MicroRNA expression and function during porcine oocyte maturation and early embryonic development

by

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NOMENCLATURE

AP-1  Activator Protein 1
cDNA  Complimentary Deoxyribonucleic Acid
CL    Corpora Lutea
COC   Cumulus Oocyte Complex
C_T   Cycle Threshold
DAVID Database for Annotation, Visualization and Integrated Discovery
DNA   Deoxyribonucleic Acid
D     Day
D12S  Day 12 Spherical
D12F  Day 12 Filamentous
D14F  Day Filamentous
dsRNA Double Stranded RNA
E2    Estradiol
EGF   Epidermal Growth Factor
FSH   Follicle Stimulating Hormone
GnRH  Gonadotropin Releasing Hormone
GV    Germinal Vesicle
GVBD  Germinal Vesicle breakdown
HSF1  Heat Shock Factor 1
HSP90α Heat Shock Protein 90α
Hr    Hour
IGF   Insulin Like Growth Factor
LH    Luteinizing Hormone
MII   Metaphase II arrested oocyte
miRNA Messenger RNA
mRNA  Messenger RNA
NC    Negative Control
nt    nucleotide
ODF   Oviductal fluid
piRNA piwiRNA
PCR   Polymerase Chain Reaction
PDCD4 Programmed cell death 4 (neoplastic transformation inhibitor)
PNA   Peptide Nucleic Acids
PTGR  Post Transcriptional Gene Regulation
PTEN  Phosphatase and Tension Homolog
PVA   Polyvinyl Alcohol
PZM3  Porcine Zygote Medium 3
QT-PCR Quantitative Polymerase Chain Reaction
RISC  RNA-induced Silencing Complex
RNA   Ribonucleic Acid
rRNA  ribosomal RNA
RT    Reverse Transcriptase
RT-PCR Reverse Transcriptase Polymerase Chain Reaction
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>SAGE</td>
<td>Serial analysis of gene expression</td>
</tr>
<tr>
<td>SCNT</td>
<td>Somatic Cell Nuclear Transfer</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error Mean</td>
</tr>
<tr>
<td>ssRNA</td>
<td>Single Stranded RNA</td>
</tr>
<tr>
<td>SPP1</td>
<td>Osteopontin</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal Transducer and Activator of Transcription</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>TN</td>
<td>Thermal Neutral</td>
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<tr>
<td>UTR</td>
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“For I know the plans I have for you, plans to prosper you and not to harm you, plans to give you hope and a future.”

- Jeremiah 29:11
ABSTRACT

Understanding oocyte maturation and embryo development in the pig is essential to improving farm animal reproduction efficiency. Many reproductive tissues are associated with a high degree of transcriptional and translational turnover that may be in part regulated by non-coding RNA such as microRNA (miRNA). The oocyte consists of a storage of maternal RNA and nutrients that are utilized to support oocyte maturation and early embryo development. MiRNA have been identified in the oocyte and throughout early embryo development in many species including the pig. MiRNA exert their biological impact on the cell through post transcriptional gene regulation (PTGR) by interacting with the 3' UTR of a specific target mRNA. MiRNA expression during oocyte maturation and embryo development up to the 4-cell stage is potentially critical as little to no transcription occurs in the oocyte following germinal vesicle breakdown until the activation of the embryonic genome. Our objectives were to 1) determine the importance of MIR21 expression and function during oocyte maturation, 2) develop and test an in vitro heat stress (HS) model for pig oocyte maturation and test the effects of HS on MIR21 expression, and 3) characterize the expression of miRNA during rapid trophoblastic elongation in the pig. Our data demonstrate that expression of mature MIR21 is increased approximately 4-fold (P = 0.001) in MII oocytes compared to GV stage oocytes. Additionally when MIR21 is inhibited during in vitro maturation, maturation rates are decreased from 55.4 ± 3.6% in control to 33.7 ± 3.6% in MIR21 inhibited. Following in vitro fertilization, MIR21 inhibited oocytes produced fewer 4-cell stage embryos (41.7 ± 12.1%) compared to the control group (73.0 ± 5.7%). While no decrease (P = 0.34) in PDCD4 mRNA (a MIR21 target) was observed, inhibition of
MIR21 resulted in increased (P < 0.05) PDCD4 protein expression in MII oocytes compared to control MII arrested oocytes. Heat stress during oocyte maturation caused alterations in both MIR21 and PDCD4 expression in MII oocytes and in 4-cell embryos created from oocytes matured during HS. In addition, other markers of HS, such as heat shock protein 90A (HSP90A), were also affected in 4-cell stage embryos created from HS oocytes. Our data demonstrated that HS during oocyte maturation caused a significant decrease in MIR21 in 4-cell stage embryos cultured in thermal neutral conditions which was associated with a significant increase in PDCD4 mRNA in the same group of embryos compared to 4-cell stage embryos created from oocytes matured in TN conditions.

Elongating pig conceptus were collected from pregnant pigs on Day 12 of gestation (spherical and elongated) and on Day 14 (elongated; n = 4 for each morphological stage). Small RNA libraries were created and subjected to massively parallel deep sequencing using the ABI SOLiD sequencing platform. Total miRNA reads for each stage of development were 7,319K, 13,831K, and 30,618K for spherical (D12S), day 12 filamentous (D12F) and day 14 filamentous (D14F), respectively. Several miRNA were selected for validation including MIR21, MIR301a, MIR23b, MIR10a, MIR200a, MIR574, MIR4057, and MIR467a. Many of these selected miRNA demonstrated significant fold changes between two or more conceptus stages and were validated within individual samples at each stage by RT-PCR. This confirmed that miRNA are temporally regulated during embryo elongation and potentially play a role in regulating cell differentiation, migration and transformation of the porcine conceptus.
CHAPTER 1
INTRODUCTION

Understanding early embryo development is essential to improve farm animal agriculture by increasing reproductive efficiency. Understanding the molecular and genetic mechanisms of early embryo development may also be useful for improving assisted reproduction technologies, such as somatic cell nuclear transfer and in vitro fertilization. Early embryo development is a unique period in development with the absence of RNA synthesis after germinal vesicle breakdown preceding a period of significant transcription at the 4-cell stage in the pig (Aiba et al., 2006). Transcriptional profiling of early pig development from germinal vesicle to 4-cell and blastocyst stage demonstrated a dynamic regulation of mRNA abundance during early embryo development (Whitworth et al., 2005b). The oocyte consists of a storage of maternal RNA and nutrients that are utilized to support oocyte development and contribute to embryogenesis prior to the activation of the embryonic genome, when the genetically unique embryo is capable of its own transcription. Complete oocyte maturation and progression through Metaphase II (MII) of meiosis may be dependent upon post-transcriptional regulation of existing mRNA. Additional regulation may also occur and through interactions with the surrounding cumulus cells (Gilchrist et al., 2004). MicroRNA (miRNA) are potent regulators of post-transcriptional gene regulation (PTGR) of mRNA and are expressed during oocyte maturation and early embryonic development in the pig. A better understanding of microRNA regulation of oocyte maturation and early embryonic development could lead to new knowledge that can be used to develop strategies to improve reproductive efficiency in pigs.
The following review focuses on oocyte maturation and early embryo development in the pig and discusses the potential for microRNA to contribute to the quality and viability of an oocyte and its ability to develop into an embryo and produce viable, quality offspring. Understanding the role of microRNA in the oocyte and developing conceptus is important for developing improvements in reproductive physiology both in vivo and in vitro.
CHAPTER II

LITERATURE REVIEW

Utilization of pigs in agriculture

The United States (US) pig breeders produce an average litter size of 10.2 piglets with a total of 28.8 million piglets per year (USDA, 2011). China is the world largest pork producer followed by the US. In 2010, 19% of US pork was exported; largely to Japan (30%), Mexico (25%) and Canada (10%) (Geisler, 2011). The US also imports 860 million pounds of pork, primarily (81%) from Canada (Geisler, 2011). Pork is one of the most widely consumed animal products in the world and demand has increased steadily over the past 3 decades (USDA-FAS, 2011). To adequately provide for increasing pork demand, production efficiency improvement will be necessary. The method of housing pigs has been a controversial topic in recent years as confinement hog operations struggle to produce more pigs at a faster pace with less space and stricter regulations (Swanson, 1995). Sow productive lifetime is a product of litter size, number of parities produced and the number of non-productive days. Improvements in sow productive lifetime can be made by providing new knowledge that can be applied to improve reproductive efficiency.

Utilization of pigs for biomedical research

The pig is a valuable biomedical model for studying human physiology and disease because of its anatomic and physiological similarities to the human. The size and composition of the porcine genome is similar to the human genome allowing the pig to also serve as a model for human genetic research (Bendixen et al., 2010). Miniature breeds of pig such as Yucatan, Hanford and Gottingen have been utilized in cases which the domestic
agricultural pig would be too large at maturity (Vodicka et al., 2005). Pigs and humans have similar cardiovascular, digestive and urinary systems. Additionally, the heart, liver, pancreas and kidney have long been considered ideal for xenographic procedures in place of human organs (Bustad and McClellan, 1966).

The physiological similarities between pigs and humans have led to the development of genetically modified pigs which mimic human disease. Transgenic pigs can be produced by somatic cell nuclear transfer (SCNT) introducing a modified donor cell into an enucleated oocyte (Niemann and Wrenzycki, 2000; Park et al., 2001; Prather et al., 2004). Somatic cell nuclear transfer is the most widely used technique for producing transgenic pigs but only has about a 1% success rate (Lai and Prather, 2003). Other methods for obtaining genetically modified pigs have been developed including oocyte transduction via viral transgene delivery (Cabot et al., 2001; Hofmann et al., 2003) and sperm mediated DNA transfer relying on the sperm’s ability to carry exogenous DNA into the pending zygote during fertilization (Lavitrano et al., 2005). Transgenic pigs derived from SCNT are valuable biomedical and agricultural models. As of 2011, 68 different genetic modifications in pigs have been successfully created for use in biomedical research and agriculture (Whyte and Prather, 2011).

Pregnancy in the pig differs from the human in several aspects however the development of the embryo is similar enough to that of the human that pigs have become a reliable model for mammalian embryology (Patten and Carlson, 1981). Fetal and neonatal pigs can be used for human perinatal research including embryology, immunology, metabolism and nutrition. The length of gestation for a pig (16.5 weeks) is less than half that of a human (40 weeks). Attachment and implantation also occur several days later in the pig
at day 14 compared to the human fetus which implants on day 6 after ovulation (Bazer et al., 1993; Finn and Martin, 1974). The placentation for a pig is classified as diffuse and epithelialchorial while the human has discoid and hemochorial placenta (Borazjani et al., 2011; Keys and King, 1990). Despite these differences in gestation and placentation, the fetal pig is more similar to the human fetus than many other mammals. The similarities in development between the pig and human fetus allow for fetal development research which can aid in the diagnosis and treatment of human development disorders (Butler et al., 2009; Welsh et al., 2009; Whyte and Prather, 2011).

