the MI stage. But as the oocytes reaches the MII arrest the mRNA translation is once again at basal levels. The lack of rRNA transcription or ribosome production is caused by the absence of a functioning nucleolus [71].

The endoplasmic reticulum's (ER) functions include lipid metabolism, protein folding and degradation, compartmentalization of the nucleus, regulation of the Ca^{2+} ion gradient, and membrane synthesis [72]. Oocyte activation during fertilization requires that Ca^{2+} be released via inositol trisphosphate (IP3) and its receptor, IP3R [73]. It is critical during maturation for the ER to undergo biochemical and structural changes for proper functioning of intracellular calcium regulation. The ER is observed to be uniformly distributed within the ooplasm of GV stage oocytes during an *in vivo* analysis of of mouse oocytes. The ER is found in cortical regions and accumulates in 1–2 μm wide clusters throughout the cytoplasm (except in the vicinity of the meiotic apparatus) the oocyte matures to the MII stage [74].

Cortical granules, known for their role in fertilization, are derived from the Golgi body [75]. As oocytes reach the MII stage of maturation, the granules are found throughout the ooplasm, close to the plasma membrane. They are strategically located to respond to spermatozoon entry and egg activation [57].

During the GV stage of maturation the organelles are spatially rearranged and organized by the cytoskeleton. The cytoskeleton forms a network within the ooplasm and nucleus, it allows organelles to move and reorganize within the membranes [76]. Microtubules are not detected in porcine GV oocytes. As maturation progresses to the stage of GVBD, where the nuclear

envelope begins to disintegrate, small asters of microtubules are observed in association with the nuclear material. The microtubules seen in conjunction with DNA have a continued presence throughout meiosis until maturation is complete at the MII stage. A cytoplasmic mesh of microtubules appears alongside the DNA associated network during IVM. Beginning in the cortex and branching into the inner cytoplasm, this network was observed at 24-28 h of IVM, but then fades. The final MII stage oocyte has no observable microtubules within the cytoplasm [59]. Oocyte graded into high and low competence groups both displayed the DNA associated microtubule networks which account for the acquisition of nuclear competence, but not necessarily cytoplasmic maturation [59].

As previously mentioned, cytoplasmic maturation entails reorganization of the oocyte internal structure, as well as accumulation of mRNA and proteins [59,60]. Expression of mRNA by the oocyte's chromosomes is dedicated to transcription, storage and processing for further translation into proteins. The proteins expressed from this mRNA do play a significant role in maturation as well as the subsequent events of fertilization and embryogenesis. Therefore it extremely important for these proteins to be stored until they are utilized [77]. In general, mRNA is protected from nucleolytic degradation through polyadenylation and remains stored until the signal for translation is generated by maturation and embryogenesis [78,79].

2.3.1.3. Protocols for in vitro maturation

There are many steps and variations in an IVM protocol. Oocytes are selected based on different criteria and are introduced into chosen IVM media that has been supplemented with a variety of compounds including gonadotropins. Then the oocytes are incubated in a suitable environment for 40-44 h before being evaluated for IVF. Each variation is meant to simulate *in vivo* events to improve IVM success, but as a result there are many different protocols currently being used.

Oocytes for IVM are generally obtained from slaughterhouse derived ovaries, the size of follicles from which the oocytes are aspirated from can influence the success of maturation.

Oocytes from larger follicles (5 mm in diameter or more) are more likely to develop into embryos following fertilization than oocytes aspirated from small follicles (less than 3 mm). The exact diameter of follicle is hard to control for when aspirating follicles from many ovaries in a short amount of time, thus for consistency most protocols recommend using oocytes from 3 to 6 mm in diameter [9].

There are limited tests that can be performed on oocytes while maintaining their ability to develop into embryos; the morphological characteristics are most commonly evaluated as a measure of the oocytes potential competence. The appearance of the cytoplasm is one morphological criterion. It has been observed that cytoplasm which is heterogenous (granular) in appearance is more likely to develop into blastocysts following IVF. These results were obtained through subjecting both *in vivo* and *in vitro* matured oocytes with heterogenous and homogenous cytoplasm to subzonal sperm injections [7].

The cumulus cell layers surrounding the oocyte are another suggested quantification for meiotic competence of the oocyte. Many protocols suggest that there be at least 3 layers of cumulus cells, however the presence of corona radiate cells are sufficient for the oocyte to complete maturation [80]. The co-culture of denuded oocytes (oocytes with no surrounding cumulus cells) with cumulus cells has proved effective as well. There are secretory factors originating from the cumulus cells that will act upon the oocyte, enabling successful maturation [80]. The expansion of the cumulus cells as well as the cumulus cell mass is an observable trait thought to signify cytoplasmic maturation. A bigger cumulus cell mass enhances the maturation success, as does a larger cumulus expansion [80].

An alternative to morphological criteria (which can be observer biased) is exposure of oocytes to brilliant cresyl blue (BCB). This staining method is a more quantitative measure of the oocytes competence. The BCB test assesses the intracellular activity of the glucose-6-phospahte dehydrogenase (G6PD), which is present in growing oocytes. As the oocyte finishes the growth phase and maturation there is decreased G6PD activity. BCB is a compound which gives a blue color to the oocytes which have matured, thus have less G6PD activity [81].

It is evident that porcine oocytes depend on follicular cells to generate signals which coordinate the growth and maturation of the oocyte [82]. And the common IVM protocols

attempt to mimic the signals that the oocyte receives *in vivo*. Intracellular GSH levels reflect the competence of oocytes, for example a high amount of GSH indicates successful maturation. GSH synthesis can be increased through cysteine supplementation of the IVM medium. The introduction of low molecular weight thiol compounds, such as β-mercaptoethanol, into the maturation medium has the same effect, increasing the blastocyst formation rate of fertilized oocytes as well as increasing the cell number within the blastocysts [16]. The supplementation of cysteamine has similar positive effects, increasing pronuclear formation and developmental competence of produced embryos [17]. Epidermal growth factor (EGF) is another addition to maturation media, whose effects are partially due to EGFs stimulation of GSH synthesis within the oocyte. EGF improves the developmental competence of blastocysts, which has been confirmed through subsequent embryo transfer and birth of piglets [18].

The introduction of retinoic acid into IVM medium increases the blastocyst formation rate, another measure of the oocytes completion of cytoplasmic maturation. The inclusion of 5 nm of retinoic acid is sufficient to improve the yield of porcine blastocyst production [19]. There are many amino acids introduced into maturation media with serums and FF. Glutamine, which is abundant in pFF, increased the rate of monospermic fertilization when included in IVM medium. The male pronuclear formation rate was also significantly increased, showing the effect of this amino acid on cytoplasmic maturation [20]. Furthermore, treatment of oocytes with estradiol has been shown to improve the rate of monospermic fertilization, blastocyst formations and blastomeres viability in *in vitro* produced embryos [25].

Most of the aforementioned supplements are included in the common maturation medias (Table 2.1). However the common media also generally add pFF as well. Many studies have proven that pFF contains substances which improve the rate of cumulus expansion, nuclear maturation, and successful fertilization and embryo development [21]. Even with the positive effects of pFF, chemically defined media should be used to standardize the IVM protocols between laboratories [6]. There has been successful piglet production from *in vitro* matured oocytes using a chemically defined system, void of any animal derived proteins [15]. A gonadotropin-free chemically defined medium has been successfully used to matured porcine oocytes [83]. Meiotic resumption and progression to MII stage were accomplished when porcine

cumulus oocyte complexes were exposed to dibutyryl cAMP and EGF-family peptides within the first half of IVM [83].

Gonadotropins are still regularly used in IVM protocols, either in the first half of IVM (the first 20 h) or throughout. Follicle stimulating hormone (FSH) and LH have beneficial effects for timing the resumption of meiosis. When treating sow cumulus oocyte complexes with FSH, either for the first 20 h or throughout IVM, there was enhanced cleavage of embryos following IVF [84]. Yet more positive effects were seen if FSH was introduced for the first 20 h of IVM, as there was a higher yield of blastocysts [84]. The effects of FSH on nuclear maturation were positive with supplementation for the entire IVM, with an enhanced nuclear maturation as well as cumulus cell expansion [84]. Meiotic resumption via gonadotropins occurs through their actions on the surrounding theca and granulosa cell layers *in vivo,* as oocytes lack gonadotropin receptors [85]. The first result of FSH supplementation is to slow the progression of the oocyte to GVBD, however after 20 h the retardation has decreased, and FSH supplementation accelerates meiosis so all oocytes reach MII at the same time [84]. LH is often supplemented along with FSH to resume oocyte meiosis [86].

The environment in which oocytes are matured also influence the success of IVM. The maturation and fertilization of porcine oocytes under low oxygen tension (5%) does not differ from those oocytes matured under high oxygen tension (20%) [10]. However, oocytes which have matured under high oxygen tension have better blastocyst formation rates [10]. In opposition, another report observed no effect on blastocyst formation, yet blastocyst cell number was increased in a low oxygen tension (5%) [11]. While the evidence is controversial, the environment in which the oocytes are matured does play a role in the success of IVM.

IVM protocols consist of oocyte selection, maturation in media with or without supplements and gonadotropins, and incubation. While these steps are common to all protocols, there are many variations which results in many protocols. A defined protocol and media would decrease the variability between labs; this is appealing as it would make IVM more efficient for commercialization and conservation strategies.

Table 2.1: Components in Common Maturation Media

Component (mM)	**TCM-199** [12]	**NCSU-23** [13]	**mWM** [14]	**NCSU-37** [13,87]	**POM** [15]
NaCl	116.36	108.73	68.49	108.73	108.88
KCl	5.36	4.78	4.78	4.78	10.00
$CaCl_2$	1.80	1.70			
KH_2PO_4		1.19	1.19	1.19	0.35
$MgSO_4 \cdot 7H_2O$	0.81	1.19	1.19	1.19	0.40
$NaHCO_3$	26.19	25.07	25.07	25.07	25.00
Glucose	5.55	5.55	5.56	5.55	4.00
Sodium lactate			25.20		
Sodium pyruvate			0.33		0.20
Calcium lactate			1.71		2.00
L-Glutamine	0.68	1.0			
Taurine		7.0			
Hypotaurine		5.0			5.00
Penicilin	100 (i.u./mL)	100 (i.u./mL)	100 (i.u./mL)	100 (i.u./mL)	
Steptomycin	50 (i.u./mL)	30 (i.u./mL)	50 (i.u./mL)	50 (i.u./mL)	
pFF	10%	10%	10%	10%	
L-Cysteine	0.57	0.57	0.57	0.55	.60
Calcium (lactate)$\cdot 5H_2O$					2.00
BME amino acids					2%
MEM non-essential amino acids					1%
Gentamicin					0.01
$CaCl_2 \cdot 2H_2O$				1.7	
D-sorbitol				12.00	
β-mercaptoethanol				50(μM)	
Insulin				5(mg/mL)	
Dibutyryl cAMP				1	

2.3.1.4.Successful IVM

Out of all the oocytes collected, few will generally attain competence. In porcine IVM, the nuclear maturation is quite successful, maturation rates of 70-95% have been seen based solely on nuclear maturation [88]. The ability of an oocyte to be fertilized shows minimum cytoplasmic maturation to allow embryonic development. Insufficient cytoplasmic maturation may decrease the competence of an *in vitro* matured oocyte., this can occur in 40% to 70% of oocytes matured [7,8]. The poor results in porcine IVM are then attributed to cytoplasmic maturation rather than nuclear maturation. A maturation rate of 70% has been observed *in vitro*; based on cumulus expansion (indicative of cytoplasmic maturation) and GVBD (evidence of nuclear maturation) [89]. In comparison to IVM rates in cattle [42] and sheep [90] where MII rates are 70-95% porcine IVM remains relatively inefficient.

2.3.1.5.Problems with IVM

Incomplete maturation of oocytes can be associated with lower cell number in blastocysts, decreased blastocysts formation, and an overall decrease in developmental potential [62]. There are certain factors within IVM that may contribute to these problems; the source of oocytes, asynchronous nuclear and cytoplasmic maturation as well as incomplete cytoplasmic maturation.

Oocytes for IVM are recovered from the ovaries of slaughtered animals, and follicles of varying sizes are aspirated to obtain oocytes. The resulting pool of oocytes is possibly from animals at varying stages of the estrous cycle. The process of collecting oocytes from unovulated follicles and attempting to mature them under *in vitro* conditions may disrupt the balance of cytoplasmic and nuclear maturation rates within the cell [91]. The different conditions within a pre-pubertal and post-pubertal ovary had a great impact on the meiotic competence of *in vitro* derived oocytes [92]. During IVM there currently is a disturbance of flow in both nuclear and cytoplasmic maturation of the oocyte [93]. The nuclear maturation and cytoplasmic maturation

must be synchronized for an oocyte to be fully competent. However, the *in vitro* matured oocyte is less synchronous than the *in vivo* counterpart, resulting in lower quality blastocysts [7]. The rate of nuclear maturation *in vivo* is equal to oocytes matured *in vitro* [94]. Therefore the asynchrony within the maturation processes, resulting in lower quality blastocysts, may be due to in adequate cytoplasmic maturation.

If the oocyte does reach MII, the possibility of being fertilized and developing into an embryo is low, incompletion of cytoplasmic maturation maybe the cause. Inadequate conditions of IVM can lead to incomplete translocation of the mitochondria to the inner cytoplasm. A difference in mitochondria movement is seen between *in vitro* and *in vivo* derived oocytes [66]. The supplements within culture media are generally of animal origin, such as serum and FF. These supplements have the possibility of introducing unknown factors into the culture media, which can decrease the repeatability of protocol.

2.3.2. IVF

2.3.2.1.Fertilization

A volume of 160-600 mL [95] of spermatozoa in seminal plasma is ejaculated into the cervix of the female to begin *in vivo* fertilization. Seminal plasma is a fluid composed of secretions from the tails of the epididymal ducts and the accessory sex glands. The ejaculation is released in fractions, pre-sperm, sperm-rich, and post-sperm-rich. The pre-sperm primarily contains the secretions of the urethral, bubourethral, and prostate glands. The sperm dominates the sperm-rich fraction. The epididymal secretions, which accompany the sperm, are diluted with more fluid from the seminal vesicle and prostate glands. The post-sperm-rich fraction does contain a few spermatozoa, but the majority of this fraction is composed of secretions from the seminal vesicles, bubourethral, and prostate glands. The bulbourethral glands secrete a floccula which coagulates the seminal plasma, and is commonly known as the “gel”. This fraction serves *in vivo* to retain all ejaculate within the uterus and prevent retrograde flow through the cervix

[96]. The order in which these fractions are released varies between boars, and the fractions make occur more than once.

After the spermatozoa have been deposited within the cervix, they are transported into the uterine lumen where the majority of the population are removed [97]. Hunter et al. reported that viable boar spermatozoa are transported to the oviducts of gilts during the pre-ovulatory period following displays of estrus. The spermatozoa which are capable of fertilization are accumulated in the caudal oviductal isthmus for 40-42 h prior to ovulation [98]. Spermatozoa have an interaction with the epithelium in the uterine tubal junction and isthmus, where they are stored as a reservoir prior to ovulation. The sperm which have contact with the epithelium maintain their membrane integrity, and following ovulation the plasma membrane in the head domain is eroded or broken as well as the acrosomal membrane [99].

During the period prior to ovulation in which the spermatozoa are stored, the decapacitating factors present within the seminal plasma are removed from the acrosomal membrane of the spermatozoa. The exposed surface of the acrosome is capable of binding luminal fluids which extract lipids from the sperm plasma membrane, creating an enhanced membrane fluidity and reorganization of the sperm plasma membrane [100]. Bicarbonate, a stimulator of adenylyl cyclise, is one effector molecule capable of stimulating lipid scrambling in the lipid bilayer of the porcine sperm plasma membrane [101]. The augmentation of the lipid bilayer is considered to be an early sign of capacitation, which is required for fertilization.

There is an alteration in the number of sperm which ascend into the tubes approximately 2 h prior to pending fertilization [98]. There are three proposed theories governing how the sperm are released from the contact with the epithelium within the reservoirs in the caudal oviductal isthmus and uterine tubal junction. There is a counter current exchange between the ovarian vein and the tubal branch of the ovarian artery; progesterone is released in proportion to the number of ovulating follicles. This progesterone could release sperm which are binding to the endothelium in the reservoirs. With more ovulating follicles there is an increase in progesterone which progressively releases sperm from the endothelium until ovulation. The amount of progesterone releases a number of spermatozoa in proportion to the amount of oocytes, maintaining a low sperm:oocyte ratio [102]. A secondary means of regulating the release of

spermatozoa is the communication of molecular messages from the cumulus oocyte complex prior to ovulation [102]. Alternatively, the cumulus cell mass could reorient the trajectories of the spermatozoa via molecular gradient, thereby guiding the spermatozoa to the unfertilized oocytes [102].

While sperm are incubating in the sperm reservoirs, MII oocytes ovulate from the tertiary follicles and are picked up though adhesion of the cilia of the infundilbulum to be transported to the ostium [103]. Adhesion between the cilia and the cumulus cells is essential for the cumulus oocyte complex to proceed to the ostium [104]. The large, expanded cumulus oocyte complex does not fit through the opening of the ostium, therefore it has to be "churned" to compact the cumulus matrix. Once the cumulus oocyte complex is small enough to pass through the opening of the ostium, it passes through to the lumen of the infundibulum and into the ampulary isthmus junction [103].

As sperm are released from the reservoirs they travel to the ampullary isthmus junction of the oviduct where fertilization will take place. Sperm which have been hyperactivated through capacitation in the female tract, use the asymmetrical beating of their flagellum to escape the oviductal epithelium [105]. Sperm infiltrate through the expanded cumulus matrix of the oocyte, to the zona pellucida, with the help of a membrane bound Hyluronidase, PH-20 [106]. The zona pellucida, which is the extracellular coating of the oocyte, is believed to contain the species-specific gamete recognition. The process of fertilization begins by binding the spermatozoa to the glycoproteins which surround the oocyte [107]. The zona pellucida is composed of three glycoproteins, referred to as zona protein 1, zona protein 2, and zona protein 3 (ZP3). ZP3 possesses the capability to bind sperm with its serine/threonine linked ooligosaccharide chains otherwise known as O-linked oligosaccharides [108]. It has been observed that the acrosome reaction releases specific components of the acrosomal matrix sequentially, exposing a potential substrate which may in turn stabilize the adhesion of the sperm to the zona pellicida [109]. A component of the mouse acrosomal matrix, sp56, has been indicated as such a substrate [110].

After the sperm bind to the zona pellucida of the oocyte, they penetrate the zona pellucida with help from the hypermotile movements of the flagellum and enter the perivitelline space. The sperm plasma membrane is exposed and has undergone some receptor changes following the

acrosome reaction which enable it to fuse with receptors on the oolemma (reviewed by [111]). This binding will stimulate a cellular pathway which increases transient intracellular calcium, a positive messenger for cortical granule release (reviewed by [112]). The cortical granules contain different substances which lead to a zona block to prevent polyspermy [113]. There are proteinases released from the cortical granules which modify the zona pellucida so it will not be receptive to sperm [114]. The newly formed fertilization envelope is also hardened through incorporation of Ovoperoxidase, an oocyte-specific protein released from the cortical granule [115,116]. N-acetylglucosaminidase has also been localized in the cortical granules, when released it deactivates the sperm binding sites on the zona pellucida [117].

As soon as the sperm enters into the ooplasm there is a rapid cascade of events leading to the resumption of meiosis in the oocyte. The male and female nuclei decondense and form pronuclei. A microtubular aster forms in the area of the sperm neck, in combination with the maternal centrosomal material. The male and female pronuclei move together to the center of the oocyte to undergo syngamy, and create a zygote [118].

2.3.2.2. Current Protocols for IVF

In vitro fertilization is the penetration of the matured oocyte by a spermatozoon outside of the female tract; the processes which occur within the tract are recreated *in vitro*. Spermatozoa are collected and introduced to *in vitro* matured oocytes. However, the simple co-incubation of male and female gametes does not result in successful IVF; there are many steps involved in preparing the oocytes and spermatozoa for the interaction. The efficiency of an IVF system is determined by the preparation of spermatozoa through co-culture, specialized fertilization media, methods of introducing oocytes and sperm, as well as how long the oocytes and sperm should interact. Ideally the most repeatable and defined system would be adopted for more standardized protocol.

Before the spermatozoon is introduced to the oocyte it needs to be prepared. Spermatozoa have been co-cultured with fluid components of the female tract in order to replicate the

incubation that spermatozoa undergo in sperm reservoirs. The pre-fertilization incubation of spermatozoa with pFF increases the success of IVF, through decreasing the number of sperm which bind to the oocyte and the number which penetrate, thus decreasing polyspermy [119]. In this study it was observed that sperm co-cultured with oviductal cells for a period of 2.5 h lead to fertilization rates of 94% and 85% in two separate trials. The co-culture reduced the incidence of polyspermy by 40% and 50% respectively. When the sperm was co-cultured for a period of 3.5 h with the oviductal cells it increased the reduction of polyspermy even more, to only 14% of the total number of fertilized oocytes, but the fertilization rate also drop to only 19% [120]. Taken together, these results indicate that incubation of spermatozoa with fluid components of the female tract have a positive effect on spermatozoa, possibly capacitating them prior to fertilization. However, as seen with the oviductal cell co-culture, the prolonged co-incubation decreases the effect, possibly over capacitating the spermatozoa until there few live or motile sperm.