The rapid growth of neonatal piglets makes them an ideal candidate for nutritional studies. Nutrient deficiencies from protein to iron have been evaluated in the growing piglet and neonate (Tumbleson et al., 1969; Wintrobe et al., 1953). Piglets have been used to evaluate infant formulas and milk substitutes because the piglet has stricter nutrient requirements than a human infant (Pond et al., 1971). The fetal pig has no circulating immunoglobulins prior to birth and remains hypogammaglobulinemic for several weeks, making the piglet an ideal subject to study the developing immune system (Butler et al., 2009). Although the pig is an excellent model to use for biomedical research, housing and other costs have limited the use of pigs at many research facilities.

**Estrous cycle**

Establishing pregnancy in swine begins with ovulation followed by fertilization of oocytes in a female during a period of sexual receptivity known as estrus. Estrus, or standing heat, is an approximately 24 to 72 hr period at the beginning of the estrous cycle. The estrous cycle is a hormonal process in which follicles are recruited for ovulation to provide
viable oocytes for fertilization and induce the uterus to prepare for fetal implantation. The porcine estrous cycle is 21 days in length and is precisely regulated by the steroid hormones estrogen and progesterone (P4) (Figure 2.1). The estrous cycle can be divided into four stages: estrus (day 0-2), metestrus (day 2-4), diestrus (day 5-16) and proestrus (day 17-19) (Senger, 1997). During prophase tertiary follicles that have avoided follicular atresia continue responding to gonadotropins (luteinizing hormone (LH) and follicle stimulating hormone (FSH)) as they mature in to Graffian follicles and begin increasing production of estradiol-17β. The elevated estrogen concentration induces estrus behavior in the gilt or sow causing her to “stand” and be receptive to the boar during the estrus phase (Hemsworth, 1985). The sow will ovulate about 55% of the way through the estrus phase (Soede et al., 2000). If fertilization is successful and two or more embryos signal their presence in each uterine horn the gilt/sow will establish a pregnancy (Christenson and Day, 1971). Metestrus occurs after ovulation with the formation of corpus hemorrhagicum (“bloody body”) located at the site of previous ovulated follicles. Estrogen concentrations decrease substantially following ovulation and the luteinization of both theca and granulosa cells results in progesterone synthesis by the developing corpora lutea (CL).

The diestrus period is dominated by the development and function of the CL which produce progesterone resulting in uterine quiescence (Christenson and Day, 1971; Knight et al., 1977). During the diestrus phase the uterus is preparing to accept embryos by producing and secreting histotroph (uterine milk) and the blood supply to the uterine endometrium increases. Progesterone concentration is highest during this phase of the cycle (Bazer et al., 1979).
**Figure 2.1.** Diagram of the 21 day estrous cycle of the pig. The estrous cycle begins with estrus and ovulation. Following fertilization, embryos continue to develop into blastocyst by d 6. The hatched blastocyst enters the uterus and begins migrating throughout the uterine horns until rapid trophoblastic elongation of the conceptus. The conceptuses rapidly elongate at d 12 and attach to the uterine endometrium at d 14. The top half of the diagram depicts the hormonal regulation of the estrous cycle beginning with estrogen production by the Graffin follicles. Progesterone is produced by the CL until prostoglandin F$_{2\alpha}$ production by the uterine endometrium causes lysis of the CL if pregnancy is not established. The green circle marks the critical period of conceptus elongation and production of estrogen by the conceptus to signal maternal recognition. The red arrow represents successful pregnancy with attachment at day 14, maintainence of the CL and a 115 d gestation.
Figure 2.1.

Estrous Cycle and Early Pregnancy

- Graffian follicles produce estrogen
- Ovulation
- Corpora Lutea produce Progesterone
- Corpora Lutea lysed via uterine PGF<sub>2α</sub>
- New follicular Growth
- Conceptus elongation and production of estrogen and IL-1β
- Corpora Lutea maintained throughout gestation
- Blastocysts Hatching and entry into the uterus
- Attachment and Implantation
- 115 d gestation

Abbreviations:
- PGF<sub>2α</sub>: Prostaglandin F<sub>2α</sub>
- IL-1β: Interleukin-1β
The presence of viable embryos at days 12-18 of gestation that are capable of synthesizing and releasing the maternal recognition of pregnancy signal (estrogen) results in the maintenance of the CL and progesterone production persists throughout gestation. The absence of viable embryos during the implantation window allows the endometrium to release the luteolytic agent, prostaglandin F2α. Prostaglandin F2α is synthesized by the uterus and when released in an endocrine fashion, results in regression of the CL and subsequent decline in circulating P4 (Bazer et al., 1979). The loss in circulating P4 results in the loss of P4 negative feedback that allows pulsatile secretion of gonadotropin releasing hormone (GnRH). GnRH induces the release of LH and FSH from the anterior pituitary which facilitates follicle recruitment during proestrus with the selection and growth of a group of tertiary follicles (Knox, 2005).

**Estrus synchronization**

Synchronizing estrus in gilts and sows is essential to maximize resources and optimize reproductive performance. Synchronizing estrus and breeding enables pig producers to rotate gestating sows through gestation facilities and optimize barn space while benefiting from the advantages of artificial insemination (AI) by narrowing the breeding window and increasing the number of sows that will be bred at one time. Timed AI can also be utilized to increases the chance for fertilization and reduces the labor of visual estrus detection (Horsley et al., 2005). In addition a single semen collection can be used to breed multiple sows. This saves the producer both labor and shipping costs adding to profit and maximum production. There are various methods for synchronizing gilts and sows based on production needs and cost. Natural estrus will generally begin 7 days after weaning in most
sows with adequate energy intake (Reese et al., 1982). Synthetic hormones such as MATRIX®, a synthetic progestogen added to feed, are used to control onset of estrus by preventing follicle formation (Flowers, 1999). Feeding MATRIX® for approximately 14 days typically results in the sow/gilt exhibiting estrus approximately 5-9 days following withdrawal (Flowers, 1999). This allows the producer to breed sows in groups, or select specific dates for farrowing.

**Follicular growth and recruitment**

The ovary contains primordial follicles until puberty when follicles are recruited to secondary and tertiary stages for ovulation at which time the oocytes continue toward maturation or undergo apoptosis. Secondary follicles contain a growing oocyte surrounded by up to 20 layers of granulosa cells (Christenson et al., 1985). The tertiary or antral follicle is visible on the surface of the ovary and contains a fluid filled antrum. Tertiary follicles with a fluid filled antrum, require approximately 83 days to form in the pig ovary and contain an oocyte surrounded by 10-30 layers of granulosa cells (Morbeck et al., 1992). The follicle is then selected for ovulation and will continue to grow for an additional 5-7 weeks until ovulation (Morbeck et al., 1992). Follicles can undergo atresia at any time during recruitment or selection.

Follicle growth occurs independently of gonadotropins until antrum formation, at which point recruitment can occur and a pool of primordial follicles are activated (Knox, 2005). Follicular response to gonadotropins contributes to selection and ovulation rate (Evans et al., 1981). In gilts, visible surface follicles are small (> 3mm), medium (3-7mm) or
large (> 7mm). Early tertiary follicles contain a fluid filled antrum which divides the granulosa cells into two groups: those closest to the oocyte are the cumulus cells and mural granulosa cells form the outer layers. At onset of estrus approximately 15 follicles are small to medium and about 15 follicles are large. By day 3 there will be approximately 30 small, 5 medium and no large. During the remainder of luteal phase, the pool of follicles increases and peaks at day 11-13 with approximately 50 small, and 30 medium with no large follicles present (Knox, 2005). By the start of the follicular phase the number of small and medium follicles rapidly decline while a pool of medium follicles is selected for ovulation. The size of the large follicles at estrus is heterogeneous but their number is reflective of the subsequent number of CL found following ovulation. Follicles do not ovulate simultaneously; however all follicles on both ovaries ovulate within a short time frame (Pope, 1988). Atresia and ovulation are the only fates for a follicle once it enters the growing pool.

The Oocyte

Female gametes originate from cells within the ovary called the oogonium. Meiotic cell division produces germ cells (oogonia and spermatogonia) by reducing the amount of genetic material by forming haploid cells following two rounds of cell divisions after only a single duplication of the chromosomes. Each round of meiosis is divided into four stages: prophase, metaphase, anaphase, and telophase. Prophase I involves the duplicated chromatin condensing with each chromosome composed of two closely associated sister chromatids. Crossing over, an event where genetic information is exchanged between chromosomes, occurs during the latter part of prophase I (Jordan, 2006). Homologous chromosomes align
to form tetrads and the distal portion of sister chromatids exchange segments in a process called gene recombination. During metaphase the condensed and highly coiled chromosomes align in the middle of the cell. Anaphase occurs when the chromosomes are separated and located at each pole of the cell (Maiato and Lince-Faria, 2010). Once the chromosomes are at each pole a nuclear envelope develops around each set of chromosomes and the cell is at telophase. The oocyte containing the newly remodeled chromosomes is then arrested at growth phase 2. The germinal vesicle (GV) oocyte remains arrested until resumption of meiosis at ovulation.

The oocyte consists of a maternal haploid chromosome, a storage of RNAs and nutrients that must be utilized to support oocyte development. The number of oocytes is established in utero with approximately $2 \times 10^5$ primordial follicles in the pig (Gosden and Telfer, 1987). Despite the extremely large number of primordial follicles few will develop to maturity as the vast majority will undergo apoptosis. A select few primordial follicles are recruited from the primordial pool during each cycle. Primary oocyte recruitment is not well understood although oocyte-cumulus cell communication is required for oocyte activation and maturation (Gilchrist et al., 2004). Bone morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9) are proteins secreted by the oocyte into the ovarian follicle during follicular development (Paulini and Melo, 2011). BMP15 and GDF9 contribute to all stages of follicular development including activation of the primordial follicles (Galloway et al., 2000; Juengel et al., 2004; Orisaka et al., 2009). Additionally, GDF9 and BMP15 are also involved in oocyte maturation via cumulus cell expansion (Dragovic et al., 2005). BMP15 and GDF9 are important in regulating oocyte maturation by communicating with the follicular environment. The communication between the oocyte and the follicular
environment is important for regulating hormonal and molecular signaling during oocyte maturation (Hawkins and Matzuk, 2010).

**Metaphase II arrest**

Meiotic cell cycle begins during fetal development and primordial follicles are arrested at prophase I at the time of birth in most mammals (Mehlmann, 2005). The oocyte will remain arrested in prophase I for weeks or years depending on species, until the female reaches puberty. The oocyte is capable of generating sufficient amounts of cAMP to maintain meiotic arrest until recruitment or apoptosis (Vaccari et al., 2008). The trigger that releases the oocyte from prophase I and selects that follicle for ovulation is independent of follicular recruitment and not well understood. Maturation promoting factor and mitogen-activated protein kinase (MAPK) regulate the cell cycle progression in the oocyte (Fan and Sun, 2004). MOS, a germ cell specific Ser/Thr protein kinase, initiates the MAPK phosphorylation cascade during oocyte maturation (Gebauer and Richter, 1997). MOS/MAPK activity in the oocyte in conjunction with endocrine signaling in the follicle are essential for reactivating the dormant oocyte. Preovulatory release of the gonadotropin LH from the pituitary induces the release of epidermal growth factor (EGF) from the granulosa cells that trigger the oocyte to resume maturation (Ashkenazi et al., 2005; Park et al., 2004). During maturation the oocyte undergoes germinal vesicle breakdown (GVBD), meiosis I and the initial phase of meiosis II. Germinal vesicle breakdown has been well characterized in Xenopus, starfish and mouse oocytes because the nucleus is clearly visible. Fluorescent 70 kDa, a fluorescent indicator which does not penetrate the nucleus of an immature oocyte, was injected into the cytoplasm of starfish oocytes in order to pin point the time and disassembly
of the nuclear envelop when the dye invaded the nuclear region (Terasaki, 1994; Terasaki et al., 2001). The breakdown of the nuclear envelope occurs during GVBD, which is an indication that the oocyte is committed to maturation.

**Germinal vesicle breakdown**

Germinal vesicle breakdown signifies oocyte commitment to maturation and precedes ovulation at which time the oocyte progresses to Metaphase II (Guthrie and Garrett, 2000; Xie et al., 1990). Transcription ceases after GVBD and will not resume in the pig until the embryonic genome is activated at the 4-cell stage. Germinal vesicle breakdown has been described as having 5 stages (GVO to GV4) based on chromatin condensation visualized with Hoechst staining (Guthrie and Garrett, 2000; Sun et al., 2004). During, GV0 chromatin is diffuse and filamentous throughout the nucleus while GV1-3 stages are characterized by condensed chromatin which gradually forms clumps within the nucleolus as maturation progresses. Lastly, GV4 stage oocytes have condensed chromatin and no nuclear membrane is visible (Sun et al., 2004). The stage of development is variable within follicles of the same size and may be related to an oocyte’s ability to complete maturation or become atretic (Guthrie and Garrett, 2000). The stage of GVBD is related to the concentrations of estradiol and P4 present in the follicle and the day of the follicular phase, GVBD is completed in the maturing oocyte after the LH surge (Guthrie and Garrett, 2000). Once an oocyte is ovulated it will travel down the oviduct and continue maturation until reaching the ampullary-isthmic junction (AIJ) where it arrests at Metaphase II until fertilization and activation of the oocyte.
In vitro vs. in vivo maturation

Porcine oocytes are generally collected for in vitro maturation from 2-6 mm follicles which are clear of blood spots or white matter, a sign of follicular atresia. The cumulus oocyte complex (COC) is then isolated from the follicular fluid and debris, washed several times and placed in maturation media at 39°C with 5% CO₂ for 42-44 hrs (Lai and Prather, 2003). Maturation media is supplemented with gonadotropin hormones LH and FSH; in addition, EGF is added and a final pH of 7.4 and osmolality of 290 mOsm/L is acquired to simulate the follicular environment. The COC contains an oocyte with an intact germinal vesicle at the time of collection and will typically mature to MII within 42 hrs. The MII oocyte is surrounded by a mass of expanded cumulus cells. The oocyte is stripped of cumulus cells at 42 hrs and mature oocytes can be identified by the presence of a protruded polar body. MII arrested oocytes can then be parthenogenetically activated or fertilized to achieve embryonic development.

In vivo oocytes begin to mature during tertiary follicle development and are highly regulated by steroid hormone concentrations. GnRH released from the hypothalamic nuclei in the hypothalamus stimulates the release of LH and FSH from the anterior pituitary which induces follicular growth and oocyte maturation. The tertiary follicle population contains developing and atretic follicles and a follicle can become atretic at any time during development (Lin and Rui, 2010). Estrogens and androgens are at their highest concentration during the preovulatory growth phase and decrease after ovulation. The ovulated COC has expanded cumulus cells and contains an oocyte that has undergone GVBD and is progressing toward MII in development (Ainsworth et al., 1980). The COC is ovulated into the infundibulum and with the assistance of the fimbria are guided toward the ampulla of the
oviduct. The oocyte is only viable for approximately 12 hrs once it has been ovulated (Hunter., 2000). The COC migrates down the ampulla assisted by ciliated cells which beat in the direction of the AIJ (Fujita et al., 2008b) at approximately 76 mm/hr (Brüssow et al., 2008). The AIJ is the site of fertilization and the oocyte will rest at this junction until sperm travel up the isthmus to fertilize the oocyte.

**Fertilization**

Fertilization is the process whereby the sperm and egg fuse together to begin the creation of a genetically unique individual whose genome is derived from both parents. Following ovulation in the pig, litter size can be limited by fertilization failure, embryonic loss, and fetal loss due to uterine space limitations or fetal loss as a result of disease.

Sperm is deposited into the cervix of the gilt/sow by artificial insemination or natural mating. Freshly ejaculated sperm must undergo capacitation before they are capable of undergoing an acrosome reaction to successfully fertilize an oocyte. Capacitation occurs *in vivo* in the uterine environment and can be induced *in vitro* with bovine serum albumin, calcium and bicarbonate (Bedu-Addo et al., 2005; Xia and Ren, 2009). Capacitation induces chemical and physiological changes in the sperm which increases pH and Ca\(^{2+}\) concentration, membrane hyperpolarization and motility (Haim, 2002; Suarez, 2008). The majority of sperm will be eliminated and only a fraction will ascend the uterus and collect in the utero-tubular junction at the posterior of the uterine horn (Rodriguez-Martinez et al., 2005). This junction can store sperm for up to 30 hrs until the time of ovulation when they will be gradually released and a limited number will continue migrating up the isthmus (Rodriguez-Martinez et al., 2005; Tienthai et al., 2004). The isthmus consists of smooth muscle and
lacks ciliated cells. The sperm swim up the isthmus with the aide of peristaltic muscle contraction and increased flow of tubal fluid which pushes the sperm up toward the AIJ (Fujita et al. 2008).

The sperm must undergo capacitation before fertilization so that it may properly bind and fertilize the oocyte. Oviductal fluid (ODF) is secreted from the oviduct epithelium and has an acidic pH and low levels of bicarbonate which capacitate the sperm. ODF is most potent for capacitation post-ovulation when bicarbonate levels are at 33-35 mM (Tienthai et al., 2004). The exocutosis of the acrosomal vesicle to release its enzymes is required for the sperm to penetrate the zona pellucida of the egg. The binding of the sperm to and subsequent entry through the zona pellucida of the egg begins the process of fertilization. Fusion of the egg and sperm cell membranes facilitates the entry of the male gamete into the egg prior to syngamy.

The act of fertilization initiates reactions within the egg cytoplasm and results in cell cycle resumption and prepares the pending zygote for early development following fusion of the male and female pronuclei. The first step of fertilization is the contact and recognition between sperm and egg that results in the acrosomal reaction. In most cases, species specific receptors ensure that only the sperm and egg of the same species are able to interact and fertilize (Burkin and Miller, 2000). These receptors and the zona pellucida regulate entry of a sperm cell into the egg. In the pig, zona pellucida receptor 3 (ZP3) has been identified as the primary sperm receptor with O-glycans mediating the interaction (Yurewicz et al., 1991). Only one sperm nucleus can ultimately unite with the egg nucleus. This is usually accomplished by allowing only one sperm to enter the egg and inhibit any others from entering. Polyspermy occurs when more than one sperm is allowed to enter the oocyte and
results in the death of the embryo. Only a small fraction of the sperm that enter the female reproductive tract make it to the site of fertilization which decreases the incidence of polyspermy (Fujita et al., 2008; Rodriguez-Martinez et al., 2005). The utero-tubular junction acts as a sperm reservoir and releases spermatozoa towards the AIJ to coincide with ovulation window (Brüssow et al., 2008). Osteopontin (SPP1) has been used in IVF in swine to reduce the incidence of polyspermy and increase fertilization (Hao et al., 2006). Once the spermatozoa have arrived at the AIJ it must penetrate the zona pellucida following the acrosome reaction and bind the oocyte plasma membrane. Once bound the oocyte engulfs the entire sperm through phagocytosis to acquire the genetic material of the sperm.

A calcium wave initiates at the point of fertilization and travels across the egg and through the sperm plasma membrane triggering the acrosome reaction (Tiwari-Woodruff and Cox, 1995). In the oocyte, Ca\textsuperscript{2+} is released from inositol triphosphate sensitive stores triggering the release of additional Ca\textsuperscript{2+} from intracellular stores. Collectively these two waves of Ca\textsuperscript{2+}, which last over 3 h, are necessary for oocyte activation (Petr et al., 2002). The maternal, and to a greater extent, the paternal DNA are demethylated as they are combine within the embryo. As the embryo begins development, the genome is remethylated in cell specific patterns as the developing embryo achieves the blastocyst stage when \textit{de novo} methylation occurs in the inner cell mass cells and trophectoderm (Fulka et al., 2006).

**Early embryo development and uterine migration in the pig**

After fertilization the male and female pronuclei fuse to form a single cell zygote. Holoblastic cleavages begin approximately 24 h after fertilization producing smaller cells with each division. Embryos that cleave earliest after fertilization are more competent and
have a developmental advantage compared to later cleaving embryos (Isom et al., 2011). The first cleavage occurs within 24-30 h in the porcine zygote which is similar to humans and many higher organisms (Bazer et al., 1993). At 60 hrs following fertilization, the pig zygote is typically at the 4-8 cell stage and has begun migrating towards the uterus. The embryonic genome becomes activated in the 4-cell stage and transcription of new mRNA, which was halted at GVBD, will resume. The embryonic genome must activate an estimated 10,000 genes for successful embryogenesis (Niemann and Wrenzycki, 2000). The embryo will develop to the morula stage by day 5 and then blastocyst formation occurs on day 6. The blastocyst will hatch from the zona pellucida when it reaches approximately 150 cells or more (Anderson, 2000). The developing embryos migrate throughout the uterine horns until trophoblastic elongation occurs around d 11-12 of gestation.

**Embryo elongation and implantation**

The pig blastocyst is unique in that it undergoes rapid elongation from a sphere (10 mm) to a long filamentous form (>150 mm) on d 11-12 of gestation (Geisert et al., 1982a; Heuser and Streeter, 1929). The trophoblast expands to increase placental surface for maternal:conceptus nutrient exchange and communication (Stroband and Van der Lende, 1990). The pig conceptus morphologically changes within hours whereas other large livestock species such as the cow or ewe take days for the conceptus to elongate and establish its space in the uterus. The pig conceptus elongates from ovoid (<10 mm) to filamentous (>150 mm) in about 4 hrs during day 11 to 12 of gestation (Anderson, 1978; Geisert et al., 1982a). All stages of embryos can be found in the uterus from days 10 through 12 (Anderson, 1978; Pusateri et al., 1990). Elongation is asynchronous and embryos that
develop slowly have the greatest probability of dying (Bazer et al., 1993). Embryo elongation involves cellular migration not cellular hyperplasia. Although both DNA and protein increase from day 9 to 13, the protein: DNA ratio decreases (Geisert et al., 1982a; Pusateri et al., 1990). During trophectoderm elongation, the diameter of the embryo decreases and there is no increase in cell number during rapid trophoblast elongation (Pusateri et al., 1990). The expansion of the trophectoderm allows the conceptus to establish sufficient uterine space for placentation and nutrient exchange throughout gestation.