Much like maturation medium, the fertilization medium (in which oocytes are introduced to spermatozoa) needs to support both cell types as well as the physiological processes. There are four common media in which spermatozoa is introduced to porcine oocytes; modified Tris-buffered medium (mTBM), IVF medium-199 (IVFm-199), Tyrode's medium (TALP) and modified Whitten's medium (mWM) (Table 2.2)[6]. The effects of mTBM, mTALP, and TCM-199 on the acrosome reaction of the sperm, the cortical reaction, and zona pellucida hardening show the importance of choosing the appropriate media. Modified TBM increased the rate at which sperm underwent the acrosome reaction, while the percentage of fertilization and cleavage were higher in the other two media, mTALP and TCM-199. The percentage of monospermic fertilization was highest in TCM-199 and mTBM, and neither of the three media affected the zona hardening [121]. The hardening of the zona pellucida prior to fertilization is important as it may prevent some of the polyspermy, as it does *in vivo*, but is not necessarily mimicked in IVM [122]. IVF media is a critical element in successful fertilization; but as the above results show, there are currently no adequate media to mimic the *in vivo* fertilization.

Caffeine is a common chemical in the aforementioned IVF media; it is introduced to simulate capacitation of spermatozoa. While caffeine does successfully induce capacitation, it also stimulates the acrosome reaction prior to the sperm binding to the oocyte [123]. As a

substitution to caffeine, hyaluronic acid can be introduced into IVF media without the negative stimulatory effects of the acrosome reaction, and it is reported to decrease polyspermy without decreasing the penetration rate [124].

To avoid variances in success, often seen with animal source fertilization media, a chemically defined porcine gamete medium (PGMtac4) was developed by Yoshioka et al. (Table 2). The combination of theophylline, adenosine, and cysteine were used to fertilize oocytes using frozen-thawed ejaculated spermatozoa [15]. The appeal in using a chemically defined media for IVM extends into IVF; and the use of a chemically consistent media with frozen thawed semen in conjunction with this system would increase the repeatability of IVF protocols. The use of frozen-thaw semen decreases the inter ejaculation variability of semen, and also the effect of the individual boar [125] as one ejaculation can be frozen and used for many experiments. The use of the reagents in the chemically defined IVF media from Yoshioka et al has been shown to minimize the differences between batches of semen to each chemical [15].

In vivo, the number of spermatozoa interacting with oocytes is regulated so polyspermy is not a problem[102], however to reduce the incidence of polyspermy during IVF the number of spermatozoa interacting with oocytes needs to be controlled. The modified swim-up method reduces the occurrence of polyspermy and therefore yields higher quality embryos with decreased chromosomal abnormalities. This technique uses a test tube of any length, and a cell strainer which is inserted into the top of the tube. The sperm are centrifuged to the bottom of the tube in a pellet. The motile sperms will swim to the upper area of the tube, eventually reaching the restricted area on top of the cell strainer where mature oocytes await. The cell strainer may also contribute to the decrease in polyspermy as it decrease the available sperm binding sites on the oocyte [126].

Table 2.2: Components in Common *In Vitro* Fertilization Media

Component (mM)	**TALP** [127]	**mTBM** [128]	**mWM** [14]	**PGMtac4** [15]	**IVF-M199** [129]
NaCl	97	113.1	100	108.00	103
KCl	3	3	4.7	10	5.37
KH_2PO_4				0.35	
NaH_2PO_4	<1				0.88
$NaHCO_3$	25		25	25	
$MgCl_2{\cdot}6H_2O$	<1		1.2		
Sodium pyruvate	<1	5		0.20	
Glucose	5	11	5.5	1.00	5.6
HEPES	10		22		25
$CaCl_2{\cdot}2H_2O$	2	7.5			1.36
BSA	6 (mg/mL)		7 (mg/ml)		
Penicillin	20 (20 u.i./mL)				
Lactic Acid (60% syrup)	3.68 (mL/L)				
Tris		20			
Lactic acid hemicalcium salt			4.8		
Pyruvic acid			1.0		
$MgSO_4{\cdot}7H_2O$				0.40	0.4
$Ca(lactate)_2{\cdot}5H_2O$				4.00	
Theophylline				2.5	
Adenosine				1.00	
L-Cysteine				0.25	
Gentamicine				0.01(mg/mL)	
Polyvinyl Alcohol				3.00 (mg/mL)	
Glutathione					0.002
L-ascorbic acid					0.003
L-glutamine					0.68
Sodium acetate anhydrous					0.61
Myo-inositol					0.0003
Antibiotic (100X)					1X

Much like the modified swim up method, the climbing over the wall technique (COW), places a barrier between the oocytes and sperm, effectively selecting for fit sperm. Spermatozoa are introduced to the outer ditch of a specialized chamber; oocytes are placed on an inner plate of the chamber. Like the modified swim-up method, only sperm with high progressive motility would be able to transverse the wall to interact with the oocytes. The decrease in available sperm to fertilize the oocytes, and selecting only high motility sperm in this method is seen to decrease polyspermy without decreasing the rate of fertilization [130].

To mimic the oviductal environment, one method of IVF uses cryopreservation straws as a site of fertilization. Only motile spermatozoa were allowed to swim to the site of fertilization and penetrate the oocyte. Compared to the traditional microdrop system, where oocytes and sperm are inserted into microdrops of IVF medium suspended within mineral oil at a pre-determined ratio, the straw IVF system improved fertilization through increasing monospermic fertilization (68% straw vs 47% microdrop) while maintain efficient penetration rates (68%) [131]. Furthermore, the zygotes produced with the straw method displayed improved developmental competence [132].

The separation of motile and nonmotile sperm is a common obstacle for most IVF systems. One system uses a microscale “chip” to isolate the motile sperm. MISS, or microscale integrated sperm sorter, is a disposable polymeric microchannel device, which utilizes a gravity driven pump system to maintain a steady flow of medium regardless of the fluid volume. MISS has the ability to isolate motile sperm from both regular semen samples as well as samples which would generally be too small for traditional separation methods [133].

A final component to the IVF system is the co-incubation time of spermatozoa and oocytes. Older IVF systems had used overnight co-incubation. To reduce the number of polyspermic embryos the amount of time given for sperm to penetrate the oocyte should be reduced. Traditional 6 h incubation still results in polyspermy, and reducing this time to as little as 10 minutes will result to similar fertilization rates. Overall, the reduction in co-incubation time from 6 h to 10 minutes did not improve the efficiency of embryo production [134]. The high concentrations of sperm within minimal IVF medium is known to produce hydrolytic radicals which can damage oocytes, thus shortening the co-incubation time may further protect oocytes

[135]. However, if the incubation time is too short there may not be sufficient time for penetration. Extended co-incubation time may also age the oocyte resulting in abnormal fertilization. Encouraging earlier capacitation of sperm so fertilization may occur sooner and co-incubation may be shortened [136]. There is a natural heterogeneity in the required time for spermatozoa to complete capacitation, and this will result in the need for different co-incubation times regardless of the chosen protocol [137].

IVF systems are meant to simulate the processes, which occur in-vivo. The media and methods above summarize a few of the improvements in IVF. Many methods have been employed to reduce polyspermy and increase the fertilization rate of IVF systems, yet there is still no standard IVF system in place.

2.3.2.3.IVF progress

The conditions of IVF systems are constantly being studied and altered to create the *in vivo* environment in an *in vitro* system. While IVF is used regularly in research application, the use of IVF in pig production is lower than that of other species [4]. The efficiency of IVF in most laboratories, evaluated through a ratio of monospermic fertilized oocytes to total oocytes inseminated remains low (30-50%) [6]. There are two contributing factors to the low success of IVF; the oocyte and the sperm.

Inadequate cytoplasmic maturation may be in part to blame for the frequent occurrence of polyspermy in porcine IVF [6]. If the oocyte is not competent, fertilization will undoubtedly fail. The source of sperm for IVF is especially important. In cattle there is a source effect of the inherent ability of the sperm to fertilize and create competent blastocysts. The differences may be due to the sperms' reaction to capacitation factors in the IVF media, but the other explanation is that specific genes within are transmitted by the bull which determine success of embryonic development [138].

The developments of tools for embryology are not meeting the demands of those interested in using them [41]. Reproductive technologies such as IVF have the possibility to be

utilized by both producers and researchers, yet the technology is not readily available to producers. Several commercial IVF labs have been developed for cattle producers around the world, but the low success of IVF in pigs has delayed this opportunity for pork producers [139]. Research is focusing on solving the IVF problems in pigs to make the technology efficient for use by both the biotechnology and agriculture industries.

2.3.2.4.Problems with IVF

The problems encountered in IVF technology are numerous, and interrelated. Polyspermy is a dominant problem in porcine IVF, and although it does occur in other species, it is especially prevalent in porcine IVF. Polyspermy results from more than one spermatozoon penetrating the oocyte's cytoplasm, and the condition is considered pathological [140]. Polyspermic penetration generally results in the developmental failure of embryogenesis in mammals [102]. If the zygotes of polyspermic origin do develop into blastocysts, they can be recognized by fewer cells within the inner cell mass, as well as aneuploidy [141,142]. The status of the oocyte's competence after IVM can determine the success of IVF, and it also contributes to the quality of embryos produced. The sperm introduced into an IVF system are under quality control, yet the variability in semen source is still capable of preventing the success of IVF.

There is a considerable variation in the rate of meiotic maturation during IVM, the varying degrees of maturation may be a factor in inducing polyspermy [143]. The source of oocytes may be a component in polyspermy, as polyspermic penetration is seen more often in oocytes derived from pre-pubertal gilts than sows [144]. The zona block occurs to prevent polyspermy [113], and it had been previously thought that IVM oocytes were inferior to their *in vivo* counterparts in that respect. Some researchers believe that this may be due to a slower zona blockage; the exocytosis of the cortical granules is thought to be slower in immature oocytes [145,146]. The zona pellucida is known to change throughout maturation and if the maturation is incomplete the zona pellucida may not be able to induce the acrosome reaction [147]. However, *in vitro* matured oocytes have been described as possessing the same abilities as *in vivo* oocytes to release corticle granules upon sperm penetration [148].

The number of sperm introduced to an oocyte is often regulated in an attempt to prevent polyspermy, and sperm is commonly introduced to oocytes at a specific concentration. However, the incidence of polyspermy does not show correlation with the sperm concentration, but rather the ratio of sperm:oocyte [149]. Intracytoplasmic sperm injection is the insertion of a single sperm into the oocyte. This technique has been used to decrease the polyspermic fertilization rate. Unfortunately the results for embryo production with intracytoplasmic sperm injection are lower than for the existing IVF methods, so it cannot be used routinely ([150] and reviewed in [151]). The use of frozen thawed sperm has proven effective at lowering the incidence of polyspermy. This success is not due to the decreased motility of the frozen sperm, as the amount of sperm introduced is adjusted for the lower motility for IVF [152].

Despite displaying similar characteristics, boar semen can penetrate oocytes at different rates depending on the source of the semen [153,154]. This effect could be because sperm sources react to sperm preparation procedures differently. IVF media supplements, such as caffeine, have varying effects on the sperm thus influencing the penetration rates. In some boars caffeine increased both the penetration rate and monospermic fertilization rate, while in others it increased penetration rate while also increasing polyspermic fertilization (90%) [155]. To avoid a boar effect such as this, frozen-thawed semen from a single source could be used to increase repeatability in experimental settings as the survival rate of frozen sperm is now above 50% for most boars (reviewed by [156]).

The use of fresh and frozen-thawed semen from Large White, Landrace, and Duroc boars demonstrated that there were significantly different penetration and polyspermic rates between breeds which led to more variability than the boar difference [152]. The success of freezing boar sperm also differs between boars, and while a boar may produce semen which freezes well, it does not necessarily mean it will have a positive effect on polyspermy [157]. Frozen-thawed ejaculates may also posses all the general criteria of a “good” sample, in that it has good motility and morphology, but does not have the potential to fertilize successfully [158]. When considering a single source of sperm, there is also variability within the ejaculations from a single boar introducing further inconsistencies [159].

In vitro maturation can determine the success of IVF, as incompletely matured oocytes contribute to polyspermy. The sperm also contributes to this prevalent problem, and can be influenced by sperm preparation, as well as sperm origin. The source of sperm can vary the success of penetration rates and monospermic fertilization rates. All attempts to regulate these problems are further complicated by the quality variability in sperm characteristics as well as freezing.

2.3.3. IVC

2.3.3.1. Embryogenesis

There are two descriptions of embryogenesis; the morphological changes which occur in the developing embryo, and the molecular changes. Structural changes during embryo development can be observed with microscopy. Other changes, such as the maternal to zygote transition are just as important, and in any way govern the morphological changes.

2.3.3.1.1. Early embryogenesis

When a sperm enters an oocytes through the zona pellucida and the membranes fuse, a small protrusion called the fertilization cone appears at the site of penetration [160]. This cone will play a role in future divisions of the embryo. The fertilization of the oocyte signals the resumption of meiosis and a one cell embryo is formed containing haploid pronuclei from both male and female gametes. These pronuclei join and replicate their DNA to produce a 2 cell embryo through mitosis [31]. The embryo undergoes a series of cell divisions; each division produces a smaller cell known as a blastomere. The size of the embryo remains the same through theses division, with each resulting blastomere being smaller than those in previous divisions.

The first two divisions take place within approximately 50 h post-insemination. The third division resulting in a 5-8 cell embryo occurs 40 h later [161]. The 8-cell embryo undergoes further divisions beforecompaction, a process in which the all the viable blastomeres flatten and adhere together. This formation of tightly associated, communicating, and polarized cells is referred to as a morulla [31].

The first cell division of the zygote is influenced by the fertilization cone, and it gives rise to the polarity in the cell. This polarity allows the development of the blastocoels on one side of the embryo and thus positions the inner cell mass on the other [162]. Blastomeres from the 32-cell stage embryos onward will differentiate, each being programmed to develop into a certain cell type. The blastomeres on the outer surface of the embryo will become the trophectoderm of the blastocyst; and those on the inside will develop into the inner cell mass of the blastocyst; further differentiating into the primitive endoderm and epiblast [162]. The fluid filled opening within the blastocyst known as the blastocoel is formed through sodium and water being pumped into the morulla and potassium being pumped out [60]. In the pig, the blastocyst forms at approximately 5 days *in vivo*, however IVP embryos can be seen to cleave at a slower rate than their *in vivo* counterparts [148,163]. Likely because of insufficient culture media [164], the IVP blastocyst forms at around 9 days after insemination [161].

2.3.3.1.2. Maternal to Zygote Transition

The maternal genes are the only known contributor to early embryogenesis in mammals before the maternal to zygote transition [32,165]. Abundant mRNA and other proteins stored during maturation are released during the rapid divisions in early embryogenesis [166]. During the first divisions for the zygote there is a silencing of the embryonic genome, and then the embryonic genome takes over the development of the embryo as maternal mRNA is depleted. Regulation of the translation of maternal mRNAs stored within the growing oocyte is through polyadenylation in mammals [167]. Polyadenylation also is responsible for stabilizing the mRNA [168] and transporting it for translation at maturation or fertilization [169]. This process is conserved among mammals [33].

From the time of GVBD in the oocyte and through the first few cleavage divisions, RNA synthesis is not observed [170]. In mammals, protein synthesis during this phase is dependent on a deposit of maternally derived mRNA [171]. At the resumption of meiosis the maternal mRNA begins to degrade [31], and transcription from the new zygotic genome occurs in two phases. The first phase of zygote activation occurs before cleavage [172], and is considered the minor activation. The minor activation is thought to occur primarily in the male pronucleus, and its transient transcription consists of a small set of peptides [172,173].

The major activation occurs after the first few cell divisions [172]. Timing of this activation is species specific, occurring at the end of the third division in pigs (for review see [174]). The major activation involves reprogramming of the gene expression, and the generation of novel transcripts not expressed in oocytes (reviewed in [175]). To identify these novel genes, which are expressed specifically in the embryo, expressed sequence tags (ESTs) have been investigated and accumulated in a public sequence database [176]. This large database will provide the means to identify genes which function specifically in embryos and oocytes and to identify stage specific genes as well [176].

2.3.3.1.3. Maternal Effect Genes

Many embryonic developmental failures have been attributed to a defect in the maternal to embryonic transition. Certain maternal effect genes, such as Mater or Stella will enable mitosis, thus allowing the embryo to cleave; if these genes are down-regulated the embryo would not develop into a competent embryo [33]. Four maternal effect genes of interest in embryogenesis are Basonuclin 1 (*BNC1*), Nucleoplasmin2 (Npm2), Zygote arrest 1 (*ZAR1*), and Tripartite motif containing 24 (*TRIM24*).

BNC1 is zinc finger protein which is known to regulate rRNA [177]. Its absence from the oocyte leads to embryonic failure at the 2-cell stage in mice [178]. The presence of *BNC1* has been observed in mouse GV oocytes through to 2-cell embryos [178]. *NPM2* is involved in nuclear and nucleolar reorganization within the oocyte and early embryo. A 1-cell *NPM2*

deficient embryo lacks normal nucleoli; and a 2-cell *NPM2* deficient embryo has decreased gene transcription and translation [179,180]. Embryos with a *NPM2* knockout can develop into live offspring; this may be due to compensatory mechanisms for the gene, meaning there is more than one gene within the embryo which has this function [179]. *NPM2* has been found in GV oocytes and progressively through to 8-cell embryos in mice [179]. *ZAR1* has been described in mice as a maternal effect gene capable of transcriptional regulation and an embryo lacking *ZAR1* will stop developing at the 1-cell stage [181]. *ZAR1* has been found in bovine GV oocyte to 4-cell stage embryos [182]. *TRIM24* transcripts mediate the nuclear receptor, ligand-dependent activation function (AF-2) [183]. Certain genes within the first wave of genome activation are also regulated through *TRIM24*, showing this gene plays a role in modulating transcription in the early embryonic genome [184]. *TRIM24* is continually expressed in the GV stage oocyte of mice through to the blastocyst stage [184]. Each of these genes is active prior to the maternal to embryo transition and can affect both *in vivo* and *in vitro* production of embryos if altered.

2.3.3.2.IVC Protocols

Often all of the presumptive zygotes are introduced into IVC, regardless of whether they were fertilized by one or more spermatozoa. As there is no consistent method of avoiding polyspermy during IVF, the selection of monospermic zygotes for IVC would increase successful blastocyst production. Visualization of pronuclei is difficult as porcine oocytes have a dark cytoplasm due to high lipid content. One method for visualization of the pronuclei was developed by Han et al [185], centrifugation of the embryos isolated the lipids of the cytoplasm which enabled visualization of the pronuclei. It has been shown that zygotes with two pronuclei developed better that those with either poly pronuclei or undetermined pronuclei [6]. This is one technique which removes the bias of polyspermy during IVC, allowing the efficiency of IVC to be evaluated.

Embryo culture media, or IVC media, has a large influence on developmental competency of embryos. Currently there are two commonly used IVC media, NSCU-23 and NCSU-37 (Table 2.3), both are considered successful for the culture of IVF embryos [13]. Both

media contain the basic components known to affect embryo culture, salts and energy substrates. The amount of NaCl was found to have more effect on blastocyst formation rate that the overall osmolarity of the IVC medium, which demonstrates the importance of salts in IVC media [186]. Culturing embryos for the first 2-3 days in a medium supplemented with pyruvate and lactate yielded higher blastocyst formations rates than culturing in either pyruvate and lactate, or glucose for the entire IVC period [11]. Amino acids also play a role in supporting embryo development. The addition of glutamine, either alone or in conjunction with glucose will support embryo development to the blastocyst stage [187]. The addition of taurine or hypotaurine is now considered an essential addition to IVC media. Supplementing media with either taurine or hypotaurine, or supplementing with both led to significantly higher blastocyst formation rates when compared to a control [188].

As with either IVF or IVM media, IVC media is developed to simulate the *in vivo* environment, which for IVC is the oviduct. Media supplemented conditioned with porcine oviductal epithelial cells not only increased the blastocyst formation rate, but also the number of cells within the blastocyst [11]. Yoshioka et al [15] had developed media resembling pig oviductal fluid, porcine zygote medium 5 (PZM5, Table 3), which has proved effective for IVC. This PZM5 is used with the other chemically defined media, thereby reducing the inconsistency caused by proteins and substrates which vary from batch to batch, and increasing repeatability of results.