The developing pig conceptuses must signal their presence to prevent CL regression and establish pregnancy. The pig conceptus does this by synthesizing and secreting estrogen during elongation on days 10 through 12 of gestation (Geisert et al., 1982b; Guthrie, 1975; Perry et al., 1973; Pusateri et al., 1990). Elongating conceptuses produce more estradiol compared to their spherical counterparts in culture (Pope, 1988). Estrogen acts on the uterus to stimulate the release of endometrial secretory products (Geisert et al., 1982b). The uterus and conceptus also communicate to prepare for implantation by increasing synthesis of IGF-I, IGF-II binding factors, protease inhibitors, interferon proteins, and the pro-inflammatory cytokine, interleukin-1β (Lee et al., 1998; Reed et al., 1998; Ross et al., 2003; Simmen and Simmen, 1990). Estrogen signals the maternal system to maintain the CL on the ovary for production of P4 by redirection the prostaglandin F2α release from the uterine endometrium. There must be four conceptuses, two per uterine horn, to produce enough signal for maternal recognition and pregnancy establishment to occur in the pig (Dziuk, 1968).

Mitotic activity is low during the initial stages of elongation (10 to 330 mm) and the morphological change is due to cell migration and restructuring not cell proliferation (Geisert et al., 1982a). Messenger RNA expression profiles have identified differential expression in
various conceptus morphological stages using microarrays (Ross et al., 2009), RT-PCR (Green et al., 1995; Wilson et al., 2000), library construction and analysis (Smith et al., 2001), cDNA library array (Lee et al., 2005) and serial analysis of gene expression (SAGE) (Blomberg et al., 2005). These different techniques are consistent at identifying gene expression and affected pathways during conceptus elongation.

The embryos are equally spaced within the uterus by day 13 of gestation and initial attachment of the trophectoderm to the uterine epithelium begins (Dantzer, 1985; Perry, 1981). The area of uterine space occupied by the conceptus is important for influencing fetal-maternal interactions including nutrient and waste exchange. Conceptus membranes do not overlap or adhere to one another until late gestation and blood supplies are not shared between adjacent conceptuses in the pig (Crombie, 1972). Trophoblast-endometrial interactions are established by day 35 of gestation and begin to develop characteristic folds (Friess et al., 1980; Knight et al., 1977). Uterine crowding can result in conceptus loss between days 30 and 40 of gestation (Knight et al., 1977; Vallet and Freking, 2007). Embryonic loss early in gestation may leave unoccupied regions of the uterus which can be utilized by the adjacent conceptus to increase their size and weight by a small percentage (Vallet et al., 2011; Vonnahme et al., 2002). However the weight of the fetus at 105 days is determined by the uterine space occupied and number of fetuses present at 35 days of gestation (Vallet et al., 2011). The conceptus must establish itself in the uterus and adhere to the endometrium for proper development during gestation.
Small RNAs in the oocyte, early embryo and elongating conceptus

RNA

Ribonucleic acid (RNA) is a polymer composed of ribonucleotides linked together by a phosphodiester bond, constructed for gene expression by RNA polymerase. Mature RNA is a single strand composed of exons transcribed from the deoxyribonucleic acid (DNA) of the gene to be expressed. RNA is synthesized by RNA polymerase which uses a DNA template to transcribe RNA (Paule and White, 2000). Coding RNA are those which are translated into proteins and include exons spliced together to form a single stranded messenger RNA (mRNA) which can then be translated into a protein. Protein synthesis takes place at ribosomes which link amino acids together according to the mRNA codon sequence. To do this, transfer RNA (tRNA) are utilized to identify the sequence specific codons and provide the corresponding amino acid. Ribosomal RNA (rRNA) and ribosomal proteins serve as the manufacturing machinery (Brenner et al., 1961). Non-coding RNA are functional RNA molecules that are not translated into proteins. MicroRNA (miRNA), snoRNA, piwiRNA (piRNA), tRNA, and rRNA are all examples of non-coding RNAs. While each of these RNA classes has distinct functions, the knowledge and understanding of miRNA function is a rapidly expanding area in biology.

MicroRNA

MicroRNA are short (18-24 nt) non-coding RNAs derived primarily from introns of coding genes in the mammalian genome (Su et al., 2010). First discovered in 1993, lin-4, a 22nt miRNA was found to negatively regulate LIN-14 protein during development in C.
elegans (Lee et al., 1993; Wightman et al., 1993). MiRNA are generally expressed in a tissue specific and spatial manner relative to a developmental stage or process. Similar to mRNA transcription, miRNA expression is also driven predominately via RNA polymerase II activity however some are expressed via RNA polymerase III (Bartel, 2004).

Primary miRNA have been coined ‘pri-miRNA’ and are transcribed by RNA polymerase II (Kim, 2005). RNase-III enzymes Drosha and DGCR8/Pasha process the pri-miRNA in the nucleus to form a ~70 nt stem-loop structure (Denli et al., 2004; Lee et al., 1993). Drosha cleavage, which requires the functional cooperation of DGCR8, due to its possession of two dsRNA binding motifs, results in the nuclear release of ~60-70 nt stem loops, referred to as miRNA precursors, or ‘pre-miRNA’. The pre-miRNA is actively exported out to the cytoplasm by Ran-GTP and Exportin-5 where it is cleaved by RNase-III enzyme, Dicer (Sontheimer, 2005). Following export of the stem loop pre-miRNA into the cellular cytoplasm, Dicer recognizes the dsRNA and typically cleaves both strands around two helical turns from the stem-loop base resulting in 18-24 nt dsRNA molecules possessing 3’ overhangs. These molecules are referred to as mature microRNA. These dsRNA duplexes are unstable and readily dissociated into ssRNA molecules, that based on their sequence complementation to mRNA may confer gene specific posttranscriptional gene silencing (PTGS).

The mature miRNA are processed and bound to a RNA-induced silencing complex (RISC) (Briscoe and Ericson, 2001) which recognizes and binds target mRNAs and inhibits or destabilizes the target RNA to prevent translation or to promote mRNA degradation (Bartel, 2004; Zamore and Haley, 2005). Argonaute proteins bind miRNA within the RISC complex to facilitate the PTGR of the specific mRNA target (Grishok et al., 2001). The
mature miRNA target mRNAs through imperfect base pairing with a seed region (6-8 nucleotides) on the 3' UTR of the target mRNA (Bartel, 2004).

Current estimations predict approximately 1,200 miRNA are present in the human genome (Griffiths-Jones, 2002; Saini et al., 2007), having the potential ability to impact approximately 30% of protein coding genes (Miranda et al., 2006). MicroRNA do not require a perfect match to their seed region of a target mRNA to be functional, allowing a multitude of mRNA targets for a single miRNA. Computer models are inaccurate at predicting miRNA to mRNA interactions that exist biologically due to the temporal and spatial control of miRNA expression (Yang et al., 2009). Some miRNA have numerous mRNA targets (Mickoleit et al., 2011) while others have few predicted targets (Brennecke et al., 2005; Rajewsky and Socci, 2004). It is unclear how a miRNA interacts with its specific target, in a cell when multiple 3' UTR of target mRNA are available and predicted to be targets. For example when twelve predicted mRNA targets of MIR21 were evaluated in breast cancer cells it was determined that only six were actually directly targeted by MIR21 (Yang et al., 2009). Proteomics are being used to evaluate the regulation of target genes by miRNA in an attempt to unravel the complex mechanism. More studies are needed to identify and define miRNA regulation and targets.

**MiRNA in the pig**

As stated above, over 1,200 mature miRNA have been identified for humans and mice while only a few hundred have been identified in the pig and most of those are predicted, not validated miRNA (Griffiths-Jones, 2002; Kim et al., 2008; Reddy et al., 2009; Xie et al., 2011). However the pig genome will likely be updated and more information
including miRNA identities will be available. MiRNA have been studied in several pig tissues such as the heart, lungs and developing brain as well as muscle groups which are important for meat production. Small RNA libraries generated from specific tissues in the pig have identified differentially expressed miRNA and novel miRNA within the pig genome. Pig heart, liver and thymus have been used to identify 120 conserved miRNA including 11 novel miRNA which displayed heart specific and liver specific miRNA expression patterns (Reddy et al., 2009). Fibroblast cells from multiple tissues were pooled and an additional six novel miRNA were identified in the pig (Kim et al., 2008). Cloning and characterization of muscle and adipose tissue were used to identify an additional 15 conserved miRNA to expand the number of known pig miRNA (Cho et al., 2010). MiRNA are differentially regulated during development and distinct patterns of expression have been observed in the same tissue at different stages of development. MiRNA in the developing pig brain were found to have differential expression during development. Within specific regions of the brain, MIR135a–135b clusters were discovered which had not been previously identified (Podolska et al., 2011). Another 332 miRNA expressed in the porcine intestine, including 201 novel miRNA, have been identified which could be used to study intestinal function and disease (Sharbati et al., 2010). MiRNA have been identified in skeletal muscle which play a role in development and regeneration as well as signal transduction, cell-cell and cell-extracellular matrix communication (Nielsen et al., 2010). MiRNA identified in the pig include MIR-206 and MIR-1 which are involved in myogenesis and contribute to skeletal muscle development by regulating connexin 43 protein during myoblast development (Anderson et al., 2006). The let-7 family, a key miRNA regulator of development, which is highly conserved across several species, includes eight members that
are found in pig skeletal muscle during development (Anderson et al., 2006). MiRNA sequences compared between breeds demonstrate that miRNA related to liver development, liver cell apoptosis and energy metabolism were differentially expressed between lean and fatty breeds of pigs (Xie et al., 2011). In the pig placenta 17 miRNA were identified to have different expression patterns on day 30 compared to day 90 (Su et al., 2010). The completion of the pig genome in the near future will allow for the identification and validation of numerous miRNA in the pig.

**MiRNA in the oocyte and early embryo**

The function of miRNA during pig oocyte maturation and early embryo development is not well understood at this time. However multiple studies in mice have shown that miRNA are present in the oocyte and are required for early embryo development (Tang et al., 2007). Maternal repertoires of mRNAs and proteins are stored in the oocyte until activation during recruitment and maturation to MII. These mRNAs must be preserved and stored until needed and then utilized by the oocyte for proper development (Tadros and Lipshitz, 2005). MiRNA play a vital role in regulating and manipulating maternal mRNA for normal oocyte maturation. Dicer is essential for oocyte maturation and when removed from mouse oocytes during maturation there is no progression past the first cell division (Tang et al., 2007). This study demonstrated that maternal genes in the oocyte are directly and indirectly regulated by miRNA and that miRNA are essential for oocyte development in the mouse (Tang et al., 2007). MicroRNA have been identified in the bovine oocyte, granulosa and COC with let-7b being the most abundant miRNA representing 26.5% of all reads for a bovine COC small RNA cDNA library (Miles et al., 2012). Let-7 had been previously identified in mouse (Reid
et al., 2008) and bovine (Tripurani et al., 2010) ovaries. Let-7 is also known to suppress tumors by interacting with MYC, a nuclear protein involved in the control of cell proliferation and differentiation (Sampson et al., 2007) but the exact role of let-7 in reproductive tissues is unknown. In cattle, MIR106a was most abundant in oocytes compared to granulosa cells which indicate it may play an important role in the oocyte development (Miles et al., 2012). A small RNA library in the pig oocyte and early embryo demonstrated that sn/sno RNA are most abundant in GV oocytes and cumulus cells, MII oocytes and cumulus cells, 4-8 cell stage embryos and blastocyst (Yang et al., 2012). MiRNA were the second most abundant RNA class in MII cumulus cells and third in GV cumulus cells (Yang et al., 2012). Chromosomal distribution of miRNA reads for the pig oocyte, and early embryo library demonstrated dynamic changes in the number of reads (Yang et al., 2012). A large number of miRNA have been identified in the mouse, bovine and pig tissues however more research is needed to elucidate the function of miRNA in oocyte maturation and early embryo development.

**MiRNA in the elongating pig conceptus**

Numerous mRNA molecules are differentially expressed during this dynamic period of conceptus growth (Blomberg et al., 2005; Ross et al., 2009). Early fetal development may involve more than 10,000 genes (Niemann and Wrenzycki, 2000) which must be regulated temporally and spatially for normal development to occur. A majority of prenatal conceptus death in the pig occurs between 12 and 18 days of gestation (Pope and First, 1985) during embryo elongation and implantation. MiRNA likely play an essential role conferring PTGR during embryo elongation and implantation. However little is known about the roles of
miRNA during embryo elongation in the pig. More research is needed to determine the roles of miRNA during embryo elongation in the pig.

**MiRNA-21**

MiRNA21 (MIR21) has been identified as a key component during cell proliferation, apoptosis and as a regulator of many cancer cell types (Bueno et al., 2008; Chan et al., 2005; Dillhoff et al., 2008; Yang et al., 2009). Carletti et al., (2010) examined the expression of MIR21 in mouse granulosa cells and suggested MIR21 may play a role in ovulation and oocyte development. They found that by inhibiting MIR21 in the mouse ovary there was increased cell apoptosis detectable by tunnel staining and ovulation rate was decreased (Carletti et al., 2010). Quantitative proteomics have been used to characterize MIR21 in breast cancer cell line MCF-7 (Yang et al., 2009). MIR21 specifically targets programmed cell death 4 (PDCD4) mRNA, and indirectly regulates activator protein 1 (AP-1), and STAT3 which have all been demonstrated to play important roles in cell fate regulation in normal development and during oncogenesis (Asangani et al., 2007; Fujita et al., 2008b; Wang et al., 2008). The transcriptional and translational regulation of MIR21 in oncogenesis may be similar to MIR21 regulation in oocyte and embryo development since both processes are characterized by an increase in cell cycle. MicroRNA in the developing embryo has not been well studied. MiRNA profiling in our lab has demonstrated a significant increase of MIR21 abundance during oocyte maturation (Yang et al., 2012, Figure 2.2). Obtaining an understanding of MIR21 and its mechanism is necessary to understand molecular regulations during oocyte maturation and early embryo development. Understanding molecular mechanisms of early embryo development will provide a more comprehensive understanding
of mammalian reproduction and development that can be fundamental in developing strategies to improve reproductive efficiency in pigs.

Programmed cell death 4 (neoplastic transformation inhibitor) is composed of 469 amino acids, with two basic domains at the N-terminus and C-terminus and two conserved α-helical MA-3 domains (Göke et al., 2002; Shibahara et al., 1995). The PDCD4 protein is localized to the nucleus and is expressed ubiquitously in normal tissues (Lankat-Buttgereit and Goke, 2009). PDCD4 was first described as a tumor suppressor which is up regulated during cell apoptosis and is negatively regulated by MIR21 (Asangani et al., 2007; Lu et al., 2008b). Apoptosis must be regulated during early embryo development for proper development (Degrelle et al., 2009). Similarly apoptosis is down regulated in most cancer cells leading to the proliferation of the cells that contribute to tumor formation (Asangani et al., 2007).
Figure 2.2. MiRNA are differentially expressed between GV and MII arrested oocytes. Microarray analysis demonstrated that several miRNA are differentially expressed during oocyte maturation from GV to MII arrest (adapted from Yang et al., unpublished data). Abundance of several miRNA either decreased or increased in MII arrested oocytes compared to GV stage oocytes. MIR21 represented the greatest difference in abundance in MII arrested oocytes compared to GV stage oocytes.
Figure 2.2.
Activator protein 1 (AP-1) activity is mediated by the down regulation of PDCD4 by MIR21 in tumorigenesis (Talotta et al., 2008). AP-1 promotes cell proliferation and regulates MIR21 through a double-negative feedback mechanism (Fujita et al., 2008). Therefore, it may be possible that by blocking apoptosis via PDCD4, MIR21 may increase cell proliferation by promoting AP-1 activity.

**Summary**

Oocyte maturation and embryo development are complex biological processes in which miRNA may play an important role as regulators of PTGR. Understanding the role of miRNA throughout these developmental processes will lead to a deeper understanding of reproduction and provide foundational knowledge that can be used for improving pig reproduction.
CHAPTER III

MicroRNA21 expression and regulation of PDCD4 during in vitro maturation of porcine oocytes.

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Cai-Xia Yang – Sample Collection

Jason W. Ross – Principle investigator, Project Design
MicroRNA (miRNA) are small non-coding RNA molecules critical in regulating cellular function including oocyte and early embryo development. MiRNA21 (MIR21) is a regulator of posttranscriptional gene regulation in several tissues including cancer, embryonic tissues and the ovary; and is associated with rapid cell proliferation and inhibition of apoptosis through interactions with \textit{PDCD4} mRNA and other targets. MIR21 interacts and suppresses PDCD4 due to the strong complementation between the MIR21 and the \textit{PDCD4} 3'UTR. The objective of this project was to examine the relationship between MIR21 and PDCD4 expression in porcine oocytes during \textit{in vitro} maturation and the impact of MIR21 inhibition during oocyte maturation on early embryo development. Additionally, we evaluated the effect of gonadotropins in maturation media and the presence of cumulus cells to determine their ability to contribute to MIR21 expression in the oocyte during maturation. To determine the regulatory role of MIR21 in the cumulus oocyte complex, antisense oligonucleotide inhibitors to prevent MIR21 function were utilized. During \textit{in vitro} maturation, expression of MIR21 increased approximately 4-fold in the oocyte and 25-fold in the cumulus cell. PDCD4 protein was 7-fold less abundant in MII arrested oocytes compared with GV stage oocytes while \textit{PDCD4} mRNA was not significantly different. Inhibition of MIR21 activity in the oocyte during \textit{in vitro} maturation using antisense MIR21 inhibitor prevented PDCD4 protein suppression and decreased embryo development to the 4-8 cell stage following parthenogenetic activation.
Introduction

Germinal vesicle breakdown (GVBD) is the first physical sign that an oocyte is committed to maturation and also represents the onset of a period of transcriptional quiescence, which persists until the activation of the embryonic genome. During this period, changes in mRNA and protein abundance within the oocyte can occur through interactions with the surrounding cumulus cells and/or through posttranscriptional gene regulation (PTGR) within the oocyte. While several mechanisms of PTGR are well described, microRNA (miRNA) represent a unique RNA class that function as potent regulators of transcription and protein abundance (Aiba et al., 2006). MiRNA are small (18-24 nt), non-coding RNA molecules that confer PTGR through several mechanisms, such as impairing translation efficiency and affecting mRNA stability following interaction with target mRNA molecules (Bagga et al., 2005; Bartel, 2004), primarily through binding recognition motifs in the 3’ untranslated region (3’UTR) (Brennecke et al., 2005; Doench and Sharp, 2004). Numerous miRNA are expressed in the mouse oocyte and developing embryo and it has been further demonstrated that knockout of dicer, a critical enzyme involved in miRNA processing, during oocyte development, significantly impairs the production of normal oocytes capable of being fertilized and developing (Tang et al., 2007). Current estimations predict approximately 1,000 miRNA are present in the human genome (Griffiths-Jones, 2002), having the potential ability to impact approximately 30% of protein coding genes (Miranda et al., 2006). Some miRNA have numerous mRNA targets (Mickoleit et al., 2011) while others have few predicted targets (Brennecke et al., 2005; Rajewsky and Socci, 2004).

Utilizing miRNA microarray analysis and deep sequencing, we have previously identified microRNA-21 (MIR21) as an up-regulated miRNA during porcine oocyte in vitro
maturation (Yang et al., unpublished data). In mice it has been reported that luteinizing hormone may increase the expression of MIR21 in mouse granulosa cells and in vivo MIR21 inhibition has a negative impact on ovulation rate (Carletti et al., 2010).

MIR21 is a well characterized miRNA that has demonstrated the ability to confer PTGR in numerous carcinomas and cell lines affecting cell proliferation by controlling apoptosis (Bueno et al., 2008; Chan et al., 2005; Dillhoff et al., 2008; Yang et al., 2009). MIR21 is expressed via RNA polymerase II, and is located in intron 10 of the transmembrane 49 (TMEM49; also referred to as VMP1) gene (Cai et al., 2004; Fujita et al., 2008a). The mature MIR21 sequence was first identified in human HeLa cells (Lagos-Quintana et al., 2002) and has since been predicted and verified in several other species, including the pig. The anti-apoptotic capabilities of MIR21 in cancer cells are primarily through the ability to suppress critical apoptotic genes such as programmed cell death 4 (PDCD4, previously referred to as neoplastic transformation inhibitor) (Asangani et al., 2007; Frankel et al., 2008; Lu et al., 2008; Qi et al., 2009). PDCD4 is composed of 469 amino acids, with two basic domains at the N-terminus and C-terminus and two conserved α-helical MA-3 domains (Göke et al., 2002; Shibahara et al., 1995). MIR21 interacts with PDCD4 through binding with complementary sequence in the 3’UTR of PDCD4 resulting reduced translation and protein abundance (Asangani et al., 2007; Lu et al., 2008). Global expression profiling of germinal vesicle stage (GV) oocytes and 4-cell stage embryos has previously demonstrated PDCD4 expression in pig oocytes (Whitworth et al., 2005). Also important, the 3’UTR of pig PDCD4 possesses a conserved MIR21 recognition sequence suggesting biologically active MIR21 during oocyte maturation may impact PDCD4 protein abundance in the oocyte following GVBD and progression to MII.
Our working hypothesis is that MIR21 expression in the pig cumulus oocyte complex (COC) can influence the abundance of PDCD4 through PTGR following GVBD. The objective of this study was to determine expression patterns of MIR21 and demonstrate its potential interactions with PDCD4 in the COC during oocyte maturation in the pig. Here we demonstrate PDCD4 protein down regulation temporal to MIR21 up regulation during in vitro oocyte maturation. These data indicate a reduced ability of MIR21 to suppress PDCD4 protein expression in the presence of a MIR21 inhibitor suggesting a biological interaction between MIR21 and PDCD4 mRNA occurs during in vitro oocyte maturation in the pig.