The ratio of embryos to media also effects the development of embryos. By increasing the numbers of embryos cultured in the same drop of the traditional micro-drop system to a 1:2 ratio with media, the blastocyst formation rate increased [189]. The Well-of-the-Well (WOW) system allows for a good ratio of embryos to media. The system employs the use of micro wells formed in the bottom of a well of a 4-well plate, with no mineral oil overlay [190]. When comparing the cleavage and blastocyst rates among the microdrop method, the open well method, and the WOW method, only the WOW method showed an increase blastocyst formation rate [191].

Table 2.3: Components of Commonly Used *In Vitro* Culturing Media

Component (mM)	**NCSU-23** [13]	**NCSU-37** [11,13]	**PZM-5** [15]
NaCl	108.73	108.73	108.00
KCl	4.78	4.78	10.00
KH_2PO_4	1.19	1.19	0.35
$MgSO_4 \cdot 7H_2O$	1.19	1.19	0.40
$NaHCO_3$	25.07	25.07	5.00
Glucose	5.55	5.55	
Glutamine	1.0		
Taurine	7.0		
Hypotaurine	5.0		
Penicillin	100 (i.u./mL)	100 (i.u./mL)	
Steptomycin	30 (i.u./mL)	30 (i.u./mL)	
Insulin	0.57		
B-mercaptoethanol	50(µM)	50 (µM)	
BME amino acids	2%		
MEM non-essential amino acids	1%		
HEPES			25
BSA		4 (mg/mL)	
Polyvinyl alcohol			3.00
Gentamicine			
$Ca(lactate)_2 \cdot 5H_2O$			2.00
Sodium pyruvate			0.20
$CaCl_2 \cdot 2H_2O$	1.7		
D-sorbitol		12	

2.3.3.3.Successful IVC

The first confirmed occurrence of the competency of blastocyst produced by IVM, IVF, and IVC was reported by Mattioli et al. [192]. These embryos developed to the 2-4 cell stage before being successfully transferred and producing live piglets. The average blastocyst formation rate for inseminated oocytes is 27.9% (reviewed by [193]). It has been suggested that quality of IVP blastocysts is lower that the *in vivo* blastocysts. Porcine embryos produced through IVM and IVF were cultured *in vivo*, in the oviduct of synchronized recipients, and *in vitro* for 2 days before transferring to a synchronized recipient were compared to *in vivo* produced embryos. The IVM-IVF embryos cultured *in vivo* had the same blastocyst formation rate as the *in vivo* embryos and the duration of time that the embryos were cultured *in vitro* decreased the blastocyst formation rate [194].

The production of piglets through embryo transfer following IVP is the only validation for the quality of blastocysts produced *in vitro*. In 2001, a percentage of piglet production of 2.5% was obtained by transferring 80 IVP embryos into 4 recipients, which resulted in one pregnancy and the birth of 2 live piglets [144]. In 2002, 150 blastocysts were transferred into 3 recipients, which resulted in 3 pregnancies, and the birth of 19 piglets total [11], this was a 12.6% piglet production. In 2011, the transfer of 18-25 blastocysts per recipient into 11 recipients had 11 pregnancies, and a 26.6% piglet production [195]. Taken together, these studies show an acceptable piglet production rate for *in vitro* technology, but are far from those rates obtained *in vivo*.

2.3.3.4.Problems with IVC

Abnormalities are seen in both *in vitro* produced embryos and *in vivo* produced embryos. It is reported that 7.3% of *in vivo* derived embryos have abnormal morphology of chromosomal numbers [196], however this rate is much lower than that for *in vitro* derived embryos. Cytogenetic analysis of porcine IVP blastocysts showed that 40% of them displayed haploidy,

polyploidy and mixoploidy chromosomal abnormalities [5]. *In vitro* derived blastocysts are also prone to low cell numbers. Blastocysts with low cell numbers are also associated with chromosomal abnormalities, specifically polyploidy [5].

The slow development rate of *in vitro* produced embryos contributes to low blastocyst quality. In one report, embryos were graded on day two of IVC into either a slow (2-cell), moderate (3-4 cell and 5-8 cell), and fast (>8 cell) embryos. The blastocyst rate was significantly greater in the moderate embryo group, and these blastocysts had many more cells per blastocyst. Embryos from the slow and fast group had a higher incidence of polyploidy [193].

Chromosomal abnormalities in blastocysts have been attributed to polyspermy during fertilization [197]. Low blastocyst formation rates as well as a high incidence of polyspermy could be caused by poor quality oocytes or incomplete maturation [4]. To improve IVC the problems in IVF must be solved. The majority of problems related to IVF and IVC can be traced back to the first step of *in vitro* production of embryos, IVM.

2.4.*Follicular Fluid in In Vitro Production of Embryos*

Follicular fluid exchanges factors from the granulosa cells of the follicles as well as the circulatory system, to the cumulus oocyte complex. The components of FF are derived from the blood outside of the follicle through filtration of plasma [198]. Follicular fluid has an abundance of steroid hormones, growth factors, cytokines and metabolites [22]. There have been numerous studies demonstrating the effects of FF and its components on *in vitro* production of embryos, most focusing on the maturation promoting effects they have on oocytes.

Table 2.1 summarizes the composition of popular maturation media; the majority contain FF. The addition of FF is a common supplement as it promotes nuclear maturation as well as cytoplasmic maturation during IVM. When introduced into the first half of IVM, nuclear maturation rates increased. Cytoplasmic maturation was supported through IVM with FF as seen by the increase in cumulus expansion, a decrease in cumulus cell apoptosis, and an increased blastocyst formation rate following IVF and IVC [199]. The beneficial effects of FF during IVM

are further emphasized as male pronuclear (MPN) formation is significantly elevated following maturation with FF [200].

The source of FF can determine its effect on maturation. Size of the follicle from which FF was obtained influences IVM. Oocytes matured in FF derived from large follicles (5-8 mm) displayed increased cumulus expansion, nuclear maturation, cleavage and blastocyst formations rates when compared to those matured in small (2-4 mm) follicle FF [201]. The age of the donor animal can also influence oocyte developmental competence, as evident by the resulting cleavage and blastocyst formation rates. Oocytes derived from pre-pubertal and adult animals were matured with FF from prepubertal and adult sources. Adult oocytes were affected by the source of FF, but pre-pubertal oocytes were not. When examined, the steroid content of the adult FF had significantly higher concentrations of progesterone and androstenedione [202]. The pre-pubertal oocytes' lack of reaction to the different FF source may have been due to its inability to respond to these steroids [202].

The components of FF have been reviewed extensively [22], and some components may explain its effects on oocyte developmental competence as well as cumulus cell survival. Follicular fluid during IVM increases the penetration rate during IVF, including polyspermic penetration. Follicular fluid meiosis-activating sterol (FF-MAS) has been isolated as one factor in FF which increased *in vitro* oocyte maturation. FF-MAS usedduring IVM decreases the polyspermic rate, as well as the rate of oocyte degeneration [203]. Protection from oxidative stress is another function of FF, further preventing oocyte degeneration. Radical scavenging enzymes are found in abundance in FF, and the introduction of these during IVM increase MPN formation and post-fertilization developmental competence [204]. Plasminogen activators and plasmin are found within FF, exerting positive effects on cytoplasmic maturation during porcine IVM as well as fertilization and early embryo development in bovine IVP [205]. In maturation media without FF additions, there is supplementation with amino acids (Table 2.1). Amino acids are abundant in FF; Glycine, Glutamic acid, Alanine, Glutamine, and Proline are prominent in FF from follicles of all sizes [20]. Insulin-like growth factor 1 plays three roles in follicular regulation. Alone it regulates cumulus cell proliferation through paracrine actions, acting additively with FSH it controls granulosa cell proliferation. Synergistically acting with FSH, Insulin-like growth factor 1 induces steroidogenic activity of the cumulus cells [206].

Maturation media is meant to support the maturation of oocytes; and as such the *in vivo* maturation medium, FF, is mimicked. The addition of FF is common in most IVM media, and certain factors found within FF have been introduced to IVM media to increase the competence of maturing oocytes. Among these factors which can influence maturation, steroid hormones such as estrogen, are abundant in FF [24].

2.5.*Estrogens' Effects*

Theca cells, in response to LH, secrete androgens, and these androgens reach the granulosa cells to be converted to estrogens via aromatization. The aromatase enzyme is present in the granulosa cells and is encoded by the CYP19A1 gene [207]. Granulosa cells and the cumulus oophorous are the primary site for androgen conversion to estrogen during folliculogenesis, and they are also present during IVM. In the granulosa cells, FSH will induce the aromatase activity, converting the theca cell androgens into estrogen [207].

Follicle stimulating hormone is included in the basic maturation media. While its effects have been shown to increase competency it is through communication with the follicle and surrounding granulosa cells in the cumulus oocyte complex [208]. The communication between the oocyte and its surrounding cells allows the oocyte to attain competence, and also enables the oocyte to affect its surrounding cells. As an oocyte matures the steroidogenic activity of the cumulus oocyte complex changes. Immature oocytes may suppress progesterone production, but through maturation they lose this ability and instead gain the ability to suppress estrogen production instead [209]. The actual response to gonadotropins and resulting steroidogenesis may be attributed to the both the oocyte and cumulus cells. Without proper maturation, the control of the steroidogenesis may be lost and further embryonic development is suspended.

Oocytes matured *in vivo* are exposed to a variety of substrates in the FF; among these is a high level of estrogen [24,210]. In some procedures, oocytes are matured *in vitro* in a combination of calf serum and pFF, both of which contain androgens and the corresponding precursors [211]. During IVM, the oocyte is surrounded by its cumulus cells and remaining

granulosa cells; the granulosa cells maintain the oocyte's aromatizing capability. Aside from androgens found in calf serum and pFF, LH and FSH are introduced during IVM; allowing granulosa cells to acquire aromatization activity. The gonadotropins induce cumulus expansion, lessening communication between the oocyte and its cumulus complex by disrupting gap junctions, and allowing the oocyte to attain competence [212]. The gradual increase in estrogen during *in vivo* maturation is substituted with an abrupt introduction of a high concentration during IVM. The negative effect of estrogen on nuclear maturation has been observed in many species including swine [26,213]. In bovine IVM, the addition of estrogen to a serum free media decreased nuclear maturation and increased nuclear aberrations [214]. Although in white-tailed deer, the addition of estrogen during IVM was seen to increase the amount of oocytes which reached MII, but this effect was only seen in high graded oocytes [215].

Many studies have investigated the effect of estrogen on reproductive functions either through inhibition of estrogen or use of estrogen receptor knockouts. It has been suggested that both paracrine and autocrine functions exist between the surrounding follicular cells and the oocyte, thus influencing oocyte competency. Mice lacking estrogen receptor α maintain the ability to ovulate following an apparently normal folliculogenesis [216]. Also, mice lacking estrogen receptor β still exhibit folliculogenesis [217]. Exogenous introduction of Arimidex (Anastrozole), an aromatase inhibitor, did not affect follicle development, ovulation and subsequent fertilization in mice [210]. In hamsters, rabbits and monkeys, folliculogenesis and ovulation were unaffected with treatment of Fadrozole, a non steroidal aromatase inhibitor [218]. With the inhibition of estrogen production due to aromatase inhibitors it showed that rising estrogen levels were not an important factor for maturation of the follicle [219]. It has been reported that estradiol was not necessary for follicular development *in vivo*, IVF, or IVC development of the embryo [210]. It has also been shown that estrogen and other steroids play no part in the nuclear maturation of oocytes in mammals [220].

While estrogen has many known endocrine actions, it also can affect gene expression. Transcripts required for cell differentiation like Glycoprotein transmembrane nmb (GPMNB), which is downregulated and Cell division cycle associated 7 like (CDCA71) which is upregulated, are altered with exposure to estrogen [221]. In the granulosa cells, the expression of inhibin α and inhibin ßB [222] as well as cyclin D2 [223], is influenced by estrogen. Estrogen

has also been observed to enhance the expression of components within the insulin-like growth factor 1 pathway and its own receptor estrogen receptor β [224]. Transcripts which are found in mammary tissue of an estrogen receptor α knock out mouse are of some of the hundreds of transcripts influenced by estrogen [221]; these may have a similar influence in ovarian tissue and deserve further investigation within the oocyte.

2.6.*Gene Expression Analysis in Oocytes*

The analysis of gene expression in oocytes and embryos in mammals faces certain challenges due to the limited quantity of genetic material available, the difficulty in normalizing results, and the number of oocyte specific transcripts. While genome databases for mouse, human, and bovine models are increasing; the mapping of the porcine genome is still limited, which further complicates the analysis of gene expression in porcine oocytes.

The primary issue in analyzing gene expression in oocytes is the minute quantity of material available. Obtaining oocytes for analysis may be difficult for *in vivo* studies using porcine models. It is estimated that an oocyte may contain only 2 ng of RNA and most transcriptome analysis protocols require 1000 times more RNA [225]. This requires amplification of cDNA produced using PCR. Through amplifying the cDNA to such an extent allows for more disturbances in the resulting product [226]. Another option is to amplify RNA using *in vitro* transcription using T7 RNA polymerase [227]. RNA amplification can be affected by both the polyadenalation status as well as the abundance of the mRNA [227]. The linearity of both amplification methods is of concern [225,226]. One way to reduce the amount of amplification needed is to increase the amount of RNA obtained by increasing the number of oocytes used.

Oocytes have variable RNA content [225], the amount of RNA present is stage specific [228]. When using just one oocyte, or a group of oocytes, it is difficult to normalize expression values. The number of oocyte may be used as a control, or the use of a normalizing gene such as *GAPDH* can be effective as it has less variability and more reproducibility [229]. The use of high

throughput arrays has allowed tens of thousands of gene expressions to be compared among each other; however it also encounters the problems of normalization due to the differing expression of oocytes at different levels of development. In array analysis, data is often normalized to the median signal which assumes that RNA content is similar [227]. Another method is to provide an endogenous control, exogenous RNA can be added to the sample at the time of RNA extraction [227].

Oocytes differ from many somatic cells in that they possess oocyte specific transcripts that are dynamically regulated. Certain genes are specific to germ cells such as Growth differentiation factor 9 (*GDF9*), which regulates normal follicle development. *GAPDH* is located in the oocyte, yet it is responsible for regulating somatic cells surrounding the oocyte [230]. In the absence of *GDF9*, the follicle does not develop fully, and as a result the oocyte degenerates within the partially formed follicle [230]. A mouse oocyte lacking the oocyte specific gene *mZP3*, which encodes for one of the glycoproteins which form the zona pellucida, will lose connections with surrounding cumulus cells and the resulting zona pellucida free oocyte is infertile [231]. Maternal effect genes are a primary example of oocyte specific genes, as they are present in the oocyte and may affect fertility. The absences of these genes which are preferentially present in the oocytes have the ability to affect reproduction through embryogenesis [232].

The Swine Genome Sequencing Consortium launched the whole genome sequencing project in 2006 [233]. The sequencing strategy uses shotgun sequencing of BAC (Bacterial artificial chromosome) clones and whole genome sequencing [234]. In 2009 a data release workshop was held by the funding agencies and Genome Canada in Toronto. The Toronto statement, which was released at this meeting, requires the producers of large genome sequencing data sets to release a statement describing their data set as well as their intent of analysis and publication of the data [235]. As so, current data sets have been released into public sequence repositories [234]. Sscrofa9 is the current assembly of the porcine genome and this data set was derived solely on BAC sequencing data [234]. Sscrofa10, which is a more complete and revised assembly, is currently being constructed using both the BAC sequencing data and the whole genome shotgun sequences [234]. A complete map of the porcine genome will allow more efficient analysis of the gene expression in oocytes.

2.7.*Microarray in Porcine Reproductive Transcriptomics*

There has been a tremendous rise in the porcine transcription data available, and several methods have been used to build the existing genebanks. Specialized techniques such as serial analysis of gene expression (SAGE), suppressive subtractive hybridization (SSH), quantitative real-time PCR (Q-PCR), and microarray based techniques have been used identify expressed sequence tags (ESTs) and create cDNA libraries [236]. Currently there are a variety of microarrays being used to evaluate different aspects of porcine reproduction.

Macroarrays are the predecessors to the microarray. They were used to evaluate tens or hundreds of genes, before the higher output microarray gained popularity. A 8009 cDNA probe macroarray evaluated the gene expression in porcine ovaries during luteinisation [237]. Human based microarrays are one option for researchers. A Human cDNA microarray was adapted for use with porcine testes tissue. This 3840 probe microarray successfully compared gene expression in pig testis with high and low levels of estrone sulphate [238]. UniGEM human chips with 7100-9100 cDNA probes were utilized in determining differential gene expression in ovaries of selected pigs [239]. Affymetrix developed a 23,937 probe porcine array, representing 20,201 genes from the porcine genome. This Affymetrix GeneChip was used to evaluate the differential expression in oocytes derived from pre-pubertal and cyclic sows [240].

Custom cDNA microarrays have been created for more specific analysis. The University of Missouri-Columbia EST projects created a pig reproductive-tissue specific 19,968 probe array derived from 27 tissue specific cDNA libraries from follicles, corpus luteum, embryos, oocytes, oviducts, endometrium, concepti, and foetal stages [241]. Gene expression of peri-implantaion concepti during elongation was assessed using a 1016 probe custom cDNA array [242]. Genes differentially expressed in the endometrium during the oestrus cycle were identified using a >14,000 probe custom cDNA array [243]. Transcriptional profiling of porcine embryogenesis from the GV oocyte until blastocyst stage was done using a >14,000 probe custom cDNA array [241].

EmbryoGENE, a network that was created to investigate embryo development in livestock, has developed a porcine transcriptomic platform along with the bioinformatics tools

necessary for analysis. The porcine transcriptomics array platform recently became available for use with EmbryoGENE projects. Data generated from processing in excess of 1 million embryo and oocyte relate gene transcription sequences was used bu Gydle Inc. when developing this array. More than 43,000 embryo/oocyte specific porcine genes and gene variants are featured on this array, referred to as a 44K array [244]. This is a large amount of probes, each specific for evaluating differential expression in porcine embryos and oocytes, will aid in assessing the effects of *in vitro* culture and improving porcine IVP.

II. GENERAL OBJECTIVES AND HYPOTHESIS

The overall goals of the research conducted for this thesis were to improve in-vitro maturation and thus improve in-vitro production of embryos. The general hypothesis was that estradiol introduced during *in vitro* maturation would have a positive effect on oocyte competency, cleavage and blastocyst production rates, and gene expression within the oocyte.

Specific Objectives and Hypothesis

Objective 1 (Chapter 3): To determine if steroids influence nuclear maturation rates of oocytes being matured *in vitro.*

Hypothesis 1 (Chapter 3): That maturation media void of steroids will have equal nuclear maturation rates as oocytes matured within medium supplemented with 10% pFF.

Objective 2 (Chapter 3): To determine the effects of estradiol supplementation to *in vitro* maturation medium on cleavage and blastocyst formation rates, and if the effects of pFF supplementation within the media are due to estradiol.

Hypothesis 2(Chapter 3): Estradiol-17β supplementation during IVM will increase the blastocyst formation rate equal to that of 10% pFF supplementation.

Objective 3 (Chapter 3): To observe the effects estradiol-17β supplementation during *in vitro* maturation has on the expression of selected maternal determinant genes within the oocyte.

Hypothesis 3 (Chapter 3): There will be a difference in gene expression between non-matured groups and matured groups, and there will be a dose dependant effect of estradiol 17-β supplementation similar to that of 10% pFF supplementation.

Objective 4 (Chapter 4): To evaluate the differential expression of porcine oocyte genes when exposed to estradiol 17-β during maturation.

Hypothesis 4 (Chapter 4): Non-matured oocytes will have differential gene expression from matured oocytes. Groups treated with estradiol-17β and 10% pFF will have similar expression.

CHAPTER 3: EFFECTS OF ESTROGEN ON *IN VITRO* MATURATION OF PIG OOCYTES

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3.1.Abstract

In vitro maturation (IVM) is often incomplete in porcine *in vitro* production (IVP) of embryo systems, which decreases blastocyst formation rates. As an additive to maturation media, pFF can affect IVM. Estrogen is found in high concentrations in pFF, and may be contributing to the effects seen when pFF is added to IVM. For this study, the effects of estrogen introduced during IVM were evaluated. Nuclear maturation of oocytes was evaluated using Lamin/Dapi staining of oocytes matured in protein-free and 10% pFF maturation media to ensure the efficiency of the protein-free media. The expression of maternal effect genes Basonuclin-1 (*BNC1*), Nucleoplasmin 2 (*NPM2*), Zygote arrest 1 (*ZAR1*), and Tripartite-motif protein-24 (*TRIM24*) were then evaluated using oocytes matured in 50 ng/ml, 100 ng/ml, or 1000 ng/ml of estradiol 17-β (E_2), 10% pFF, or protein-free maturation media. Cleavage and blastocyst formation rates were examined following *in vitro* fertilization of oocytes matured in 100 ng/ml E_2, 10% pFF, or a protein-free maturation medium to investigate the effect of estradiol on IVP embryos. Protein-free and 10% pFF media mature oocytes at similar rates (91% and 89% respectively). The ΔCt expression of *TRIM24* in 0 ng/ml E_2 maturation medium and the 10% pFF maturation medium were significantly different ($p<0.05$) from the non-matured control, the other maternal determinant genes did not differ in their expression under any treatment. When observing cleavage and blastocyst formation rates both the E_2 and pFF additions increased the blastocyst formation rate (14.6% and 12.4% versus 3.5%) without altering the cleavage rates (60.2% and 55.7% versus 54.9%) from the control. The combined results from these experiments suggested that E_2 has a similar effect as pFF during *in vitro* maturation of porcine oocytes.