Materials and methods

In vitro maturation

Sow ovaries were obtained from a local abattoir for isolation of COCs. Follicles (3-5 mm) were aspirated and COC were collected and washed in TL-Hepes with 0.1% polyvinyl alcohol (PVA) (Lai and Prather, 2003). Cumulus oocyte complexes were cultured in maturation media (Tissue Culture Media 199 (TCM-199)) containing 0.57 mM L-cysteine, follicle stimulating hormone (0.5 µg/mL), luteinizing hormone (0.5 µg/mL), and epidermal growth factor (10 ng/mL) for 42-44 hours at 39.0°C in 5% CO₂. Prior to in vitro maturation, an aliquot of GV stage oocytes for each replication were randomly selected from the COC pool. GV stage oocytes used for analysis were stripped of cumulus cells via vortex (6 to 8 min) in 1% hyaluronidase in TL-Hepes-PVA. Following in vitro maturation oocytes were stripped of cumulus by vortexing 5-6 min in TL-Hepes-PVA supplemented with 1% hyaluronidase and Metaphase II (MII) arrested oocytes were identified by the presence of an extruded polar body. Cumulus cells before and after maturation (n=4 per maturation stage)
and GV and MII oocyte pools (25 oocytes per pool, n = 4 per stage) were snap frozen in liquid nitrogen and stored at -80°C until used for quantitative reverse transcription PCR (RT-PCR). Pools of GV and MII arrested oocyte pool from the same replications (50 oocytes per pool, n=4) were utilized for Western blot analysis.

**MIR21 expression in oocytes with and without LH and FSH during in vitro maturation**

To determine the effect of LH and FSH on MIR21 expression in oocytes during *in vitro* maturation, COCs were matured in maturation media as described above with LH and FSH or lacking LH only, lacking FSH only or lacking both. COCs were washed four times and cultured in the designated hormone treatment in groups of 80-90 COCs per well. COCs were cultured for 42 hrs, denuded of cumulus cells by vortexing as described above and MII oocytes were identified by the presence of a polar body. This experiment was repeated four times. Maturation rates were recorded and MII arrested oocytes from each treatment and replication were collected for MIR21 expression analysis as described above.

**MIR21 expression in oocytes cultured with and without cumulus cells**

To determine the effect of cumulus cell presence on MIR21 expression in oocytes during *in vitro* maturation, we subjected COCs to one of three treatments 1) normal *in vitro* maturation (intact COC), 2) *in vitro* maturation following cumulus cell removal and then co-cultured with denuded oocytes or, 3) denuded oocytes matured without cumulus cells. Cumulus cells were removed by gentle vortex in 1% hyaluronidase in TL-Hepes. Cumulus cells returned to cell culture were centrifuged at 600 x g for 5 minutes washed twice in 200 µL of maturation media and then resuspended in 200 µL of maturation media and added to
the *in vitro* maturation culture plates which contained 300 µL maturation media and the
denuded oocytes. Final volume of culture media for all plates was 500 µL and each well
contained 75-85 oocytes. This experiment was repeated four times. Maturation rates were
recorded and MII arrested oocytes from each treatment and replication were collected for
MIR21 expression analysis as described above in the *in vitro* maturation section.

**MIR21 inhibition during in vitro maturation**

Peptide nucleic acids (PNA) are artificially constructed oligonucleotides with a
strong affinity and specificity to endogenous nucleotides while resistant to nuclease making
them ideal for miRNA inhibition (Oh et al., 2009). We used an anti-MIR21 PNA (Panagene
Inc., Daejeon, Korea) designed to specifically bind to and prevent MIR21 activity. A
scrambled PNA with no predicted targets was used as a negative control (Panagene Inc.,
Daejeon, Korea). PNA oligonucleotides were diluted in maturation media at a stock
concentration of 100 nM/µL and then added to maturation media on the day of COC
collection to acquire a final concentration of 2.0 nM and 0.2 nM. A control group without
PNA was used to evaluate the potential toxicity of the PNA. GV oocytes and cumulus cells
were collected at the time of COC collection and MII oocytes and cumulus cells were
collected at 42-44 hrs of maturation.

Parthenogenetic activation of MII oocytes was performed with 50 oocytes from each
treatment to determine developmental competence up to 60 hrs. MII arrested oocytes were
washed in a high calcium activation media (Mannitol 0.28M, CaCl₂ 1.0 mM, MgCl₂ 0.1mM,
HEPES 0.5mM and BSA 1mg/mL), then placed between two electrodes covered with
activation media and activated by two consecutive 30 µsec pulses at 1.2 kv/cm. Following
activation, zygotes were washed and cultured in porcine zygote medium-3 (PZM3) at 39°C in 5% CO$_2$ (Lai and Prather, 2003). At 60 hours embryos were evaluated for development and the number of embryos with 4 or more uniform blastomeres was recorded.

**Quantitative RT-PCR of pooled porcine oocytes**

Oocytes were collected and denuded of cumulus cells as described above. Oocytes from each stage of development and treatment were collected in pools of 25 oocytes in 10 µL of PBS. Both PDCD4 and MIR21 analysis were analyzed from the same sample lysis. TaqMan™ Gene Expression Cells-to-Ct™ Kit (Applied Biosystems, Carlsbad, CA) was used to lyse oocytes and prepare samples for RT-PCR. Lysis solution and DNase from the Cells-to-Ct kit were added to each pool at 4.95 and 0.05 µL, respectively, and incubated at RT for 5 min. Stop solution (0.5 µL) was added, incubated for an additional 2 min and placed on ice. Two µl of the sample lysis was added to each RT-PCR reaction. PDCD4 forward (5’- ACAGTTGGTGGCCAGTTTATTGC-3’) and reverse (5’- CTTTGCACCTCCACCTTAGACA- 3’) primers were used to determine mRNA abundance of PDCD4 within each pool. QuantiTect® SYBR® Green RT-PCR Kit (Qiagen, 204243) was used for the RT-PCR reaction with PDCD4 according to manufacturer’s recommendations. The standard cycling conditions were 50°C for 30 min, 95°C for 15 min followed by 45 cycles of 95°C for 15 sec, 60°C for 30 sec, and 72°C for 30 sec followed by, melting curve analysis.

MIR21 was quantified using TaqMan® MicroRNA Reverse Transcription kit (Applied Biosystems, Carlsbad,CA) for the RT reaction and the primers and probe used were TaqMan® MicroRNA Assay for hsa-MIR21 (Applied Biosystems, Carlsbad, CA) according
to manufacturer’s recommendations. The RT reaction was 20 µL consisting of 13 µL master mix, 3 µL primers, and 4 µL sample lysis. Reverse transcription conditions were 16°C for 30 min, 42°C for 30 min and 85° for 5 min. The final volume for all RT-PCR reactions was 20 µL which include 1.33 µL of the RT product, 1 µL TaqMan MicroRNA Assay (20x), 10 µL TaqMan 2x Universal PCR Master Mix and 7.67 nuclease free water. The thermal cycling conditions for the TaqMan MicroRNA RT-PCR were 95° for 10 min, followed by 45 cycles of 95°C for 15 sec and 60° for 60 seconds. Fluorescent data acquisition was during the 60°C extension step. For analysis of MIR21 expression in cumulus cells, total RNA was extracted from cumulus cells of GV stage and MII arrested oocytes using the mirVana RNA isolation kit (Life Technologies, Grand Island, NY), 10 ng of total RNA was utilized for the RT reaction conducted the same way as the oocyte samples. For cumulus cells, cycle threshold (C_T) values were normalized to RNU43 RNA expression prior to comparison between stages and statistical analysis. All samples were assayed in duplicate. The comparative C_T method was used to calculate relative fold changes between samples as previously described (Ross et al., 2003).

PDCD4 Western blot analysis

Pools of 50 denuded GV and MII oocytes within a replication were collected, washed in PBS and stored at -80°C until used for Western blot analysis. Oocyte pools were lysed in 2.5 µl of 5x SDS (total sample volume 12.5 µL) at 95°C for 4 min followed by 1 min on ice and then centrifugation at 1000 rpm for 1 min at RT. Samples were then loaded into a 4-20% Tris glycine gel (Lonza PAGEr® Gold Precast Gels). BioRad Mini PROTEAN Tetra System was used to run the gel at 60 volts for 30 minutes followed by 120 volts for 90
minutes. The gel was transferred to a nitrocellulose membrane for 1 hr at 100 volts at 4°C. Membrane blocking was conducted using 5% milk in PBST (PBS with 0.5% Tween 20) for 1 hr at RT. A rabbit anti-PDCD4 monoclonal antibody (Abcam, ab79405) was added (1:1000 dilution) to the membrane in 0.5% milk in PBST overnight at 4°C and a negative control membrane lacking primary antibody was also conducted. Following primary antibody incubation, the membranes were washed with PBST three times at RT for 10 min each. Donkey anti-Rabbit IgG (Amersham™ ECL™ NA934) was incubated (1:2000) with the membrane for 1 hr at RT. The membrane was then washed three times for 10 min each at RT. Horse–radish peroxidase substrate (Millipore, Billerica, MA) was added to the membrane for 1 min in the dark. The membrane was then exposed to x-ray film and developed for visualization. Average pixel intensity for the protein corresponding to 52 kDa (PDCD4 molecular weight) was conducted using Image J (Abramoff et al., 2004).

**Immunostaining of GV and MII oocytes**

Oocytes were placed in 4% paraformaldehyde overnight and preserved in PBS until staining. Oocytes were transferred into 0.5% Triton X100 in PBS for 30 minutes at RT followed by blocking with 3% BSA in PBS for 45 minutes at room temperature. Rabbit anti-PDCD4 monoclonal antibody (1:500 dilution) in 3% BSA overnight at 4°C was incubated with each treatment and replication of oocytes. Oocytes were then washed in 0.05% PBST twice for 30 minutes and placed in secondary antibody, Alexa 674 anti-rabbit (1:250, Millipore, Billerica, MA) for 1 hr at room temperature. Two washes with 0.05% PBST for 30 minutes removed excess antibody and background. Oocytes were post-fixed in 2% paraformaldehyde for 10 minutes at RT, washed twice in 0.05% PBST and mounted with
anti-fade medium containing DAPI for visualization of the nucleus. Negative controls for both Alexa674 and DAPI were performed to establish base line setting for microscope imaging. ImageJ was used to determine pixel intensity for each oocyte and at least four oocytes were measured per replication (n=4) for each treatment.

**In situ hybridization of MIR21 in the developing follicle**

Gilt ovaries were preserved in 4% paraformaldehyde and utilized for in situ hybridization to determine MIR21 expression. Ovary sections (5µm) were then mounted on slides for analysis. Each section was subjected to CitriSolv® twice for five minutes, rehydrated in 2 changes of 100% ethanol for 3 minutes, followed by 95% ethanol for one minute and finally 80% ethanol for one minute. Slides were then immersed in heated citrate buffer (95˚C) for 30 minutes and then cooled to RT. Once at RT slides were blocked for 30 minutes with 5% BSA. Slides were then placed in hybridization solution for 1 hr at 65˚C. The probe was then added and slides were incubated in high humidity overnight at 65˚C. On day 2 slides were washed in standard saline citrate (SSC) solution and PBST at RT. Anti-fade DAPI was added and a cover slip was placed over each section. Primary and secondary follicles were imaged at 400x and tertiary follicles were imaged at 200x with a Leica microscope.

**Statistical Analysis**

PROC MIXED of the Statistical Analysis System was used to determine statistical differences of all data including percentage maturation and differences in C_T value for RT-PCR data. Significance (P < 0.05) was determined for the model and least-square means was
used to determine significant differences between samples. The effect of oocyte stage on MIR21 and PDCD4 expression ($C_T$) was determined. Treatment effect for the inhibitor, presence or absence of gonadotropins, and the presence or absence of cumulus cells during in vitro maturation on MIR21 expression was evaluated. Replication was included as a covariate. Percentage graphs are set to reflect the GV stage as 100% expression and all other treatments are relative to GV. Data are displayed as mean ± SEM.