3.2.Introduction

The *in vitro* production (IVP) of porcine embryos is not as successful as in other livestock species and many challenges remain to be solved [4,6]. Poor embryo culture conditions contribute to the low number of viable embryos [245] and polyspermy can occur in 27% to 59% of fertilized porcine oocytes [134]. IVP embryos when compared to *in vivo* produced embryos often display chromosomal abnormalities, abnormal blastocysts development and decreased cell number within the blastocyst, and a change in the expression of genes related to mitochondrial function [93,146,246]. To increase the efficiency of *in vitro* maturation (IVM) of oocytes, cytoplasmic maturation and nuclear maturation must occur within the ovum for IVP to be successful [77]. Nuclear maturation requires the oocyte to reach MII of meiosis which can be attained through current IVM systems at rates of 75% to 85% [245,247]. On the other hand, MII can be completed through culture without the oocyte ever achieving full competence [57]. Nuclear and cytoplasmic maturation are separate events, although the processes do depend on one another [248]. Cytoplasmic maturation entails the accumulation of mRNA and proteins, reorganization of the oocyte internal structure, and changes in the oocytes metabolism [59,249]. The oocyte accumulates stable mRNA until the end of the follicular growth phase [71,77] and oocyte mRNA levels have been linked to the oocytes competence [29]. These oocyte transcripts can be influenced, during IVM, through supplementation of maturation media [29,250]. Without proper accumulation of maternal mRNA during cytoplasmic maturation, including maternally derived genes responsible for early embryogenesis, a fertilized zygote will not develop any further [77].

In the pig IVM system, the use of porcine pFF in the maturation medium is proven to be effective, even with the inherent variability of pFF composition [6,201]. However, oocytes can successfully mature in a chemically defined medium in the absence of pFF [15]. The concentration of estrogen can be very high in pFF, with published values varying from approximately 8 ng/ml to 266 ng/ml [24]. This steroid does not appear to play a part in the nuclear maturation of oocytes in mammals [220]**.** However, the addition of estrogen to a serum free medium decreased nuclear maturation and increased nuclear aberrations in bovine oocytes [214]. Interestingly, some studies showed a positive effect of estrogen on IVM in cattle [251]

and sheep [252]. In porcine embryos, estrogen may have an inhibitory effect on the nuclear maturation of the oocyte [213]. Along with the known endocrine actions of estrogen, some gene transcripts required for cell differentiation like Glycoprotein transmembrane nmb (GPMNB), and Cell division cycle associated 7 like (CDCA71) are altered with exposure to estrogen [221]. For this study, we hypothesize that the addition of estradiol 17-β (E_2), which is prevalent within pFF, can affect the maturation of porcine oocytes; and in turn influence the overall success of porcine IVP.

3.3.Materials and Methods

3.3.1. General Experimental Design

All chemicals were obtained from Sigma-Aldrich Canada Chemical Company unless otherwise noted.

3.3.1.1.Evaluation of nuclear maturation with protein-free IVM media

Nuclear maturation of porcine oocytes was evaluated with the use of a protein-free maturation media. Cumulus oocyte complexes (COCs) were screened for grades 1 and 2 [253], those with evenly granulated cytoplasm and three or more layers of cumulus cells. COCs were conveniently assigned to a non-matured group, and groups matured in a 10% pFF, or a protein-free maturation medium. The protein-free maturation medium was composed of Medium-199 (12340-030, Invitrogen, Canada) with 0.1% polyvinyl alcohol (P8136), 3.05 mM D-glucose (G7021), 0.91 mM sodium pyruvate (P4562), antibiotics 1X (15240-062, Invitrogen, Canada), 10 ng/ml EGF (E4127), 0.57 mM L-cysteine (W326305, SAFC/Sigma, Canada), and 0.01 U/ml Lutropin-V® (Bioniche, Canada), 0.01 U/ml Folltropin® (Bioniche, Canada). The pFF was obtained from aspiration of antral follicles of pre-pubertal and mature gilts/sows and centrifuging

at 3000 X g for 15 min then collecting and filtering the supernatant with a 0.22 µm filter. Oocytes from the matured and non-matured control groups were denuded in 0.3% hyaluronidase (H3884) in Medium-199. The oocytes then underwent LAMIN and DAPI staining to evaluate the nuclear maturation rates (Fig 3.1). This was done in sequential biological replicates.

3.3.1.2.Effects of estradiol and pFF addition on expression of maternal effect genes

Expression of maternal effect genes Basonuclin-1(*BNC1*), Nucleoplasmin 2 (*NPM2*), Zygote arrest 1 (*ZAR1*), and Tripartite-motif protein 24 (*TRIM24*) (Table 3.1) were evaluated before and after *in vitro* maturation of oocytes exposed to different concentration of estradiol 17-β (E2758) or pFF (Fig 3.2). The grade 1 and 2 [253] COCs were conveniently allocated into groups of 40 for culture with the following added into protein-free maturation medium: 0 ng/ml E_2, 50 ng/ml E_2, 100 ng/ml E_2, 1000 ng/ml E_2, or 10% pFF. After maturation these oocytes and a non-matured control were denuded and stored at -80°C for RNA extraction. This was done for three biological replicates, with IVM being completed for each group on a weekly basis. Gene expression was evaluated using Q-PCR, comparing the matured groups to the control group. There were 3 replicates for each group and each was run in technical triplicate.

3.3.1.3.Effects of estradiol addition on in vitro production of embryos

Grade 1 and 2 COCs [253] were divided and conveniently assigned to a maturation group. COCs were matured in the protein-free maturation media containing either 0 ng/ml E_2, 100 ng/ml E_2, or in 10% pFF (Fig 3.3). After IVM, the oocytes were denuded and underwent *in vitro* fertilization (IVF) and *in vitro* culturing (IVC). Cleavage rate was counted on Day 2, and blastocysts formation rate was counted on Day 9 (Day 0=IVF). This was repeated for a total of three biological replicates done sequentially.

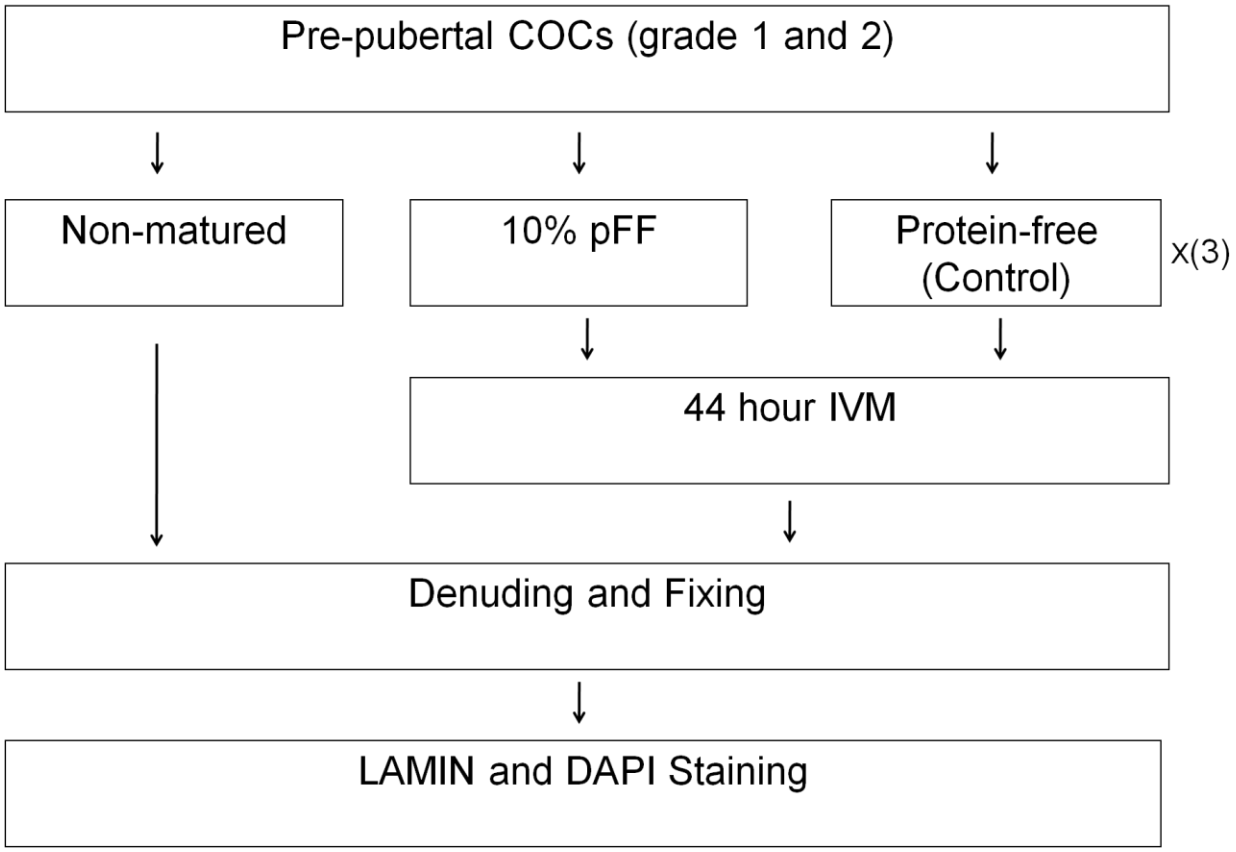

Figure 3.1: Experimental design for the DAPI and LAMIN staining to determine meiosis status of oocytes. COCs were collected and after screening for grades 1 and 2, then were evenly divided into each treatment group for IVM with a minimum of 50 COCs per group; this was repeated for 3 biological replicates.

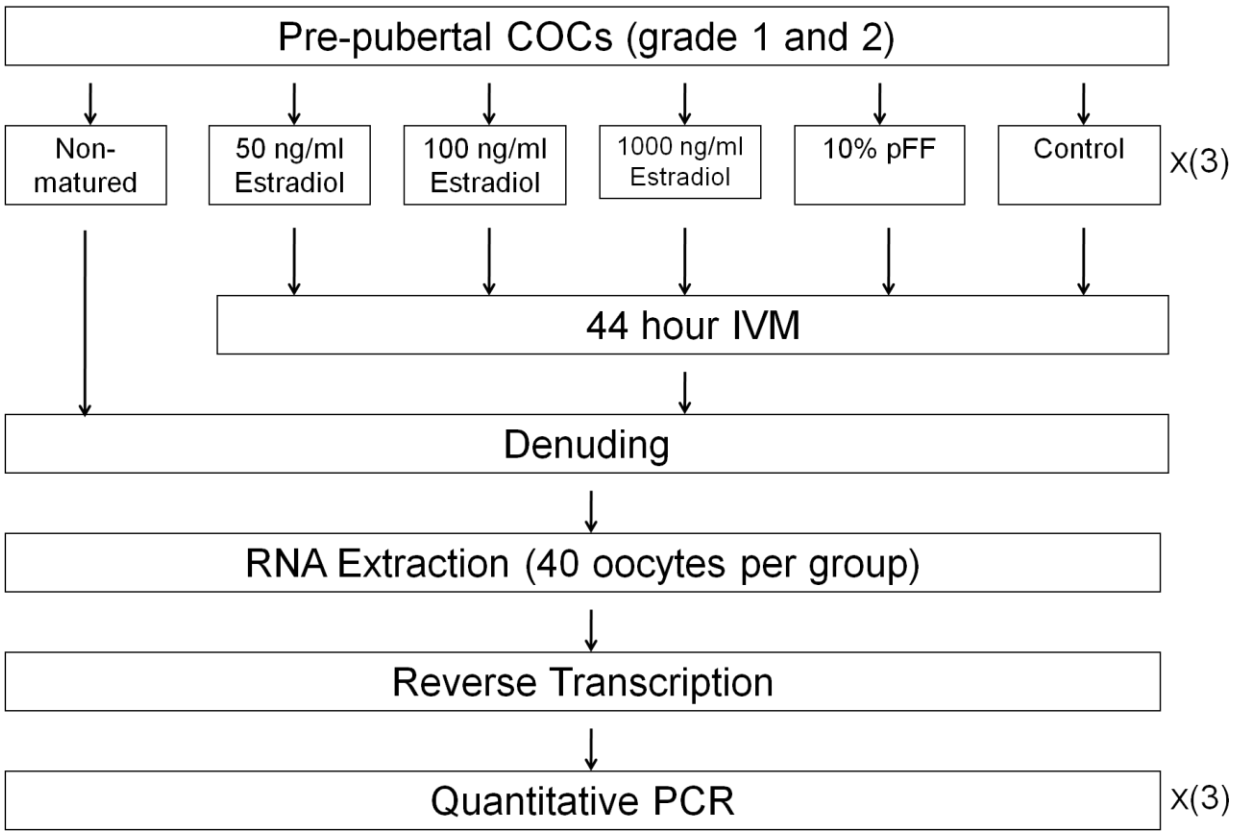

Figure 3.2: Experimental design for the analysis of maternal determinant gene expression in oocytes incubated in different conditions. COCs were collected and after screening for grades 1 and 2, then the total amount were evenly divided into each treatment group for IVM. This was repeated for three biological replicates and the QRT-PCR was done in technical triplicate.

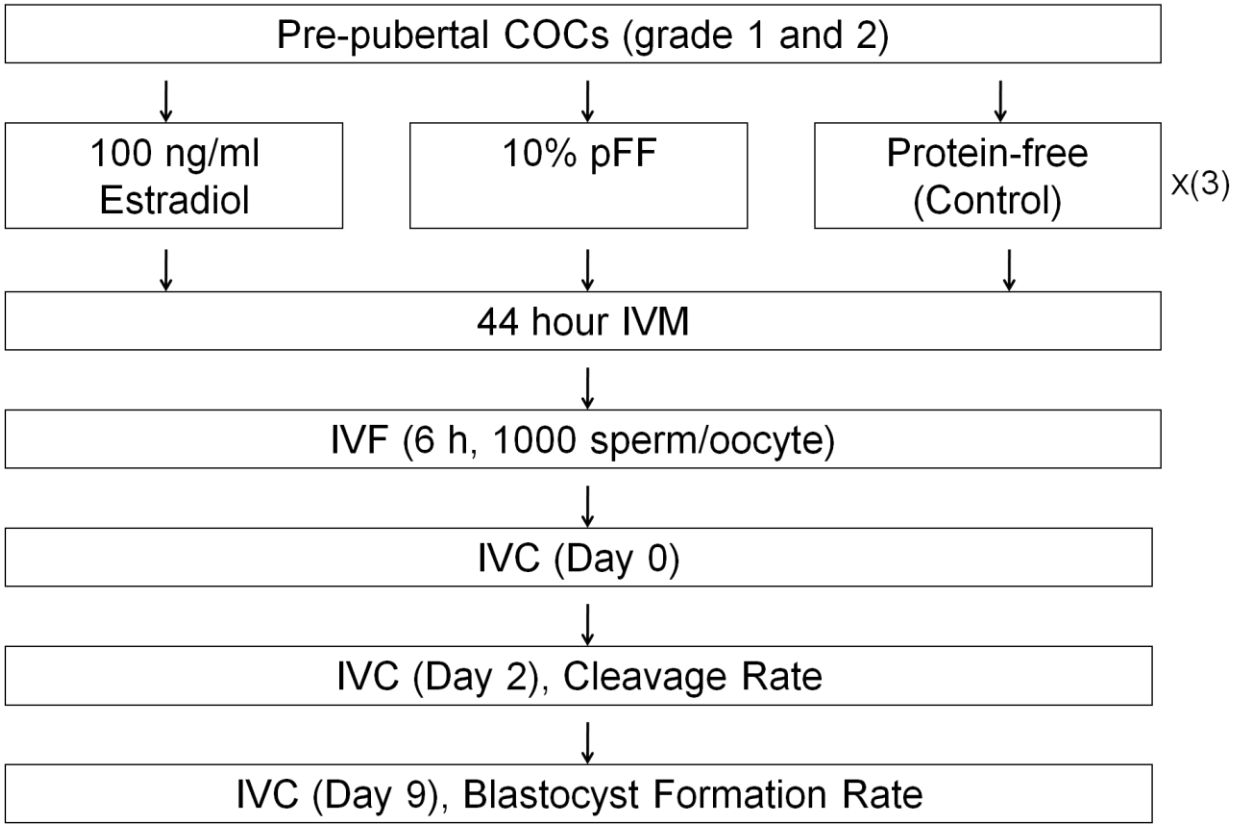

Figure 3.3: Experimental design for the evaluation of cleavage and blastocyst formation rates. COCs were collected and after screening for grades 1 and 2, then were evenly divided into each treatment group for IVM with a minimum of 50 oocytes per group for a total of three biological replicates.

Table 3.1: Primers and GenBank accession numbers for Maternal Determinant Genes.

Primer	Sequence	Origin	Product Size (bp)	Annealing Temperature (°C)	GeneBank Accession Numbers
GAPDH	TCGGAGTGAACGGATTTGCCTGGAAGATGGTGATG	PORCINE	219	51	AF_017079
BNC1	CTGCCCTTTCTTTCTCCTGAATCCAACGTCGTGTTTGACAT	MURINE	284	60	NM_007562
NPM2	CCTTTTGACTTGTTTGACAGGGCTCAGGACCTGTGTTCCTC	BOVINE	126	60	XM_617403
ZAR1	TGTCTGCTCCCTTAGCACCTGCGTGGAGGATATCACCTGT	MURINE	148	59	AY_262114
TRIM24	CAGCCAAGAGTGTGCAGAGAGCTTCTGCATTGTCCTCTACAG	PORCINE	226	60	NM_001129956

BNC1 : Basonuclin-1, *NPM2*: Nucleoplasmin 2, *ZAR1*: Zygote arrest 1, and *TRIM24*: Tripartite-motif protein-24. bp: base pairs

3.3.2. Collection of oocytes and IVM

Ovaries from pre-pubertal gilts were obtained from an abattoir and were transported to the laboratory in an insulated container. After washing the ovaries with physiological saline, follicles of 2-5 mm in diameter were aspirated using 18G needle and 5 ml syringe, and COCs were screened and graded. COCs were selected and transferred into a final maturation medium in one well of a NUNC 4-well plate (Thermo Scientific, USA); without mineral oil overlay to avoid absorption of steroids. The COCs were incubated in a 5% CO_2, 5% O_2, and 90% N_2 humidified environment for 40 h.

3.3.3. In Vitro Fertilization (IVF) and In Vitro Culturing (IVC)

After IVM, IVF was completed using modified Tris-Buffered Medium (mTBM) [128] supplemented with 1 mM caffeine (C0750) and 0.4% BSA (Minitube, Canada). Fresh semen collected using the gloved-hand method was washed by centrifuging (500 X g for 4 min) three times with mTBM then re-suspended to the original volume in mTBM. Ova were introduced into the mTBM in a 4-well plate without mineral oil overlay followed by sperm at a concentration of 1000 sperm cells per oocyte (Day 0). The cells were incubated for 6 h in a 5% CO_2, 5% O_2, and 90% N_2 humidified environment at 38.5°C. After IVF, the zygotes were then washed three times in a NCSU-23 [13] supplemented with 0.4% BSA culture medium (Zenith Biotech, USA). These zygotes were incubated in a 4-well plate without mineral oil in a 5% CO_2, 5% O_2, and 90% N_2 humidified environment at 38.5°C until evaluated.