**Results**

*MIR21 expression is temporally regulated during porcine cumulus oocyte complex maturation*

To determine the relationship between MIR21 and its putative target, PDCD4, during in vitro maturation, quantitative RT-PCR and Western blot analysis were utilized to evaluate expression of MIR21 and PDCD4 in GV and MII stage oocytes and cumulus cells. During the transition from GV to MII, MIR21 was up-regulated approximately 4-fold in oocytes ($P = 0.001$, Figure 3.1A) and approximately 25- fold in cumulus cells ($P = 0.003$, Figure 3.1B). In the same samples *PDCD4* mRNA abundance was not statistically different ($P = 0.34$, Figure 3.1A). Western blot analysis demonstrated PDCD4 protein abundance was reduced ($P = 0.02$) in oocytes during the transition from GV stage to MII arrested oocytes (Figure 3.1B and C). To verify this, immunostaining of individual oocytes for PDCD4 protein was performed and the pixel intensity was measured for each oocyte and compared between GV stage and MII arrested oocytes (Figure 3.2A and B). Similar to the Western blot analysis, PDCD4 protein expression, as determined by immunostaining, was greatest in GV stage
oocytes compared with MII arrested oocytes which had 21 ± 2.1% less PDCD4 protein compared to GV stage oocytes.

Gonadotropins in maturation media influence maturation rate but not MIR21 expression in MII arrested oocytes

To determine the effect of gonadotropins, LH and FSH, on MIR21 expression during in vitro oocyte maturation, we matured COCs with and without gonadotropins. Maturation rates were not different between media lacking FSH (62.7 ± 1.4%) compared with control maturation (61.7 ± 1.2%, Figure 3.3A). However, progression to MII arrest was significantly decreased (P < 0.001) when COCs were cultured in maturation media lacking LH (47.8 ± 2.5%) or both FSH and LH (47.4 ± 0.9, Figure 3.3A). MIR21 expression in MII arrested oocytes was not significantly different from the control and treatment groups (P = 0.68) although GV oocytes had significantly less (P = 0.03) MIR21 abundance compared with MII arrested oocytes cultured with LH and FSH or LH only (Figure 3.3B).

Cumulus cells influence oocyte maturation but not MIR21 expression in MII arrested oocytes

To determine the impact of cumulus cells on MIR21 expression in the oocyte during in vitro maturation we compared MIR21 expression in MII arrested oocytes following maturation of intact COCs, denuded oocytes cultured in the presence of cumulus cells and denuded oocytes cultured without cumulus cells. Maturation rate for control COCs was 58.4 ± 1.5% which tended to be greater than denuded oocytes cultured with cumulus cells (51.2 ± 4.0%, P = 0.07). However, denuded oocytes cultured without cumulus cells had significantly
lower (42.5± 2.0%) maturation rates than intact COCs \((P < 0.05)\) and denuded oocytes cultured with cumulus cells \((P < 0.05, \text{Figure 3.4A})\). MIR21 expression in MII arrested
**Figure 3.1** MIR21 expression is significantly increased in the cumulus cells of MII oocytes compared to cumulus cells of GV oocytes and has an inverse relationship with PDCD4 protein in the oocyte during maturation from GV to MII. (A) Quantitative RT-PCR analysis for MIR21 and PDCD4 was compared among GV and MII oocytes (n=4). (B) Quantitative RT-PCR analysis for MIR21 in GV and MII cumulus cells (n = 4). (C) PDCD4 Western blot analysis. Pixel intensity was quantified with ImageJ and demonstrates a decrease in PDCD4 protein abundance in MII oocytes compared with GV oocytes (n = 3). (D) Representative Western blot of PDCD4 protein for three reps of oocytes. a,bMeans ± SEM with different superscripts are different (P < 0.05).
Figure 3.1.

(A) MIR21 and PDCD4 mRNA expression in GV and MII oocytes

(B) MIR21 expression in GV and MII cumulus cells
(C)

PDCD4 protein expression in GV and MII oocytes

(D)

GV  MII  GV  MII
**Figure 3.2** PDCD4 immunostaining to detect protein expression demonstrated a decrease in PDCD4 protein during oocyte progression from GV to MII arrest. Oocytes were co-stained with DAPI to visualize the nucleus and identify any remaining cumulus cells. Signal intensity for each oocyte was quantified using ImageJ software. (A) Representative GV oocytes immunostained for PDCD4 protein (n=18 from 3 replications). (B) Representative MII oocytes immunostained for PDCD4 protein (n=14 from 3 replications). (C) PDCD4 immunostaining intensity presented as a percentage of mean PDCD4 abundance in GV stage oocytes. a,b Means ± SEM with different superscripts are different (P < 0.05).
Figure 3.2

(A) (B) (C)

PDCD4 protein expression as determined by immunostaining of GV and MII oocytes

<table>
<thead>
<tr>
<th>Stage of Oocyte Maturation</th>
<th>Relative Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>GV</td>
<td>a</td>
</tr>
<tr>
<td>MII</td>
<td>b</td>
</tr>
</tbody>
</table>
Figure 3.3 Luteinizing hormone and follicle stimulating hormone affect oocyte maturation to MII arrest and the lack of FSH decreased MIR21 expression in MII oocytes. (A) Maturation rates for oocytes cultured with both LH and FSH, without FSH, without LH and without LH and FSH. (B) MIR21 relative expression for GV oocytes and MII oocytes cultured with LH and FSH, without FSH, without LH and without LH and FSH. a,b Means ± SEM with different superscripts are different ($P < 0.05$).
Figure 3.3.

A) 

Maturation to MII in the presence or absence of LH and FSH during IVM

B) 

Oocyte MIR21 Expression
**Figure 3.4** Oocyte maturation was affected by the absence of cumulus cells; however MIR21 expression was not affected by the presence or absence of cumulus cells. (A) Maturation rates for intact COC, denuded oocytes cultured with cumulus cells, and denuded oocytes. (B) Expression of MIR21 in GV oocytes, and MII arrested oocytes from intact COC, denuded oocytes cultured with cumulus cells, and denuded oocytes. a,b Means ± SEM with different superscripts are different (P < 0.05).
Figure 3.4.

A) 
Maturation to MII arrest with and without cumulus cells during IVM

B) 
Oocyte MIR21 Expression
Figure 3.5 (A) Antisense MIR21 FAM labeled PNA added to maturation media to demonstrate translocation of PNA during *in vitro* maturation into cytoplasm of cells in the cumulus oocyte complex (top left) and denuded oocyte (bottom right). Exposure 1.2 sec (20x). (B) Control oocytes lacking detectable autofluorescence.
Figure 3.5.

(A)  
(B)
oocytes was not affected by the presence or absence of cumulus cells during maturation ($P = 0.82$). GV oocytes had lower MIR21 expression ($P < 0.05$) compared to MII arrested oocytes for all treatments (Figure 3.4B).

**Inhibition of MIR21 affects oocyte maturation and PDCD4 protein expression**

Using a fluorescent labeled PNA, we demonstrated the PNA’s ability to translocate into both the cumulus cell and the oocytes (Figure 3.5). While some variability in the intensity of the fluorescence between oocytes existed, all oocytes exposed demonstrated a measurable level of fluorescence compared with oocytes treated with a non-fluorescently tagged PNA.

Anti-MIR21 antisense oligonucleotides were added to maturation media during oocyte maturation to determine the effects of MIR21 inhibition on maturation rate and PDCD4 expression in MII arrested oocytes. Consistent with results presented in Figure 3.1, MIR21 was significantly increased ($P < 0.05$) in control MII oocytes compared with GV oocytes (Figure 3.6A). Mature MIR21 expression was variable between in the MIR21 inhibited (0.2 nM) and NC-scramble (2.0 nM) oocytes but remained significantly greater compared to MIR21 expression in GV stage oocytes ($P < 0.05$, Figure 3.6A). MII arrested oocytes in the presence of MIR21 inhibitor (2.0 nM) demonstrated intermediate MIR21 expression compared to GV and MII arrested control oocytes ($P = 0.13$). *PDCD4* mRNA expression was significantly less in MII arrested oocytes compared with GV stage oocytes ($P < 0.05$, Figure 3.6B). MII arrested oocytes from MIR21 inhibited (2.0 nM) and NC treatments had similar *PDCD4* mRNA abundance compared to the control MII arrested oocytes and significantly lower expression compared to ($P < 0.05$) GV stage oocytes. As
**Figure 3.6** Oocytes cultured with MIR21 inhibitor for 42 hrs during *in vitro* maturation. Quantitative RT-PCR of MIR21 (A) and *PDCD4* (B) presented as relative expression.

Samples include untreated GV and MII oocytes, PNA treated oocytes with a MIR21 inhibitor at 0.2 and 2.0 nM and negative control PNA at 2.0 nM. $^{a,b}$Means ± SEM with different superscripts are significantly different ($P < 0.05$).
Figure 3.6.

(A)

**MIR21 expression in oocytes cultured with MIR21 inhibitor during IVM**

![Graph showing MIR21 expression levels with treatments GV, MII, MIR21-I (0.2nM), MIR21-I (2.0nM), NC (2.0nM).](image)

(B)

**PDCD4 mRNA expression in oocytes cultured with MIR21 inhibitor during IVM**

![Graph showing PDCD4 mRNA expression levels with treatments GV, MII, MIR21-I (0.2nM), MIR21-I (2.0nM), NC (2.0nM).](image)
**Table 3.1** Summary of oocyte development to MII arrest and 4-cell stage of embryonic development at 60 hrs following parthenogenetic activation.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Total Oocytes Matured</th>
<th>Percentage MII Arrested Oocytes</th>
<th>Percentage 4-cell or greater at 60 hrs</th>
<th>Average blastomere # at 60 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1646</td>
<td>55.4 ± 3.6%</td>
<td>73.0 ± 5.7%</td>
<td>5.8 ±1.2 (n=16)</td>
</tr>
<tr>
<td>Negative Control (2.0nM)</td>
<td>1136</td>
<td>49.0 ± 2.5%</td>
<td>60.2 ± 15.8ab</td>
<td>6.3 ±1.4 (n=12)</td>
</tr>
<tr>
<td>MIR21Inhibitor (2.0nM)</td>
<td>1639</td>
<td>33.7 ± 3.6%</td>
<td>41.7±12.1b</td>
<td>4.8 ±1.4 (n=12)</td>
</tr>
</tbody>
</table>

a,b Values with different superscripts in the same column are significantly different ($P < 0.05$).

1 Treatment groups include control, NC-PNA (2.0 nM), MIR21 inhibitor (2.0 nM).

2 Total number of GV oocytes matured for each treatment from five replications.

3 Percentage of MII arrested oocytes from each treatment. Mean ± SEM.

4 Percentage of embryos achieving 4-stage or greater within 60 hours following parthenogenetic activation of MII arrested oocytes. Mean ± SEM.