3.3.4. Lamin and DAPI Staining

Oocytes were stained with DAPI following a modified procedure by Arnault et al., 2010 [254]. Oocytes were washed in 1X Dulbecco's phosphate buffered saline (DPBS;

14040,Invitrogen, Canada) + 5% fetal calf serum (CS; 10438034, Invitrogen, Canada) then partially denuded by washing in Ca++/Mg++ free DPBS (14190,Invitrogen, Canada). Oocytes were pipetted up-and-down 80 times in 0.3% hyaluronidase for final denuding, following by three washes in DPBS + 5% CS then washed twice in DPBS + 0.1% PVA (polyvinyl alcohol). Denuded oocytes were fixed using 4% paraformaldehyde and kept at 4°C for a maximum of 1 week. Fixed oocytes were washed with DPBS+ 0.1% PVA before the membranes were permeabilized using 0.5% Triton-X 100 in DPBS for 30 min at room temperature. Membranes of the oocyte were re-permeabilized with 0.05% Tween-20 in DPBS for 30 min at room temperature, then the oocytes underwent three washes in DPBS + 0.1% PVA. Permeabilized oocytes were incubated in DPBS with 2% BSA (heat shock fraction V) for 1 h at room temperature while shaking. The first antibody, mouse anti-lamin A/C (sc-7292,Santa Cruz, USA; 1:300 dilution ratio) in DPBS + 2% BSA was introduced to the oocytes for 1 h at room temperature while gently shaking and then washed in DPBS + 0.1% PVA. After three washes, the secondary antibody Alexa 488 labelled anti-mouse IgG (A11029, Invitrogen, Canada; 1:200 dilution ratio) in DPBS+ 2% BSA was introduced to the oocytes at room temperature while shaking in the dark for 1 h. A final wash in DPBS+ 0.1% PVA of the oocytes was done before mounting the labeled oocytes using Vectashield Mounting Medium with DAPI (Vector Laboratories, Canada) and evaluated with an epifluorescence microscope. Following this labeling procedure the oocytes were individually classified as germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), or metaphase II (MII) (Fig 3.4). The rate of nuclear maturation was obtained by taking the ratio of MI and MII over the total number of cells.

3.3.5. RNA Extraction, Reverse Transcription and Q-PCR

The RNA extraction was done using Qiagen RNeasy Plus Mini Kit (74134,Qiagen, Canada). Immediately after extraction of the 40 oocytes, 10 ng of RNA was reverse transcribed using RevertAid™ H Minus First Strand cDNA Synthesis Kit (K1631, Fermentas Canada) using random hexamer primers. Q-PCR was performed using Brilliant SYBR® Green QPCR Master Mix (600548, Applied biosystems, USA), and the 20 pmol of primer per reaction listed in Table

3.1 (Alpha DNA, Canada), then run on the Stratagene MxPro 3005 Q-PCR machine. The temperature profile was 3 minute initial denaturation at 95 °C, then 40 cycles of 95°C for 20 seconds, 59°C for 30 seconds, and 72°C for 1 min, with a final elongation of 72°C for 7 min. The house-keeping gene *GAPDH* was used as a reference and the level of other mRNA was measured in relation to *GAPDH*. PCR amplicons were sequenced and confirmed the gene of interest was amplified.

3.3.6. Statistical Analysis

For the nuclear maturation experiment, a 2-sample test for equality of proportions with continuity corrections was used. For the expression of maternal effect genes the ΔCt (cycle threshold) values obtained from the difference between the Ct values of the normalizing gene (*GAPDH*) and the genes of interest, were compared among treatment groups using a Kruskal-Wallis Rank Sum Test, this included the ΔCt values for the non-matured control. A Post hoc pairwise comparison was done using the Wilcoxon Rank Sum test on any genes showing significance ($p \leq 0.05$). Values of ΔCt were analyzed in place of fold change to avoid data manipulation. In the IVP experiment, the cleavage and blastocysts formation rates were analyzed using a glm model to ensure that the biological replicates or the treatment did not have significant interactions. The proportions cleaved cells as well as the proportion of blastocysts formed were compared among the treatment groups using a 2-sample test for equality of proportions with continuity corrections. All statistics were analyzed within the R environment, a statistical software program [255].

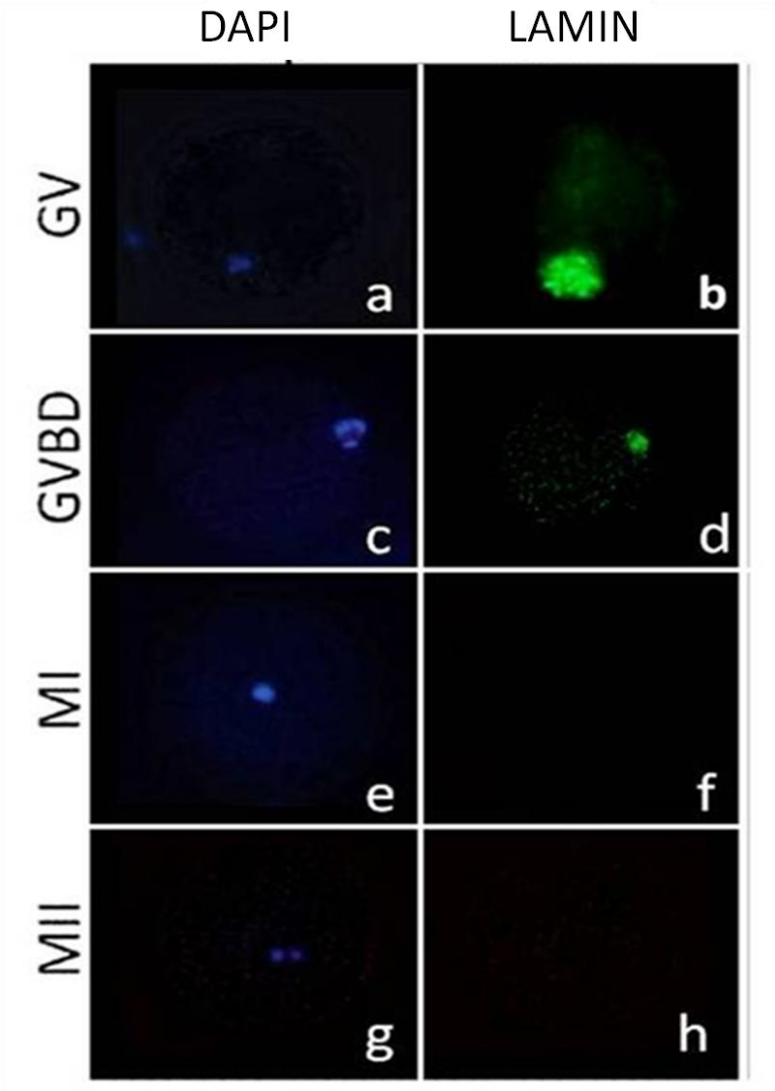

Figure 3.4: DAPI and LAMIN staining examples of oocyte stages: Germinal Vesicle (GV), Germinal Vesicle Breakdown (GVBD), Metaphase I (MI), and Metaphase II (MII). a: DAPI staining of GV, b: LAMIN staining of GV, c: DAPI staining of GVBD, d: LAMIN staining of GVBD, e: DAPI staining of MI, f: LAMIN staining of MI, g: DAPI staining of MII, h:LAMIN staining of MII.

3.4.Results

3.4.1. Evaluation of nuclear maturation of pig oocytes in different IVM media

The purpose of this investigation was to determine if the protein-free maturation medium allowed the oocytes to undergo nuclear maturation as well as the commonly used 10% pFF media. The percentage maturation (MI+MII/n) of 10% pFF and protein-free maturation media did not differ statistically (Table 3.2; $p>0.05$). Both these media promoted maturation and increased the number of oocytes in the MI (10% pFF: 37%, protein-free: 42%) and MII (10% pFF: 54%, protein-free: 47%) stages, with few oocytes remaining in the GV (10% pFF: 6%, protein-free: 9%) and GVBD (10% pFF: 3%, protein-free: 2%) stages. The non-matured group had a significantly lower ($p\leq0.05$) percentage of maturation at 6% (16/251), than the 91% (282/309) of the common 10% pFF maturation media or the 89% (188/211) of the protein-free maturation media. Most of the oocytes within the non-matured group were of GV or GVBD stages; with a few in the MI stage (Table 3.2).

3.4.2. Effects of E_2 on expression of maternal determinant genes

Different levels of E_2 were added into the maturation media and most levels of E_2 showed no significant effect (Fig 3.5) on the expression of the maternal determinant genes when analyzing the ΔCt values. *TRIM24* had a significant difference comparing the treatment groups ($p\leq0.05$). The post hoc comparison indicated that the mean Δ Ct for *TRIM24* differed between the non-matured and the 0 ng/ml E_2 group, and the non-matured and 10% pFF group. The average ΔCt for the 0 ng/ml E_2 group (3.33 ± 1.66) is lower than the non-matured average ΔCt (4.00 ± 0.63), and the average ΔCt for the 10% pFF group (4.75 ± 0.74) is higher than the average non-matured ΔCt (Fig 3.5).

3.4.3. The effects of E_2 on in vitro production of embryos

Cleavage rates for the E_2 (n= 252; 60.2%) or 10% pFF (n= 256; 55.7%) additions to the maturation media did not differ when compared to the protein-free maturation media (n=264; 54.9%, Table 3.3). There was no statistical difference between the blastocyst formation rates of the 10% pFF (n=256; 12.4%) and E_2 (n=252; 14.6%) groups (Table 3.3). However both 10% pFF and E_2 groups had significantly higher blastocyst formation rates ($p \leq 0.05$) than the protein-free maturation media (n=264; 3.5%).

Table 3.2: Nuclear maturation rates in different maturation media.

Treatment	Germinal Vesicle (GV)	Germinal Vesicle Breakdown (GVBD)	Metaphase I (MI)	Metaphase II (MII)	Total Cell Number (n)	Percentage Maturation (MI+MII/n)*100
Non-matured	**219 (87%) [a]**	**16 (6%) [a]**	**16 (6%) [a]**	**0 (0%) [a]**	**251**	**6% [a]**
Protein-free (Control)	**19 (6%) [b]**	**8 (3%) [b]**	**115 (37%) [b]**	**167 (54%) [b]**	**309**	**91% [b]**
10% pFF	**18 (9%) [b]**	**5 (2%) [b]**	**89 (42%) [b]**	**99 (47%) [b]**	**211**	**89% [b]**

Groups with different letters denote significance ($p \leq 0.05$) within each column.

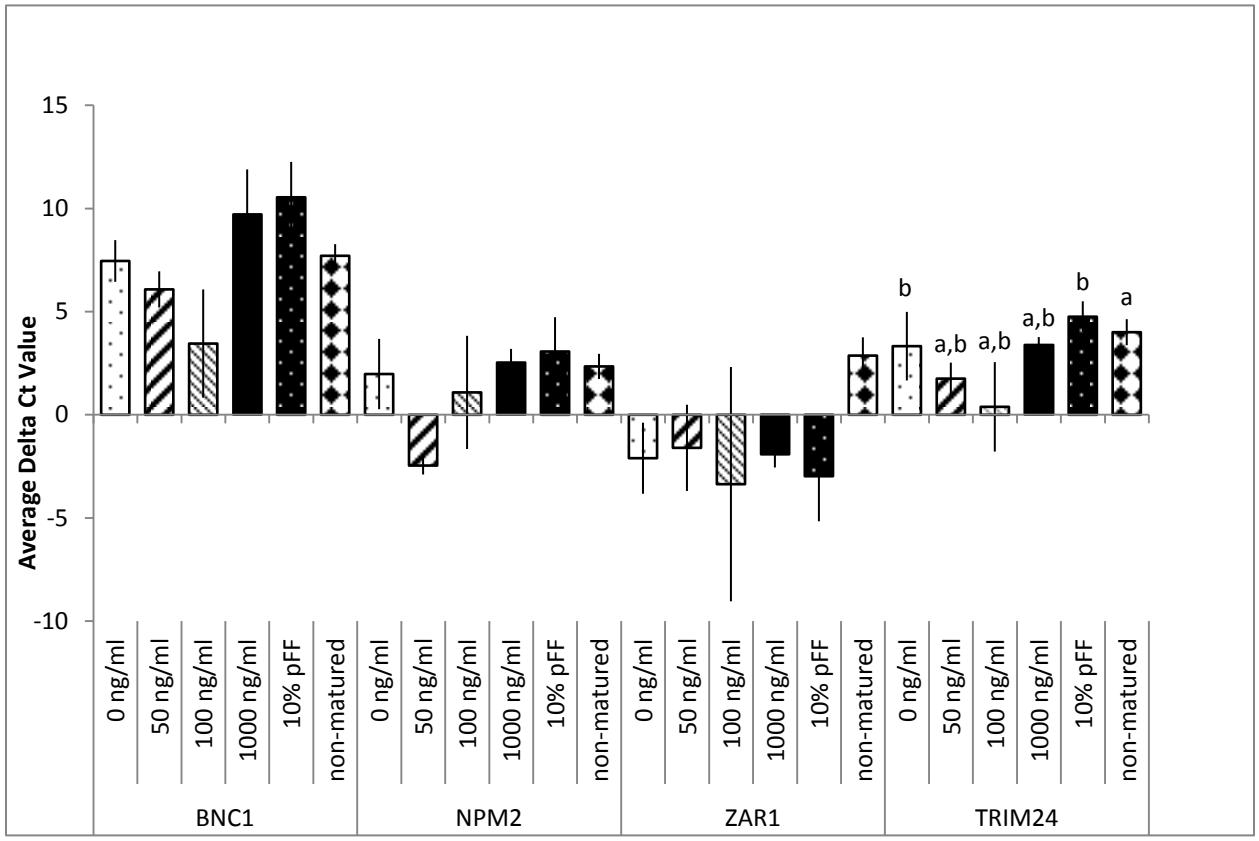

Figure 3.5: Average ΔCt values with SEM of selected maternal determinant genes in porcine oocytes matured in protein-free maturation media supplemented with 0 ng/ml E_2, 50 ng/ml E_2, 100 ng/ml E_2, 1000 ng/ml E_2, or 10% pFF, and a non-matured group. Groups with different letters denote significance ($p \leq 0.05$).

Table 3.3: Cleavage and blastocyst formation rates

Treatment	Cleaved Cells	Blastocysts	Total Cells	Cleavage Rate	Blastocyst Formation Rate
100 ng/ml Estradiol	**154**	**36**	**252**	**61%** [a]	**14%** [a]
Protein-free (Control)	**141**	**9**	**256**	**55%** [a]	**4%** [b]
10% pFF	**145**	**33**	**264**	**55%** [a]	**12%** [b]

Groups with different letters denote significance ($p \leq 0.05$) within each column.

3.5.Discussion

The physiological processes involved in IVM have been classified as nuclear maturation, and cytoplasmic maturation. Follicular fluid as an additive to maturation media has proved beneficial in bovine [256] ovine [257], buffalo [258], equine [259], and porcine [260] IVM systems. However, FF composition varies greatly from sample to sample which decreases the consistency of IVP protocols [6]. Here, the protein-free maturation medium, which was used as a control medium, induced full nuclear maturation at the same rate as the commonly used 10% pFF maturation media when MI and MII stages were observed. This indicates that the protein-free media was a sufficient control to induce nuclear maturation. These results are supported by the development of defined pFF free medium which have successfully produced embryos [245]. As there was no observed effect of pFF supplementation on nuclear maturation of the oocytes, the beneficial effect of pFF in an IVP system must be on molecular cytoplasmic maturation of pig oocytes as there was an observed effect on blastocyst formation rate.

LAMIN/DAPI staining is a newer method of evaluating nuclear maturation in oocytes [254]. LAMIN A/C is located in the nuclear envelope, and as the oocyte resumes meiosis the nuclear envelope containing LAMIN A/C is dismantled. The process of nuclear envelope break down, known as GVBD, is seen through the staining with LAMIN A/C antibodies. DAPI is used to stain the progress of the chromatin, and as the oocyte reached MII two clearly visible spots are seen within the oocyte; one bright spot being the nucleus and the other being the first extruded polar body. Aceto orcein staining has also been used in many studies to confirm nuclear maturation rates in mammalian oocytes [261], but this technique is dependant on the users experience and ability to recognize the different stages of meiosis. Aceto orecin does stain chromatin, however the nuclear status cannot always be determined in the cytoplasm of oocytes with dark lipids [262]. By using LAMIN/DAPI staining there is no cell in which the nuclear envelope or chromatin are masked by the cytoplasm possibly making LAMIN/DAPI staining more efficient. The different nuclear maturation rates seen in other reports [15,247] may not correlate exactly with LAMIN/DAPI evaluation of nuclear maturation as they are two separate techniques for measuring nuclear maturation rates of oocytes.

The maternal to zygote transition is the process in which the embryos genome begins to control embryogenesis, and the maternal mRNA stored within the oocyte during oogenesis degrades [31]. Some recent work suggests that the oocyte is not transcriptionally silent throughout maturation. Transcription gradually declines from GV to MII stages, and there is accumulation of cytoplasmic polyadenylated mRNA at GVBD stage [71]. In cattle, the maternal effect gene transcript basic fibroblast growth factor (bFGF) along with other transcripts (Na+/K+ ATPase α-1 isoform, copper/zinc superoxide dismutase, and cyclins A and B) have been found to change with different IVM medium supplements [29] indicating that the oocytes mRNA may still be affected during this transcriptionally silent stage. Some of the maternal effect genes are known to be directing embryogenesis before the maternal to zygote transition like *BNC1* [178], *NPM2* [179], *ZAR1* [181], and *TRIM24* [184]. *TRIM24* is a transcriptional modulator for nuclear receptors [183] and also regulates certain genes within the first wave of genome activation in mice [184]. It is expressed in the GV stage oocyte of mice to the blastocyst stage, however most embryos lacking *TRIM24* do not develop to the blastocyst stage [184]. Its expression was significantly different in the 0 ng/ml E_2 and 10% pFF groups than the non-matured control; indicating that this gene has an altered expression through IVM (Fig 3.3). Interestingly, the different levels of E_2 within the media had no effect on the expression of maternal determinant genes. The lack of exogenous E_2 in the media seems to have altered the expression from the non-matured control. The non-matured group did not differ statistically from any of the E_2 supplemented groups, indicating the gene expression in the E_2 supplemented groups is most similar to the non-matured cells. Meiotic progression is reported to be suspended with E_2 supplementation during the first half of IVM, and with the removal of E_2 meiosis resumes [25]. Accordingly the oocytes which are exposed to E_2 throughout IVM may not fully mature, and as such it could be hypothesized that the reason no significant difference was observed between the E_2 groups and the non-matured groups is that a higher proportion of E_2 matured oocytes remain at the GV and GVBD stage than the oocytes matured in the 10% pFF or protein-free media. The lower ΔCt values for the 0 ng/ml E_2 could mean that the lack of any estradiol or steroids may increase the degradation of *TRIM24* transcripts through maturation. Addition of 10% pFF during maturation may not only protect the transcripts from being degraded but also releases more from stores, increasing availability of transcripts in the MII stage; this is seen by the increase in ΔCt values.

It has been observed that the introduction of external steroid hormones, including E_2, does not affect nuclear maturation, or some aspects of cytoplasmic maturation such as male pro-nuclear formation in pigs [263]. However, IVP has been shown to be both positively [251] and negatively [214] influenced in cattle with the introduction of E_2. The results of our work illustrate that the introduction of 10% pFF or 100 ng/ml of E_2 during IVM has no influence on the cleavage rate on day 2 of IVC (55.7%, 60.2%, 54.9% respectively). The difference in blastocyst formation rates demonstrate that both pFF and E_2 supplementations to a protein-free maturation medium increase blastocyst formation rates on day 9 of IVC. This supports the idea that pFF increases IVP success, and the positive effects of E_2 are also supported by studies in the literature in cattle and sheep [251,252] as well as in pigs. The composition of pFF is known to be widely inconsistent due to the source from which it is derived [6,201], thus if E_2 can be used as a substitute, it can eliminate the variability in previously accepted protocols.

In summary, E_2 does affect IVM of porcine oocytes. Although nuclear maturation rates did not seem to be affected by the absence of pFF, the effect of pFF may be on the cytoplasmic aspect of maturation. *TRIM24* is one maternal effect gene, which changes levels of mRNA through maturation in a medium devoid of E_2. Although the nuclear maturation rate with E_2 supplementation was not directly studied, it is hypothesized that pFF and E_2 supplemented media have differing effects on nuclear maturation. The similar gene expression of oocytes matured with E_2 supplementation as the non-matured oocytes indicates they may be more meiotically similar than the oocytes matured in 10% pFF or protein-free media. If the E_2 matured groups do have a higher proportion of GV and GVBD stage oocytes, they may also have the same MII rate as they had equivalent blastocyst production rates.

There was an observed change in gene expression through maturation for one gene of interest. Understanding the changes in gene expression throughout maturation as well as the influence of estrogen added to the IVM media will likely increase our success in porcine IVP. E_2 and pFF both increase blastocyst formation rates while having no difference on cleavage rates. Based on this study, the supplementation of E_2 into IVM could replace the pFF in the *in vitro* production of pig embryos.

3.6.Acknowledgements

This work was supported by the Canadian Animal Genetic Resource Program from Agriculture and Agri-Food Canada. The authors would like to thank Alex Pasternak, and Dr. Michael Dyck for assistance with experimental design and analysis; Gillian Gratton and the Animal Care Unit Staff at the University of Saskatchewan for help with animals and the collection of ovaries. Also, Fast Genetic^{TM} (Saskatchewan, Canada) for their in-kind support by providing semen to perform this study.

CHAPTER 4: ESTRADIOL'S INFLUENCE ON GENE EXPRESSION OF PORCINE OOCYTES THROUGH *IN VITRO* MATURATION

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4.1.Abstract

In vitro maturation (IVM) is the possibly the most important step when producing porcine embryos *in vitro*. During the oocyte growth phase maternal RNA and proteins are stored, to be used during maturation and embryogenesis. The transcripts within the oocyte can be altered based on the *in vitro* maturation environment. In other species, thousands of differentially expressed genes are seen through maturation, yet this has not been studied in the pig. In porcine IVM a high amount of estrogen is added to maturation medium through the supplementation of IVM media with pFF. For this study, estrogen is hypothesized to influence gene expression of porcine oocytes through maturation. Oocytes were matured in a control protein-free media and the protein-free media supplemented with 100 ng/ml estradiol 17-β or 10% pFF. A non-matured sample was also obtained, and all four groups of oocytes were denuded and prepared for microarray analysis. The groups were compared in a reference design with a pooled reference representing the population of matured and non-matured oocytes. Microarray data was transformed and statistically analysed, and thresholds were assigned. The control, 100 ng/ml estradiol 17-β, and 10% pFF groups were compared to each other, and against the non-matured groups. Genes were not differentially expressed among the matured groups with the outlined threshold values of $-2 \geq \log2(\text{fold change}) \geq 2$, and adjusted p-value ≤ 0.05. A total of 16 differentially expressed genes between the non-matured and all matured groups exceeded this threshold. Of these genes, 6 are novel transcribed regions with evidence of being an embryonic EST, and 1 is a novel protein-coding gene. The other genes are FBJ murine osteosarcoma viral oncogene homolog (Fos), Vimentin (*VIM*), Capthesin C (*CTSC*), Selenium binding protein 1 (*SELENBP1*), Poly(A) binding protein cytoplasmic 1 (*PABPC1*), Tissue factor pathway inhibitor 2 (*TFPI2*), Cysteine-rich, angiogenic inducer 61 (*CYR61*), Acyl-CoA synthetase long-chain family member 6 (*ACSL6*), and Phospholipase A2 group VII (*PLA2G7*). There was an increased expression of *FOS*, *VIM*, *SELENBP1*, *PABPC1*, *CYR61*, *ACSL6* and *PLA2G7*. There was a decreased expression of *CTSC* and *TFPI2*. The changes in gene expression throughout maturation for these genes indicate they play a role in maturation; but the supplementation of maturation medium with estrogen did not influence the gene expression of porcine oocytes through maturation.

4.2.Introduction

In vitro maturation (IVM) is the first step in *in vitro* production of embryos, and it prepares the oocyte for all subsequent steps. Without proper accumulation of maternal mRNA during growth of an oocyte, a fertilized zygote will not develop any further [77]. Interestingly, the oocyte transcripts can be influenced, during IVM, through supplementation of maturation media [29,250]. Early embryonic development in mammals, prior to the embryonic genome activation is regulated by maternal mRNA and proteins stored during the oocyte growth phase [170]. There is a downregulation of RNA transcription in the immature oocyte of the mid-antral follicle [264], correlating to the quiescent GV stage oocyte [170]. It is essential for the maturation of the oocytes and the developmental potential of the embryo that prior to the GV stage there is optimal storage of stable mRNA (reviewed by [59]).

While the transcription within the oocyte is most active in the growing phase, and decreases after GVBD, there are differences in the global mRNA transcripts between GV and MII oocytes [27,28]. Over 800 genes have differential expression during meiotic maturation in the bovine oocyte [70]. In the human, 1200 genes are differentially expressed between the GV/MI and the MII stages of maturation, which suggests that major modifications in gene transcripts occur between these stages [28]. The changes in expression is due to selective degradation or utilization and poly-A-tail elongation of maternal mRNA transcripts during maturation not transcription itself (reviewed by [59]). The increased abundance of expressed gene transcripts at the GV stage suggests that these transcripts play a role prior to meiotic completion [30].

Oocytes matured *in vivo* are shown to have superior competence when compared to *in vitro* matured oocytes, therefore the maturation process has the ability to affect the competence of the oocyte. Bovine oocytes recovered at the pre-ovulatory surge and matured *in vitro* were compared to the *in vivo* matured oocytes after *in vitro* fertilization and *in vitro* culturing. An increased blastocyst formation rate was observed with the *in vivo* matured oocytes [265]; this shows that the *in vitro* environment affects oocyte competency. In swine, *in vivo* and *in vitro* derived oocytes were subjected to subzonal fertilization and the major difference seen was with

blastocyst formation, again emphasizing the influence of the *in vitro* environment of oocyte competency [7]. If the oocytes differ developmentally, they may differ at a molecular level. Gene transcripts in bovine oocytes matured *in vivo* and *in vitro* were compared, and a difference in relative mRNA abundance of several developmentally important genes was perceived [266]. This illustrates that the maturation environment influences the expression of oocyte gene transcripts.

Oocyte maturation media has been shown to influence levels of oocyte gene transcripts. The abundance of gene transcripts was also correlated with oocyte competence [29]. Porcine FF supplementation in the maturation medium is proven to be an important factor [201]. Interestingly, the concentration of estrogen can be very high in pFF [24]. Transient estrogen has been shown to improve maturation of porcine oocytes as well as blastocyst formation following IVF [25]. For this study, we hypothesize that the addition of estradiol 17-β (E_2) to maturation medium can affect the gene expression in porcine oocytes.

4.3.Materials and Methods

All chemicals were obtained from Sigma-Aldrich Canada Chemical Company unless otherwise noted. The microarray used was the EmbryoGENE Porcine Transcriptomics Microarray which was developed as a part if the EmbryoGENE NSERC Strategic Research Network was printed and supplied by Agilent Canada.

4.3.1. Experimental Design

The experimental designed is outlined in Fig 4.1. The cumulus oocyte complexes (COCs) were conveniently allocated into groups of 40 for culture with the following added into protein-free maturation medium: 0 ng/ml E_2 and 0% pFF (control), 100 ng/ml E_2, or 10% pFF. After maturation these oocytes and a non-matured control were denuded and 10 of the oocytes from each group (test sample) were stored at -80°C for extraction. The remaining 30 oocytes from

each group (reference sample) were stored in separate tubes at -80°C; these oocytes would be combined after extraction to be used as the reference. Five separate maturations were completed to comprise 5 biological replicates.

Each sample underwent extraction separately, the best RNA from four test samples and four reference samples for each group was chosen for further steps. Then equal amounts of RNA from reference samples were pooled into two groups. Each test sample was labelled, and the two pooled reference groups were combined then separated into 8 reactions to be labelled. The 8 labelling reactions for the pooled reference sample were combined, and equal amounts were added to the hybridization reactions with the test samples.

4.3.2. Oocyte Collection and Maturation

Ovaries from pre-pubertal gilts were obtained from an abattoir and were transported to the laboratory in an insulated container. After washing the ovaries with physiological saline, follicles of 2-5 mm in diameter were aspirated using 18G needle and 5 ml syringe, and COCs were screened and graded. Grades 1 and 2 COCs were selected and transferred into a final maturation medium in one well of a NUNC 4-well plate (Thermo Scientific, USA); without mineral oil overlay to avoid absorption of steroids. The protein free maturation medium was composed of Medium -199 (12340-030, Invitrogen, Canada) with 0.1% polyvinyl alcohol (P8136), 3.05 mM D-glucose (G7021), 0.91 mM Sodium Pyruvate (P4562), Antibiotics 1X (15240-062, Invitrogen, Canada), 10 ng/ml EGF (E4127), 0.57 mM L-Cysteine (W326305, SAFC/Sigma, Canada), and 0.01 U/ml Lutropin-V® (Bioniche, Canada), 0.01 U/ml Folltropin® (Bioniche, Canada). The pFF used to supplement of the media was obtained from aspiration of antral follicles and centrifuging at 3000 X g for 15 min then collecting and filtering the supernatant. Estradiol 17-β (E2758) was added to one medium after being dissolved into 100% ethanol. The COCs were incubated in a 5% CO_2, 5% O_2, and 90% N_2 humidified environment for 40 h. Oocytes from the matured and non-matured groups were denuded in 0.1% hyaluronidase (H3884) in Medium-199.

4.3.3. RNA Extraction

Following IVM, samples were washed in $MgCl_2$ free phosphate buffered saline (PBS) and stored at -80°C in a minimal amount of $MgCl_2$ free PBS. The Arcturus® PicoPure™ RNA Isolation Kit (KIT0204, Applied Biosystems, Canada) was used for the extraction. Following the first wash of the RNA purification column, DNase mix was prepared according to instructions from the RNase-Free DNase Set (79254, Qiagen, Canada), added to the purification column and incubated at room temperature for 15 min. The RNA was eluted from the column using nuclease free water and each sample was run on the Agilent 2100 Bioanalyzer (Agilent Technologies, USA) for quality control verification.

4.3.4. RNA Amplification

The RNA which met the quality control verification underwent amplification using the 1647 Arcturus® RiboAmp® HSPlus RNA Amplification Kit (KIT0525, Applied Biosystems, Canada). There was 1000 to 3000 pg of RNA from each test sample was used for amplification. The variation in starting material was due to availability of RNA after extraction. An amount of 5000 pg of combined RNA was used for amplification of the pooled reference samples. During the 1st strand cDNA synthesis, SuperScript™ III Reverse Transcriptase (18080-093, Qiagen, Canada) was added to the 1st Strand Synthesis components prior to incubation. Elution buffer was used to elute the aRNA, and it was stored at -80°C. Quality control verification was run using the Nanodrop™ 2000 (Thermo Scientific, USA) to insure the amplification was successful, and that the aRNA was pure.

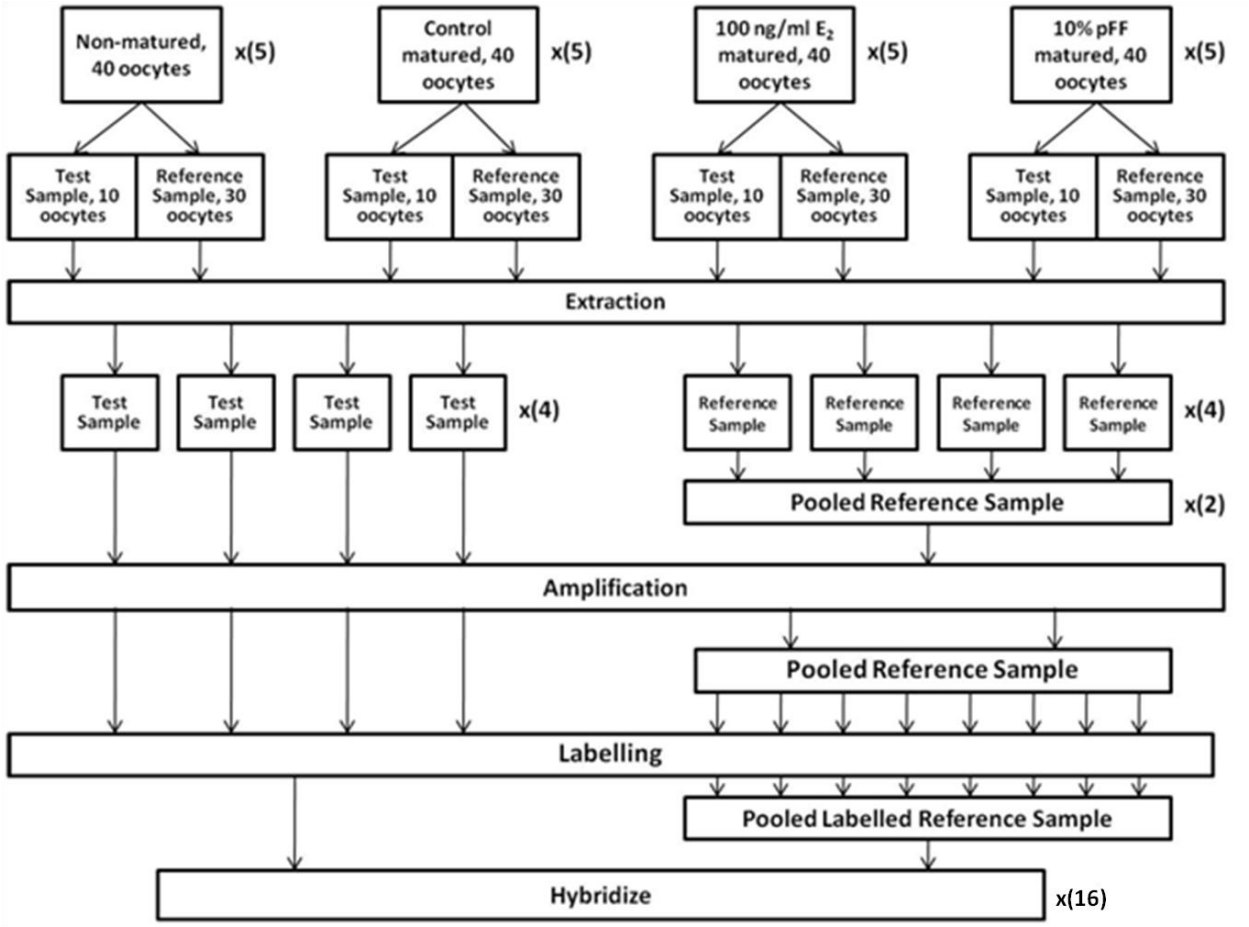

Figure 4.1: Experimental design to evaluate gene expression using microarray. Oocytes were collected and assigned into groups; these groups were further divided after maturation for reference and test samples. For each step reference samples were pooled or separated based on the number of reactions which needed to be preformed to obtain the required amount of material for the hybridization.

4.3.5. aRNA Labelling

Only aRNA of high quality was used for labelling. A 2 µg sample of aRNA was used for the ULS™ Fluorescent Labeling Kit for Agilent arrays (with cyanine 3 and cyanine 5) (EA-021, Kreatech, Netherlands). To remove any unlabelled aRNA, the PicoPure™ RNA Isolation Kit (KIT0204, Applied Biosystems, Canada) was used. The labelled aRNA was run on the Nanodrop™ 2000 to ensure the appropriate concentration as well as the labelling efficiency of aRNA. The test samples were labelled with cyanine 3 (Cy3) and the reference sample was labelled with cyanine 5 (Cy5).

4.3.6. Hybridization

Fragmentation of labelled aRNA was performed before hybridizing on the microarray slides. The Agilent spike and aRNA labelled with Cy3 and Cy5 were used for Gene Expression Hybridization Kit (5188-5242, Agilent, Canada) protocol.

Equal volumes of the 2x GEx Hybridization Buffer HI-RPM and fragmentation mix are combined and a volume of 110 µl was added to the gasket side of the Agilent slide containing 4 of the 44K EmbryoGENE Porcine Transcriptomics Microarrays. Using the Agilent SureHyb Hybridization chamber (G2534A, Agilent, Canada), the array was hybridized for 17 h at 65°C while rotating.

After 17 h the chamber was removed from the hybridization oven and disassembled in Gene Expression Wash Buffer 1 (5188-5325, Agilent, Canada) at room temperature. Then the slides were washed in Gene Expression Wash Buffer 1 for three minutes at room temperature while being stirred with a magnetic stir bar. The slides were then transferred to Gene Expression Wash Buffer 2 (5188-5326, Agilent, Canada) for 3 minutes at 42°C while stirring. To protect against ozone degradation of Cy5 dyes, the slides were transferred to an acetonitrile wash for 10 seconds then to the Stabilization and Drying Solution wash (5185-5979, Agilent, Canada) for 30 seconds at room temperature while stirring.

4.3.7. *Microarray Images*

The microarrays were scanned using the GenePix 4200 Autoloader (Molecular Devices, USA) using the GenePix Pro Acuity 4.0 Microarray Informatics Software (Molecular Devices, USA). Each array was scanned individually using Auto PMT, and a pixel size of 5 µm.

4.3.8. Statistical Analysis

The microarray intensity files were uploaded to EmbryoGENE LIMS and Microarray Analysis (ELMA), EmbryoGENE's web based application (http://elma.embryogene.ca/login). Using this application, files were exported to Flexarray [267]. A simple background correction was performed on the raw data, and the Loess normalization method was applied to normalize dye-bias within each array. To compare the groups among each other the Limma package was used, as there was no ANOVA function in Flexarray. Limma is a package for analysing differential expression from microarray experiments. False discovery rate, Benjamini–Hochberg–Yekutieli, was applied to the differentially expressed genes. The threshold was set at $-2 \geq \log2(\text{fold change}) \geq 2$, and adjusted p-value ≤ 0.05.

4.3.9. Gene Functions

The biological pathways of all genes, which were differentially expressed, were analysed using the NCBI BioSystems database [268]. A literature review was completed to evaluate whether each of the genes played a role in reproduction.

4.4.Results

There were no genes differentially expressed between the groups matured in 100 ng/ml E_2, 10% pFF, or the control media when the threshold values were applied. When comparing each of the matured groups to the non-matured groups, there were differences in gene expression with threshold values set at $-2 \geq \log2(\text{fold change}) \geq 2$, and adjusted p-value ≤ 0.05. The results for differentially expressed genes are summarized in Tables 4.1 (Non-matured vs Control), 4.2 (Non-matured vs 100 ng/ml E_2), 4.3 (Non-matured vs 10% pFF), and Fig 4.2.

There was a significant difference in gene expression from all matured groups when compared to non-matured oocytes. All maturation groups when compared to the non-matured group showed 16 genes expressed differently (Fig 4.3), 7 are Novel genes. There are 6 Novel transcribed regions with evidence of them being an embryonic expressed sequence tag (EST). The other genes which showed a differing expression were FBJ murine osteosarcoma viral oncogene homolog (*FOS*), Vimentin (*VIM*), Capthesin C (*CTSC*), Selenium binding protein 1 (*SELENBP1*), Poly(A) binding protein cytoplasmic 1 (*PABPC1*), Tissue factor pathway inhibitor 2 (*TFP12*), Cysteine-rich, angiogenic inducer 61 (*CYR61*), Acyl-CoA synthetase long-chain family member 6 (*ACSL6*), and Phospholipase A2 group VII (*PLA2G7*) (Table 4.4). All of these genes play a role in reproduction.

There were 16 differentially expressed from the non-matured groups when compared to all 3 matured groups. Tables 4.1, 4.2, and 4.3 list the genes which differ between each matured group and the non-matured group. The genes differing from all matured groups and the non-matured group are denoted by bolding, italicizing and underlining. In each of these tables and within Fig 4.2 there are many other genes which seem to not be common among all matured groups; this is due to the imposed threshold limitations. Genes that did not meet $-2 \geq \log2(\text{fold change}) \geq 2$, and adjusted p-value ≤ 0.05 were not considered common among all matured groups for this study, however the fold change value for these genes was slightly lower than threshold but was still differentially expressed from the non-matured group. The global differential gene expression may be investigated in further studies to form a complete picture of changes in gene expression through maturation.

Table 4.1: Differentially expressed genes in control matured group when compared to non-matured group. Gene symbols which denoted with (*) are common to all matured groups.

Gene Symbol	Gene Description
NULL	Novel Transcribed Region; evidence: embryonic ESTs
NULL	17-beta-hydroxysteroid dehydrogenase type 1 [Source:UniProtKB/TrEMBL;Acc:B2X0A4]
FOS*	FBJ murine osteosarcoma viral oncogene homolog [Source:HGNC Symbol;Acc:3796]
CTSC*	cathepsin C [Source:HGNC Symbol;Acc:2528]
HTRA1	HtrA serine peptidase 1 [Source:HGNC Symbol;Acc:9476]
NULL	suprabasin [Source:HGNC Symbol;Acc:24950]
NULL	Aldehyde dehydrogenase, mitochondrial Precursor (EC 1.2.1.3)(ALDH class 2)(ALDH-E2) [Source:UniProtKB/Swiss-Prot;Acc:Q2XQV4]
VIM*	vimentin [Source:HGNC Symbol;Acc:12692]
NULL*	Novel Transcribed Region; evidence: embryonic ESTs
SPINK6	serine peptidase inhibitor, Kazal type 6 [Source:HGNC Symbol;Acc:29486]
NULL*	Novel Transcribed Region; evidence: embryonic ESTs
NULL*	Novel Gene
FST	follistatin [Source:HGNC Symbol;Acc:3971]
CYR61*	cysteine-rich, angiogenic inducer, 61 [Source:HGNC Symbol;Acc:2654]
NULL	Novel Transcribed Region; evidence: embryonic ESTs
HNRNPA3	heterogeneous nuclear ribonucleoprotein A3 [Source:HGNC Symbol;Acc:24941]
PLEKHH2	pleckstrin homology domain containing, family H (with MyTH4 domain) member 2 [Source:HGNC Symbol;Acc:30506]
NULL	Novel Transcribed Region; evidence: embryonic ESTs
PABPC3*	poly(A) binding protein, cytoplasmic 1 [Source:HGNC Symbol;Acc:8554]
NULL	Novel Transcribed Region; evidence: embryonic ESTs
NULL	Novel Transcribed Region; evidence: embryonic ESTs
NULL	regulator of G-protein signaling 3 [Source:HGNC Symbol;Acc:9999]
NULL*	Novel Transcribed Region; evidence: embryonic ESTs
PABPC3*	poly(A) binding protein, cytoplasmic 1 [Source:HGNC Symbol;Acc:8554]
GKN1	gastrokine 1 [Source:HGNC Symbol;Acc:23217]
NULL	Novel Transcribed Region; evidence: embryonic ESTs
PLA2G7*	phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma) [Source:HGNC Symbol;Acc:9040]
NULL	cytochrome P450 2C49 [Source:RefSeq peptide;Acc:NP_999585]
VIM	vimentin [Source:HGNC Symbol;Acc:12692]

NULL	Novel Transcribed Region; evidence: embryonic ESTs
TFPI2*	tissue factor pathway inhibitor 2 [Source:HGNC Symbol;Acc:11761]
ACSL6*	acyl-CoA synthetase long-chain family member 6 [Source:HGNC Symbol;Acc:16496]
NULL*	Novel Transcribed Region; evidence: embryonic ESTs
SELENBP1*	selenium binding protein 1 [Source:HGNC Symbol;Acc:10719]
NULL*	Novel Transcribed Region; evidence: embryonic ESTs
NULL	17-beta-hydroxysteroid dehydrogenase type 1 [Source:UniProtKB/TrEMBL;Acc:B2X0A4]
ITM2A	integral membrane protein 2A [Source:HGNC Symbol;Acc:6173]
NULL*	Novel Transcribed Region; evidence: embryonic ESTs
SNX7	sorting nexin 7 [Source:HGNC Symbol;Acc:14971]

Table 4.2: Genes which are differentially expressed between the non-matured group and the group matured in 100 ng/ml E_2. Gene symbols which denoted with (*) are common to all matured groups.

Gene Symbol	Gene Description
NULL	Novel pseudogene
NULL*	Novel Transcribed Region; evidence: embryonic ESTs
PABPC3*	poly(A) binding protein, cytoplasmic 1 [Source:HGNC Symbol;Acc:8554]
NULL	Novel Transcribed Region; evidence: embryonic ESTs
ACSL6*	acyl-CoA synthetase long-chain family member 6 [Source:HGNC Symbol;Acc:16496]
VIM*	vimentin [Source:HGNC Symbol;Acc:12692]
SELENBP1*	selenium binding protein 1 [Source:HGNC Symbol;Acc:10719]
VIM*	vimentin [Source:HGNC Symbol;Acc:12692]
FOS*	FBJ murine osteosarcoma viral oncogene homolog [Source:HGNC Symbol;Acc:3796]
NULL*	Novel Transcribed Region; evidence: embryonic ESTs
CTSC*	cathepsin C [Source:HGNC Symbol;Acc:2528]
NULL	Novel Transcribed Region; evidence: embryonic ESTs
TFPI2*	tissue factor pathway inhibitor 2 [Source:HGNC Symbol;Acc:11761]
HTRA1	HtrA serine peptidase 1 [Source:HGNC Symbol;Acc:9476]
NULL*	Novel Gene
NULL*	Novel Transcribed Region; evidence: embryonic ESTs
PLA2G7*	phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma) [Source:HGNC Symbol;Acc:9040]
CYR61*	cysteine-rich, angiogenic inducer, 61 [Source:HGNC Symbol;Acc:2654]
NULL*	Novel Transcribed Region; evidence: embryonic ESTs
NULL*	Novel Transcribed Region; evidence: embryonic ESTs
NULL*	Novel Transcribed Region; evidence: embryonic ESTs
PABPC3*	poly(A) binding protein, cytoplasmic 1 [Source:HGNC Symbol;Acc:8554]
NULL	Novel Transcribed Region; evidence: embryonic ESTs
NULL	Novel Transcribed Region; evidence: embryonic ESTs
NULL	Novel Transcribed Region; evidence: embryonic ESTs

Table 4.3: Genes which are differentially expressed between the non-matured group and the group matured in 10% pFF. Gene symbols which denoted with (*) are common to all matured groups.

Gene Symbol	Gene Description
NULL	Aldehyde dehydrogenase, mitochondrial Precursor (EC 1.2.1.3)(ALDH class 2)(ALDH-E2) [Source:UniProtKB/Swiss-Prot;Acc:Q2XQV4]
NULL	Novel pseudogene
NULL	cytochrome P450 2C49 [Source:RefSeq peptide;Acc:NP_999585]
NULL*	Novel Transcribed Region; evidence: embryonic ESTs
OPCML	neurotrimin [Source:HGNC Symbol;Acc:17941]
ACSL6*	acyl-CoA synthetase long-chain family member 6 [Source:HGNC Symbol;Acc:16496]
NULL	Translation elongation factor eEF-1 alpha chain Fragment [Source:UniProtKB/TrEMBL;Acc:Q29837]
SELENBP1*	selenium binding protein 1 [Source:HGNC Symbol;Acc:10719]
VIM*	vimentin [Source:HGNC Symbol;Acc:12692]
FOS*	FBJ murine osteosarcoma viral oncogene homolog [Source:HGNC Symbol;Acc:3796]
NULL*	Novel Transcribed Region; evidence: embryonic ESTs
CTSC*	cathepsin C [Source:HGNC Symbol;Acc:2528]
NULL	Novel Transcribed Region; evidence: embryonic ESTs
TFPI2*	tissue factor pathway inhibitor 2 [Source:HGNC Symbol;Acc:11761]
NULL	Novel protein coding
NULL*	Novel Gene
NULL*	Novel Transcribed Region; evidence: embryonic ESTs
PLA2G7*	phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma) [Source:HGNC Symbol;Acc:9040]
CYR61*	cysteine-rich, angiogenic inducer, 61 [Source:HGNC Symbol;Acc:2654]
NULL*	Novel Transcribed Region; evidence: embryonic ESTs
HNRNPA3	heterogeneous nuclear ribonucleoprotein A3 [Source:HGNC Symbol;Acc:24941]
NULL*	Novel Transcribed Region; evidence: embryonic ESTs
NULL*	Novel Transcribed Region; evidence: embryonic ESTs
NULL	Translation elongation factor eEF-1 alpha chain Fragment [Source:UniProtKB/TrEMBL;Acc:Q29837]
NULL	Novel Transcribed Region; evidence: embryonic ESTs
PABPC3*	poly(A) binding protein, cytoplasmic 1 [Source:HGNC Symbol;Acc:8554]
NULL	Glutathione S-transferase Fragment (EC 2.5.1.18) [Source:UniProtKB/TrEMBL;Acc:Q29418]
U6	U6 spliceosomal RNA [Source: RFAM;Acc:RF00026]
GKN1	gastrokine 1 [Source:HGNC Symbol;Acc:23217]
NULL	suprabasin [Source:HGNC Symbol;Acc:24950]

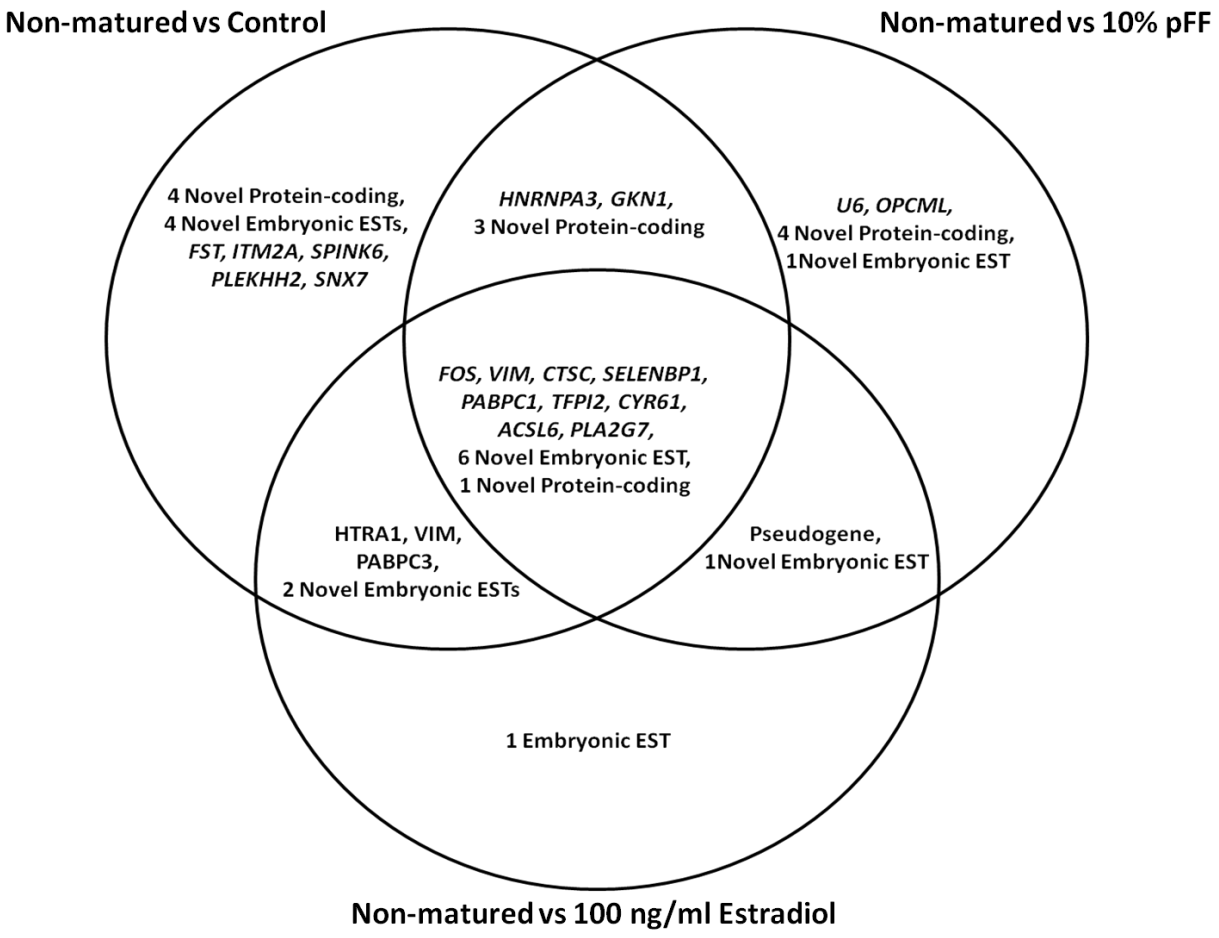

Figure 4.2: A Venn diagram showing the distribution of differentially expressed genes from the non-matured group for each treatment.

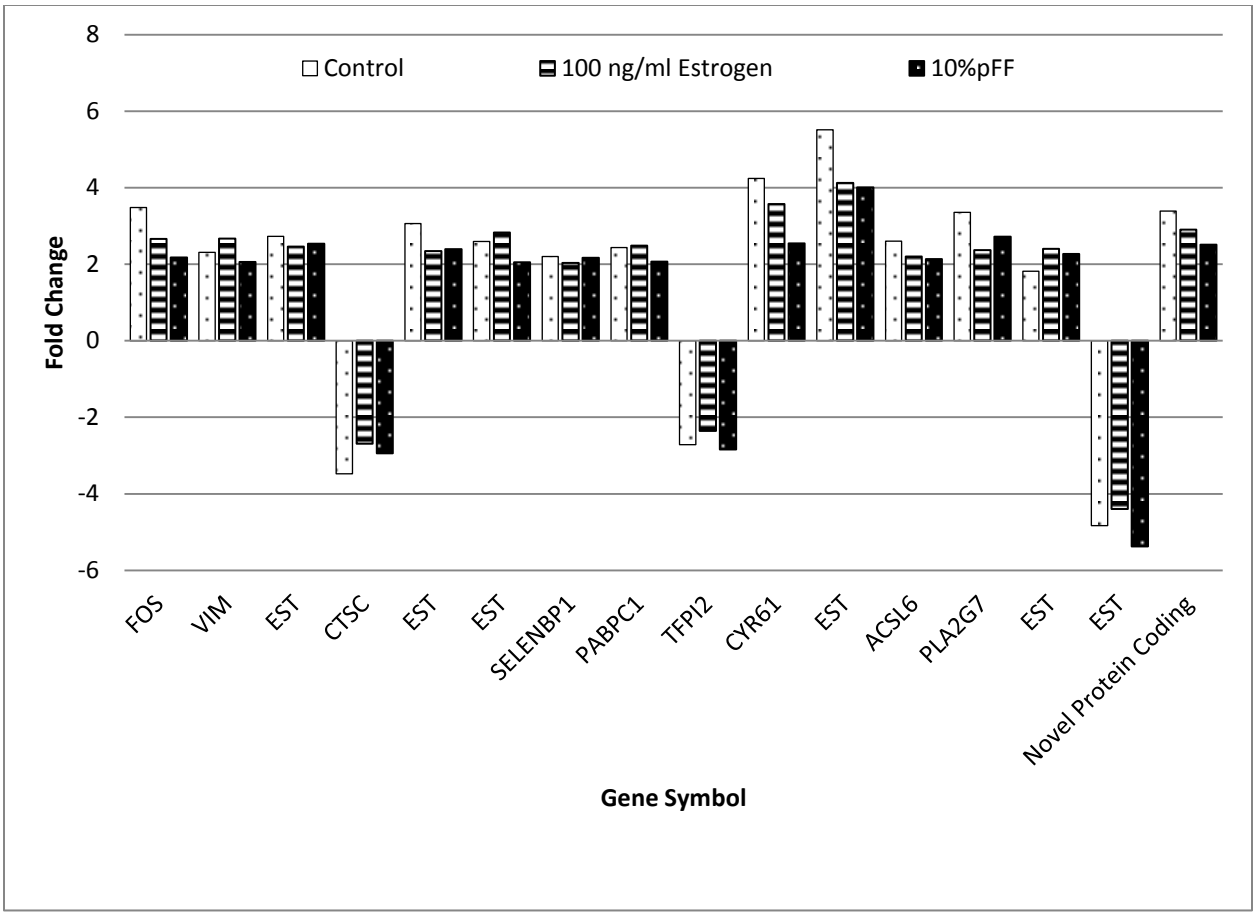

Figure 4.3: Fold change values of differentially expressed genes from the non-matured group when compared to the matured groups. These genes are common to the control, 100 ng/ml E_2, and 10% pFF matured groups.

Table 4.4: Biological pathways of the common genes which change throughout maturation. It is noted whether these genes have been shown to play a role in reproduction.

Gene Symbol	**Gene Name**	**Does this Gene play a Role in reproduction**	**Biological Pathways**
FOS	FBJ murine osteosarcoma viral oncogene homolog	Yes	Colorectal cancer (from KEGG)
			Chagas disease (American trypanosomiasis)(from KEGG)
			B cell receptor signaling pathway (from KEGG)
			Activation of the AP-1 family of transcription factors (from REACTOME)
			Activated TLR4 signalling (from REACTOME)
			Innate Immunity Signaling (from REACTOME)
			Leishmaniasis (from KEGG)
			MAP kinase activation in TLR cascade (from REACTOME)
			MAPK signaling pathway (from KEGG)
			MAPK targets/ Nuclear events mediated by MAP kinases (from REACTOME)
			MyD88 cascade initiated on plasma membrane (from REACTOME)
			MyD88 dependent cascade initiated on endosome (from REACTOME)
			MyD88-independent cascade initiated on plasma membrane (from REACTOME)
			MyD88:Mal cascade initiated on plasma membrane (from REACTOME)

			NFkB and MAP kinases activation mediated by TLR4 signaling repertoire (from REACTOME)
			Osteoclast differentiation (from KEGG)
			Pathways in cancer (from KEGG)
			Rheumatoid arthritis (from KEGG)
			Signaling in Immune system (from REACTOME)
			T cell receptor signaling pathway (from KEGG)
			TRAF6 Mediated Induction of proinflammatory cytokines (from REACTOME)
			TRAF6 mediated induction of NFkB and MAP kinases upon TLR7/8 or 9 activation (from REACTOME)
			Toll Like Receptor 10 (TLR10) Cascade (from REACTOME)
			Toll Like Receptor 2 Cascade, organism-specific biosystem (from REACTOME)
			Toll Like Receptor 3 (TLR3) Cascade (from REACTOME)
			Toll Like Receptor 4 (TLR4) Cascade (from REACTOME)
			Toll Like Receptor 5 (TLR5) Cascade (from REACTOME)
			Toll Like Receptor 7/8 (TLR7/8) Cascade (from REACTOME)
			Toll Like Receptor 9 (TLR9) Cascade (from REACTOME)
			Toll Like Receptor TLR1:TLR2 Cascade (from REACTOME)
			Toll Like Receptor TLR6:TLR2 Cascade (from REACTOME)

			Toll Receptor Cascades (from REACTOME)
			Toll-like receptor signaling pathway (from KEGG)
VIM	vimentin	Yes	Caspase-mediated cleavage of cytoskeletal proteins (from REACTOME)
			Apoptotic cleavage of cellular proteins(from REACTOME)
			Apoptotic execution phase(from REACTOME)
			Apoptosis(from REACTOME)
			Striated Muscle Contraction(from REACTOME)
			Muscle contraction(from REACTOME)
			Amoebiasis (from KEGG)
			Lysosome(from KEGG)
			Systemic lupus erythematosus(from KEGG)
			Renin-angiotensin system(from KEGG)
			Neuroactive ligand-receptor interaction(from KEGG)
CTSC	cathepsin C	Yes	Lysosome (from KEGG)
SELENBP1	selenium binding protein 1	Yes	Metabolic pathways (from KEGG)
			Amyotrophic lateral sclerosis (ALS)(from KEGG)
			Selenocompound metabolism(from KEGG)
			Fat digestion and absorption(from KEGG)
			Huntington's disease(from KEGG)
			PPAR signaling pathway(from KEGG)
			Aminoacyl-tRNA biosynthesis(from KEGG)
			Methane metabolism(from KEGG)
			Glyoxylate and dicarboxylate metabolism(from KEGG)

			Arachidonic acid metabolism(from KEGG)
			Glutathione metabolism(from KEGG)
PABPC1	poly(A) binding protein, cytoplasmic 1	Yes	RNA degradation (from KEGG)
			RNA transport (from KEGG)
			mRNA surveillance pathway (from KEGG)
TFPI2	tissue factor pathway inhibitor 2-	Yes	Complement and coagulation cascades (from KEGG)
CYR61	cysteine-rich, angiogenic inducer, 61	Yes	RNA transport (from KEGG)
ACSL6	acyl-CoA synthetase long-chain family member 6	Yes	Adipocytokine signaling pathway (from KEGG)
			Peroxisome (from KEGG)
			PPAR signaling pathway (from KEGG)
			Fatty acid metabolism (from KEGG)
			Metabolic pathways (from KEGG)
PLA2G7	phospholipase A2, group VII	Yes	Metabolic pathways (from KEGG)
			Ether lipid metabolism (from KEGG)

4.5.Discussion

The introduction of pFF in maturation media has proven effective for both cytoplasmic and nuclear maturation [201]. Among the many components of pFF, the concentrations of steroids, namely estrogen is high [24]. While the transient supplementation of estrogen in maturation media has improved maturation and oocyte developmental competence [25], we did not observe any effects of pFF or estrogen on oocyte gene transcripts through maturation illustrated by no significant differences in gene expression between the matured groups. There was no differential gene expression between groups of oocytes matured in these three media once the threshold values of $-2 \geq \log2(\text{fold change}) \geq 2$, and adjusted p-value ≤ 0.05 were applied. There may have been differences before the false discovery tests were performed, but these differences could not be included into this study.

In previous research (data not shown) we had shown the 100 ng/ml of E_2 and 10% pFF additions to maturation media did lead to an increased blastocyst formation rate when compared to a control. Following the same protocol for supplementing the media with E_2 for the entire maturation period there was no effect seen during this experiment on differential expression of genes with the microarray we used. Kim et al, 2011 used a transient supplementation of E_2 within the maturation media and found that E_2 introduced only during the first half of maturation increased maturation as well as embryo development [25]. Further investigation as to the effects of timing the E_2 introduction into media should be undertaken.

FOS has been shown to inhibit the actions of 17α-hydroxylase 17,20 lyase (CYP17) within the theca cells of the ovarian follicle [264]. In response to LH, theca cells produce androgens which are transported to granulosa cells to form steroids in response to FSH [269]. CYP17 is one of three enzymes involved in androgen production in the theca cells. The Protein kinase C (PKC) pathway is a principal androgenic pathway in the theca cell which through *FOS* can control CYP17 to regulate androgen production [264]. *FOS* has been localized in abundance within the granulosa cells, and in small amounts in the theca cells [269]. As the PKC pathway is capable of functioning in the granulosa cells [270], it can be supposed that the high levels of *FOS* within the granulosa cells is responsible for inhibiting CYP17 and thus inhibiting the granulosa

from producing androgens independent from the theca cells [264]. The increased abundance of *FOS* gene transcripts, which are involved in androgen production, indicates that mature oocytes are less responsive to androgens than the non-matured oocytes. This conclusion is in support of current research [202].

VIM is known to code for an intermediary filament protein, this protein is present in the oocyte [271] as well as lymphocytes [272],smooth muscle [273], and hepatic cells [274] . *VIM* proteins are expressed dynamically throughout embryonic development [275]. *VIM* protein, vimentin, can be used as a marker for cells which will develop into mesenchymal cells within the embryo [276]. Determining the timeline of differentiation in pig embryos could be of importance for future stem cell lines. The increased expression of *VIM* transcripts through maturation in this study, may indicate that the mature oocyte responds more appropriately to cytoskeletal remodelling, which takes place through cytoplasmic maturation and fertilization.

CTSC is a gene highly conserved among mammalian and non mammalian species [277]. In the mub crab, the expression of *CTSC* was significantly higher in ovarian than testicular tissues [278]. In the kuruma prawn, *CTSC* expression was also high within the ovary, and is supposed to regulate ovarian growth [279]. Also in the kuruma prawn, *CTSC* was upregulated in the final stages of oocyte maturation, especially during the cortical rod stage [277]. In contrast to the kuruma prawn, *in vitro* matured porcine oocytes had decreased expression of *CTSC* transcripts than the non-matured oocytes. *CTSC* is identified within the lysosome (Table 9), and during maturation lysosomes are found to be redistributed during the breakdown of the nuclear envelope [280]. The decreased expression of *CTSC* transcripts in this study indicates that they may be utilized to produce proteins during this step of organelle reorganization.

SELENBP1 has been found to be expressed in many human tissues [281]. Reduced expression this gene has been reported in some human malignancies. In colorectal cancer, *SELENBP1* overexpression resulted in a suppression of cell proliferation, decreasing cell migration and an increasing apoptosis [282]. The proteins of *SELENBP1* are also found in the surface epithelium of the ovary [283]. There is a conjugated peptide of SELENBP1, SP56, which when given to mice in a preclinical trial for a contraceptive vaccine, reduced fertility by 50% [284]. Sperm protein 56 (sp56) has been indicated as a potential substrate which stabilizes the

adhesion of sperm to the zona pellucida of the oocyte and in turn promotes fertilization [110]. It is supposed that autoantibodies of SELEBP1 may have a detrimental effect on fertility, although little is known of the function of *SELENBP1* [285] deficient sperm oocyte binding resulting from the auto antibodies of sp56 may cause a decrease in fertility. There in an increase in the expression of *SELENBP1* in matured oocytes; and as it has been related to sp56, an increase in sp56 receptors on the zona and increased fertilization capacity of the oocyte may result from upregulation of this gene.

PABPC1 is known to influence mRNA translation and decay [286]. In some mammalian cells, it shuttles between the nucleus and the cytoplasm [287]. Within the nucleus, *PABPC1* protein will bind to the poly (A) tail of a newly synthesized transcripts [288]. In oocytes of Xenopus and mouse, *PABPC1* was not expressed until the maternal to zygote transition, and embryonic poly(A) binding protein (EPAB) functions as PABPC1 before this time [289,290]. Increased expression of *PABPC1* transcripts in mature oocytes signifies that mature oocytes may be able to respond to changes in cellular status better than non-matured oocytes; as *PABPC1* is involved in mRNA translation, the increased abundance of *PABPC1* transcripts in matured oocytes could mean that the immature oocytes do not have the appropriate levels of transcripts required for the proper cell function and embryonic development [240].

TFPI2 is thought to be involved in the initial placental development, as the endometrium is invaded by cytotrophoblasts during implantation [291]. More specifically, *TFPI2* regulates the expression of serine proteases and matrix metalloproteinases to degrade the extracellular matrix during implantation [291]. Ratios of *TFPI2* expression in biparental and parthenote foetuses were examined there was a greater expression in parthenote foetuses which only contain maternal expression, indicating that this gene is a maternally expressed gene [292]. *TFPI2*'s expression is imprinted, according to the parental conflict theory, the maternally imprinted genes conserve maternal resources for long-term reproductive fitness of the mother [293]. This gene is expressed from the maternal allele only, and it acquires this imprinting expression during pre-implantation development [294]. In this study, the decreased expression of this genes transcripts in mature oocytes may reflect the general purpose of maternally imprinted genes, acting to restrict fetal growth in an effort to conserve maternal resources rather than promoting enhancement of offspring development and fitness [293]. Reduction of gene transcripts may

increase developmental potential of the oocyte, and the level of this gene could then be used a marker for potential embryo development.

CYR61 is important for embryonic development, a loss of function mutation of *CYR61* in a mouse leads to embryonic death due to decreased vessel integrity and failed chorioallantoic fusion [295]; and this gene maintains pluripotency of embryonic stem cells in the mouse, through inhibition of cell differentiation [296]. *CYR61* encodes for a protein with heparin binding properties, associated with the cell surface and extracellular matrix [297]. In human, this gene has been shown to promote proliferation, migration and adhesion of endothelial and fibroblast cells by acting as an extracellular matrix signalling molecule [298]. In mouse foetuses, the spatiotemporal expression pattern observed in the endothelium also suggests a role for *CYR61* in the development of the external genitalia [299]. In this study we found an increase of *CYR61* transcripts through maturation, which may encourage early embryo development through promoting proliferation and maintaining pluripotency.

The *ACSL6* gene was first characterized in human erythrocytes, and is a member of the acyl-CoA synthetase family [300]. It is known to have physiological functions in fatty-acid catabolism [301]. A defect in *ACSL6* haplotype is thought to contribute to premature ovarian failure through its role in lipid metabolism in ovarian tissue [302]. The metabolites formed by of acyl-CoA from saturated free fatty acids can lead to apoptosis of granulose cells [303]. *ACSL6* is involved in metabolic pathways (Table 4.4), and metabolic rates of oocytes has been related to their developmental competence [304]. The increased transcript abundance in matured oocytes seen in this study may mean matured oocytes are more capable of undergoing fertilization and embryo development.

The final gene, whose expression is considered to be related to reproduction is *PLA2G7*. *PLA2G7* proteins are a member of the phospholipase A2 family, which are generally responsible for releasing amino acids from membrane phospholipids (review [305]). Platelet activating factor (PAF)-acetyhydrolase is one proteinenzyme produced by the Pla2g7 gene. PAF-acetyhydrolase is known to inactivate PAF by converting it to Lyso-PAF (review [306]). PAF itself is involved in ovulation, fertilization, implantation and parturition through various pathways (review [306]).

The increased expression of *PLA2G7* may mean the oocyte following maturation is more capable at regulating PAF in later developmental processes such as fertilization.

An increased expression of *FOS*, *VIM*, *SELENBP1*, *PABPC1*, *CYR61*, *ACSL6* and *PLA2G7* is possibly due to increased number of transcripts present or being used during the end stages of maturation as the majority of oocytes matured would be at MII stage. Stored RNA and transcripts are being used, or translated through maturation; the change in expression of these genes shows they play a role in later oocyte development such as fertilization of embryogenesis. *CTSC* and *TFPI2* show a decreased expression, meaning these transcripts are more abundant before resumption of meiosis, and they are utilized or degraded during the maturation processes. The changes in gene expression throughout maturation for these genes signify that they play a role in maturation.

There are 16 genes that were found to differentially expressed throughout maturation, and aside from the NOVEL genes, 9 of these are known to play a role in reproduction. Although the maturation media seemed to not affect the gene expression, under different statistical restrictions there may be more subtle differences. In conclusion, the supplementation of estradiol 17-β in maturation media did not alter the expression of genes within the oocyte.

4.6.Acknowledgments

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CHAPTER 5: GENERAL DISCUSSION AND CONCLUSIONS

In vitro production (IVP) of embryos in swine remains relatively inefficient when compared to other livestock species [4]. This technology could be used to manage herd genetics more selectively, reduce the impact and threat of disease on a herd's genetics, as well as conserve the genetic diversity of swine. However there are problem in porcine IVP systems; chromosomal abnormalities and decreased cell numbers in blastocysts during *in vitro* culturing (IVC) [5], low monospermic fertilization rates with *in vitro* fertilization (IVF) [6], and incomplete maturation from *in vitro* maturation (IVM) [7,8]. As IVM is the first of the three IVP processes, it has the ability to affect each following processes. Insufficient IVM has been a contributing factor to the elevated polyspermic rates following IVF, as well as low quality embryo development during IVC.

Oocyte matured *in vivo* are more competent that those matured *in vitro* [7], showing that the *in vitro* maturation environment is deficient in some respect. Different maturation media and the supplementations to that media [16-20] have been shown to increase the competence of oocytes matured *in vitro*. Porcine FF is commonly added to maturation media [12-14], as it increases both nuclear and cytoplasmic maturation as well as improving IVF and IVC success [199,200]. There are many maturation promoting factors within pFF, including FF-MAS [203], plasminogen activators and plasmin [205], amino acids [20], insulin-like growth factor 1 [206], and steroid hormones [24]. Estrogen is present in pFF, in both high and low concentration depending on the donor animal, follicle size and the stage of estrus the donor animal was in [23,24]. Estrogen is reported to negatively affect nuclear maturation in bovine IVM [214], but in white tail deer supplementation of IVM media with estrogen improved nuclear maturation rates [307]. In porcine IVM systems, estrogen supplementation has had controversial effects as some report estrogen to reduce nuclear maturation rates [26,213], while others found an improvement in nuclear maturation as well as blastocyst formation [25].

Nuclear maturation rates were observed for oocytes matured in a common pFF supplemented media as well as a control maturation media containing no sera, proteins, or steroid hormones (Chapter 3). Nuclear maturation is the resumption of meiosis from prophase I

arrest. During prophase I the nuclear envelope can be visualized, and the oocyte is referred to as a GV stage. As meiosis resumes the nuclear envelop dissolves and the oocyte is staged as GVBD. When no polar body or nuclear envelope can be observed, the oocyte is classed as MI. Telophase I is marked by extrusion of the first polar body; the oocyte arrests before fertilization in MII stage.

The Lamin Dapi staining in this study revealed that both maturation media matured oocytes equally well when MI and MII rates were considered together (Chapter 3). Protein-free media was a sufficient control to induce nuclear maturation; this is supported by the development of defined maturation medium which has successfully produced embryos [245]. The different maturation rates observed between this study and others [88] using aceto orcein staining, is that Lamin Dapi does not depend as heavily on the users experience. In aceto orcein staining the different stages of meiosis are evaluated independently by the user; however Lamin Dapi staining allows easier visualization of each stage and thus removes much of the user bias.

The equal maturation rates between the protein-free and 10% pFF supplemented media indicated that the nuclear maturation was not significantly improved by the addition of pFF. It is hypothesized that the beneficial effects that are seen with pFF supplementation are on cytoplasmic maturation. Cytoplasmic maturation is the reorganization of the oocyte's cytostructure, and accumulation of RNA and proteins [59,60]; this can be evaluated through intracellular glutathione (GSH) content, cumulus expansion, ability of the oocyte to be fertilized and the resulting cleavage and blastocysts formation rates [4,61,62]. There are many changes to internal organelles and cytoskeleton, but the utilization of stored RNA and proteins is needed for the cytoplasmic changes. The proteins expressed from this mRNA do play a significant role in maturation as well as the subsequent events of fertilization and embryogenesis [77].

In vitro production of embryos is both positively [251] and negatively [214] influenced in cattle with the introduction of E_2. In porcine IVP, E_2 supplementation in maturation has been reported to have no effect on fertilization and positive effects on fertilization and embryo development [25,263]. The results of our work illustrate that the introduction of 10% pFF or 100 ng/ml of E_2 during IVM has no influence on the cleavage rate; however the difference in blastocyst formation rates between the protein-free control, and the pFF and E_2 supplemented

media, demonstrate that E_2 may positively influence IVP. This supports the idea that pFF increases IVP success, and the positive effects of E_2 are also supported by the literature in cattle and sheep [251,252] as well as in pigs. The effect of these supplementations may be on cytoplasmic maturation as there was no observed difference in nuclear maturation when comparing the pFF supplemented medium to the protein-free medium. The effect of E_2 cannot be concluded from this study as the nuclear maturation rate using E_2 supplementation was not directly compared to each of the maturation media. Future investigation, using Lamin/Dapi staining, into the effect of E_2 on nuclear maturation rates may show more conclusive results.

When using pre-pubertal oocytes, there is expected differences from oocytes derived from adult animals, as pre-pubertal oocytes show reduced blastocyst formation rates [308]. Also pre-pubertal oocytes have higher incidence of polyspermy, showing that the cells may not be competent following maturation [144]. Developmental competence of pre-pubertal oocytes increase with increasing follicle size. Oocytes from 3 mm follicles have a significantly lower blastocyst formation rate when obtained from pre-pubertal ovaries; this differs from oocytes in 3 mm follicles from adult sow ovaries [309]. There is a high percentage of small follicles found within a pre-pubertal ovary, and therefore the majority of oocytes aspirated from pre-pubertal ovaries are from 3 mm follicles [309]. The pre-pubertal oocyte has less innate competency than adult oocytes, and when using pre-pubertal oocytes it is expected to have lower blastocyst formation rates [309].

The *in vitro* environment effects the competence of oocytes, as oocytes matured *in vivo* are shown to have superior competence when compared to *in vitro* matured oocytes [265]. When comparing bovine *in vitro* and *in vivo* matured oocytes, differentially expressed genes were observed [265]. The maturation environment clearly influences gene expression within the oocyte; the maturation media is also shown to affect the abundance of gene transcripts which is further correlated with oocyte competence [29]. Embryonic failure has been attributed to defects in some maternally derived genes [33], therefore the alteration in gene transcripts through supplementation with estrogen during maturation may contribute to low IVP success rate.

The maternal to zygote transition occurs when an embryos genome initiates its control over embryogenesis, and the maternal genome's regulation decreases as the store maternal RNA

degrades or is utilized [31]. Recent work suggests that the oocyte is not transcriptionally silent throughout maturation as is traditionally thought. Transcription was observed to decline from GV to MII stages of maturation, and there is accumulation of cytoplasmic polyadenylated mRNA at GVBD stage [71]. Either with, or without transcription, the transcripts within the oocyte are altered through maturation and embryogenesis. The difference in gene expression of *TRIM24* was significantly different in the 0 ng/ml E_2 and 10% pFF groups than the non-matured control in this study; indicating that this gene has an altered expression through IVM (Chapter 3). The expression of all other maternal effect genes evaluated showed no differences between the non-matured groups or amongst the different supplementations indicating that their expression was not influenced through maturation. The non-matured group did not differ from any of the E_2 supplemented groups, indicating the gene expression in the E_2 supplemented groups is most similar to the non-matured cells. As exogenous E_2 is reported to retard meiosis during the first half of IVM [25], the oocytes which are exposed to E_2 throughout IVM may not fully mature. An increased proportion of E_2 matured oocytes may remain at the GV and GVBD stage than those oocytes matured in the 10% pFF or protein-free media.

Understanding the changes in gene expression through maturation likely would improve porcine IVP. Transcription is most active in the oocyte growth phase and after GVBD it declines [71], a difference in abundance of gene transcripts is observed when GV and MII oocyte are compared [28]. Hundreds of genes are differentially expressed through the maturation process in bovine [70] and human [28] suggesting that major modifications of gene transcripts occur through maturation. In this study (Chapter 4) a difference in gene expression from all matured groups was seen when compared to non-matured oocytes. Gene expression of porcine oocytes did change through maturation, and this is in support of previous research. Of the 16 differentially expressed genes through maturation, 8 (excluding the novel genes) are known to play a role in reproduction.

Some of these genes give us information to develop a more successful maturation system, as well as providing useful indicators of a competent oocyte. The increased abundance of *FOS* transcripts in matured oocytes supports the idea that androgens would affect early maturation, as the matured oocytes may be less responsive to androgens than non-matured oocytes. Maturation systems should than introduce any androgens, known to have a positive effect on oocyte

competence in the first half of IVM. SELENBP1 is associated with its conjugate peptide sp56 which is known to play a role in the adhesion of sperm to the zona pellucida [110], and antibodies of this peptide reduce fertility [284]. As indicated by this study, the increase in expression of *SELENBP1* may be a marker for the oocytes fertilization capacity. *ACSL6* is involved in metabolic pathways, specifically fatty acid catabolism [301]. The presence of free fatty-acids is known to induce apoptosis of granulosa cells which are important for oocyte maturation [303], thus increased expression of *ACSL6* may be a marker for oocyte competency, as metabolic rates of oocyte have been related to oocyte competency [304]. The above genes provide much needed insight into the specific markers of competent oocytes obtained through IVM as well as possible changes that can be made to IVM systems.

While the traditional idea of transcriptionally silent oocytes is being challenged, the changes in transcript abundance through maturation can also be explained by selective degradation or utilization and poly-A-tail elongation of maternal RNA transcripts during maturation (reviewed by [59]). Each of these activities is controlled by specific sequences in the 3'- end of mRNA, and mRNA translation is regulated by changes in the length of 3'poly-A-tail [310]. During maturation, the oocytes maternal mRNA polyadenylation is temporally regulated [310]. This accounts for increased abundance of transcripts during different periods of oocyte maturation. The increased abundance of expressed gene transcripts at the GV stage suggests that these transcripts play a role prior to meiotic completion [271]. The increased number of transcripts seen following maturation implies the transcripts are used during the end stages of maturation or for the following fertilization and embryo development.

We did not observe any effects of pFF or estrogen on oocyte gene transcripts through maturation with threshold values of $-2 \geq \log2(\text{fold change}) \geq 2$, and adjusted p-value ≤ 0.05. There may have been differences in fold change before the false discovery tests were performed, but these differences could not be included into this study. By lowering the threshold values or looking at the global gene expressions in the groups differentially expressed genes may be found between media and this should be explored at a later date. Further investigation as to the effects of timing the E_2 introduction into media should be undertaken to evaluate its effects on gene expression in porcine oocytes; as transient supplementation of E_2 within the maturation media

found that E_2 introduced only during the first half of maturation increased nuclear maturation as well as embryo development [25].

Based on the work conducted in this thesis we confirm that nuclear maturation is successful in porcine oocytes without the supplementation of pFF or hormones. Estradiol and pFF supplementation positively influence the production of blastocysts following IVF in a porcine IVP system, and based on this study the common pFF supplementation may be replaced by E_2 to create a serum free media for a more standardized IVM system. The response of maternal effect genes through maturation is varied, with only *TRIM24* displaying differential expression, and E_2 possibly hindering meiosis as there was no statistical difference between the E_2 and non-matured groups. In global gene expression analysis no effect of E_2 supplementation was seen with the applied threshold levels. Although differential gene expression was observed for all maturation media through maturation; indicating that gene expression changes temporally through maturation in porcine oocytes.

Future directions in porcine IVM research should investigate E_2 supplementation in IVM systems as evaluated by nuclear maturation rates through Lamin and Dapi staining to provide a more conclusive answer to E_2's effects in IVM. The timing of E_2 supplementation in IVM systems deserves investigation as seen by other studies [25], it does effect oocyte maturation and subsequent embryo development, however by evaluating these effects using Lamin Dapi may give different results, as well as using a protein-free maturation media. The increased abundance of *FOS* in this study further supports investigation into the timing of E_2 introduction into IVM systems.

Further research into gene expression of porcine oocytes may provide markers for oocyte quality. Using Q-PCR the current study's results (Chapter 4) may be confirmed, and the genes which show differential expression should be examined as markers for oocyte competence. Investigation into other pathways they may be involved in within the oocyte could be undertaken. By removing the thresholds when analysing the current study's microarray data further examination may be preformed to obtain a global picture of gene expression of porcine oocytes through maturation, and more genes which influence reproduction and oocyte competence may be realized. The evaluation of porcine cumulus cell gene expression through

IVM may also provide needed markers to evaluate COC quality, as many of the current techniques are qualitative rather than quantitative, and removing any observer bias would supply a more standardized IVM system. The differences in gene expression should also be evaluated by comparing in vivo and in vitro oocytes, as in vivo would act as the ultimate control to see if the in vitro system is changing gene expression.

If E_2 can be used to supplement pFF, its effects will have to be tested through cryopreservation of the resulting blastocysts. The standardization of IVM media using E_2 may be valuable for research purposes, but unless the created blastocysts may be cyopreserved they may not be as useful for protecting the genetic diversity of swine. A final confirmation of the competence of an oocyte is the production of live offspring, and the production of blastocysts produced using E_2 supplementation in the maturation media should be confirmed through embryo transfer. The only method to conclude that $E_2$is a successful replacement for pFF in porcine IVM systems is to produce healthy offspring using E_2 supplemented IVP blastocysts.

The studies presented in this thesis may lead to further investigation into estradiol's influence on maturation as well as investigation into porcine oocyte gene expression. There is a need for successful porcine IVM to allow producers and researchers the opportunity of using IVP technology and future research is needed for this technique to be applicable in porcine as it is in other livestock species

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