5 Average number of blastomeres at 60 hrs based on a subset of embryos stained with nuclear stain DAPI.
shown in table 3.1 inhibition of MIR21 during oocyte maturation decreased ($P < 0.01$) the percentage of oocytes achieving MII arrest after 42 hrs of culture. Oocytes cultured in the presence of the NC PNA (2.0 nM) had similar maturation rates compared to control maturation conditions ($P > 0.05$) and higher ($P < 0.05$) maturation rates compared to the MIR21 inhibited oocytes (Table 3.1).

The effect of MIR21 inhibition on PDCD4 protein abundance was analyzed by Western blot with pools of oocytes and immunostaining of individual oocytes within each treatment and presented as a percentage of GV PDCD4 expression. Western blot analysis of pools of 50 oocytes per sample demonstrated PDCD4 abundance was not different between GV and MIR21 inhibited samples ($P > 0.05$, Figure 3.7A and B) at both 0.2 and 2.0 nM concentrations. MII arrested oocyte PDCD4 abundance was numerically lower compared to GV ($P = 0.10$) while NC-PNA (0.2 and 2.0 nM) had significantly lower ($P < 0.05$) PDCD4 abundance compared to GV and MIR21 inhibited oocytes. Immunostaining of oocytes demonstrated decreased ($P < 0.01$) PDCD4 protein expression in MII arrested oocytes relative to GV stage oocytes, however unlike the Western blot analysis, the MII arrested oocytes for the NC-scramble treatment were similar ($P > 0.05$) to GV stage oocytes and MII arrested oocytes matured in the presence of the MIR21 inhibitor at a concentration of 2.0nM (Figure 3.8A and B).
**Figure 3.7** PDCD4 protein expression between samples following analysis and quantification of band intensity. PDCD4 expression abundance is presented relative to PDCD4 expression in GV oocyte samples (n=3). From left to right: GV oocytes, control MII arrested oocytes, MII arrested oocytes in vitro matured in the presence of low concentration (0.2 nM) of the PNA MIR21 inhibitor or scrambled PNA, and MII arrested oocytes in vitro matured in the presence of high concentration (2.0 nM) of the PNA MIR21 inhibitor or scrambled PNA. Data are presented following normalization to the mean GV stage oocyte band intensity. a,b Means ± SEM with different superscripts are different (P < 0.05). Inset is a representative Western blot of PDCD4 protein expression.
Figure 3.7.

(A)
**Figure 3.8** PDCD4 immunostaining of oocytes following *in vitro* maturation. (A) Representative images of PDCD4 immunostaining of individual oocytes (n = 55) from 3 replicates. Oocytes were stained with DAPI (blue) to visualize nuclei of the oocyte and cumulus cells. (B) Fluorescent intensity of normal MII arrested oocytes, negative control-PNA (2.0 nM) and MIR21-I (2.0 nM) relative to GV oocytes. 

\[ a^{,b} \text{Means} \pm \text{SEM with different superscripts are different (P < 0.05).} \]
Figure 3.8.

(A)

GV  MII  NC (2.0 nM)  MIR21-I (2.0 nM)

(B)

Relative Expression

Treatment

GV  MII  NC (2.0 nM)  MIR21-I (2.0 nM)
MIR21 inhibition during in vitro maturation impacts parthenogenetic embryo development

Metaphase II arrested oocytes were parthenogenetically activated following in vitro maturation to test the hypothesis that MIR21 inhibition during in vitro oocyte maturation negatively impacts early embryo development prior to activation of the embryonic genome at the 4-cell stage of development. The number of embryos achieving the 4-cell stage of development or greater within 60 hours post activation was significantly \( P < 0.05 \) affected by MIR21 inhibition during in vitro maturation (Table 3.1). Development to the 4-cell stage or greater within 60 hours post activation was greatest for control oocytes \((73.0 \pm 5.7)\) compared with oocytes matured in the presence of a MIR21 inhibitor \((41.7 \pm 12.1)\) or in the presence of a negative control PNA \((60.2 \pm 15.8)\).

MIR21 expression in the developing pig follicle

In situ hybridization of MIR21 demonstrated expression throughout the granulosa cells and the oocyte (Figure 3.9). In primary follicles expression is most abundant in the oocyte with no detectable expression in the surrounding cells. Secondary follicles express MIR21 abundantly in the oocyte as well as the surrounding cumulus cells. MIR21 expression in the oocyte was greatest in the tertiary follicle and the granulosa cells lining the follicular cavity of tertiary follicles displayed MIR21 expression throughout all cell layers.
**Figure 3.9** *In situ* hybridization of MIR21 in the gilt ovaries to identify MIR21 expression during follicle development. Primary and Secondary oocytes were imaged at 400x and tertiary follicles were imaged at 200x. MIR21 expression is green with DAPI (nuclear) staining in blue.
Figure 3.9

MIR21

Primary

Secondary

Tertiary

Negative Control
Discussion

Despite their presence in the developing oocyte and mouse ovary (Bracken et al., 2011; Carletti et al., 2010), miRNA expression and function during pig oocyte maturation has not been well characterized. The ability of MIR21 to interact with PDCD4 leading to posttranscriptional gene regulation of PDCD4 protein expression in the maturing pig oocyte as demonstrated in this manuscript has also been described in several types of cancer cells (Asangani et al., 2007; Dillhoff et al., 2008; Frankel et al., 2008; Mudduluru et al., 2007; Qi et al., 2009; Yang et al., 2009). The interaction between MIR21 and PDCD4 appears to be conserved across species as is the MIR21 target recognition sequence in the 3’UTR of human and pig PDCD4 are 97% similar with 100% similarity in the seed sequence.

We hypothesized that increased MIR21 expression in the maturing cumulus oocyte complex is associated with posttranscriptional regulation of PDCD4 expression in the oocyte and that interference of MIR21 function would compromise subsequent embryonic development.

Utilizing miRNA microarray and massively parallel deep sequencing we have previously demonstrated the expression of numerous miRNA in the developing cumulus oocyte complex (Yang et al., unpublished data). In this communication, we have further characterized the temporal relationship between increased mature MIR21 expression and decreased PDCD4 protein abundance during in vitro maturation of pig oocytes. We expanded the characterization of MIR21 expression in cumulus cells and demonstrated a significant 25-fold increase in MIR21 expression during in vitro maturation. This is consistent with data previously reported in mice demonstrating increased MIR21 expression in granulosa cells in response to LH (Carletti et al., 2010). Increased MIR21 expression in
response to gonadotropins in mice provides a potential mechanism for the increased MIR21 expression observed in the pig oocyte during maturation. We tested the effects of both LH and FSH on MIR21 expression during in vitro maturation. We were unable to demonstrate differential expression of MIR21 as a result of gonadotropins during in vitro maturation although oocyte maturation was decreased when LH was removed from the maturation media.

An additional potential mechanism of MIR21 transport into oocytes may be the result of increased expression in the cumulus cells followed by transport into the oocyte. Other factors have been demonstrated to be able to translocate between the maturing oocyte and the surrounding cumulus oophorus (Hawkins and Matzuk, 2010). Oocyte - cumulus cell communication is bi-directional and required for normal cumulus cell gene expression and oocyte maturation (Hunter et al., 2005). Gap junctions allow the transit of molecules less than 1000 Da including ATP, sodium, chloride, calcium ion and cAMP (Arellano et al., 2002; Mattioli et al., 1998). It may be possible that miRNA are transported to the oocyte from surrounding granulosa cells. In addition to gap junctions, small molecule transport between cells has also been demonstrated to occur through exosomes and other microvesicles that can be secreted and taken up by neighboring cells (Turchinovich et al., 2011; Wang et al., 2010a). This was tested as a possible mechanism of MIR21 up regulation in the oocyte by culturing denuded oocytes in the presence or absence of cumulus cells. While a reduction in maturation rate was observed, as has been previously demonstrated (Zhang et al., 2009), MIR21 expression in MII arrested oocytes was not different. It may be possible that the increased expression in oocyte MIR21 occurs during the initial portion of in vitro maturation prior to GVBD.
Functionally, *in vivo* inhibition of MIR21 in mice ovaries reduces ovulation rate (Carletti et al., 2010). We utilized a MIR21 inhibitor during *in vitro* maturation and demonstrated loss of function in the ability to suppress PDCD4 protein expression in MII arrested oocytes. Metaphase II arrested oocytes matured in the presence of the MIR21 inhibitor demonstrated reduced maturation rate and ability to achieve development to the 4-cell stage or greater within 60 hours following activation. These findings taken together suggest the potential for MIR21 and miRNA in general to impact protein expression in the maturing oocyte that is also associated with developmental ability. Oocyte maturation begins prior to antral follicle development and it is unclear when MIR21 activation occurs or what mechanism underlies this observation. We observed large variation between GV oocytes and MII arrested oocytes which may be a reflection of oocyte quality or other factors prior to IVM. There was a limited response with gonadotropin and cumulus cell treatments on MIR21 expression. The COCs we collect from 3-5 mm antral follicles prior to GVBD may be capable of transcribing primary MIR21 (pri-MIR21) transcript that can be further processed into mature MIR21 during maturation. Additionally, while not examined in this study, future analysis of pri-MIR21 abundance prior to and during oocyte maturation may yield insight into the mechanisms contributing to the current observations.

Several promoters have been identified upstream to the pri-MIR21 transcription start site, containing predicted consensus sequences for binding of AP-1 and signal transducer and activator of transcription 3 (STAT3) (Fujita et al., 2008a). In cancer, the interaction between MIR21 and PDCD4 is necessary for maximal AP-1 activation. AP-1 activation is suppressed by PDCD4, however, AP-1 induction of MIR21 and subsequent posttranslational regulation of PDCD4 allows further and more sustained AP-1 activation (Fujita et al., 2008a; Talotta et
Phorbol 12-myristate 13-acetate (PMA) induced transcription of both TMEM49 and MIR21 through AP-1 action (Fujita et al., 2008a), however, STAT3 has also been documented to induce pri-MIR21 transcription (Loffler et al., 2007). It is possible that STAT3 could be related to the expression of MIR21 during oocyte maturation and early embryo development. Leptin (an adipokine) has been demonstrated to increase STAT3 expression during bovine embryo culture and is associated with reduced apoptosis in bovine blastocysts (Boelhauve et al., 2005). In addition, leptin has been shown to increase STAT3 expression in both cumulus cells and oocytes during in vitro maturation and enhance the oocytes ability to complete meiosis (Paula-Lopes et al., 2007).

We evaluated the potential relationship between MIR21 and PDCD4 during in vitro maturation of the porcine oocyte. Other potential targets of MIR21 include phosphatase and tension homolog (PTEN) (Meng et al., 2007) which is expressed in the cumulus oocyte complex (Reddy et al., 2008). PTEN has been shown to be involved in PI3K signaling in oocytes during follicle recruitment (Jagarlamudi et al., 2009; Reddy et al., 2008).

In summary we have demonstrated that MIR21 and PDCD4 have a reciprocal, temporal relationship during oocyte maturation, and that inhibition of MIR21 results in an increased abundance of PDCD4 protein. MIR21 expression in MII oocytes matured in vitro was not affected by gonadotropins or the presence of cumulus cells during in vitro maturation. It is possible that MIR21 expression increases in the oocyte earlier during oocyte activation or early maturation prior to GVBD and warrants further investigation (Figure 3.9). Obtaining an understanding of MIR21 and its mechanism is necessary to further understand molecular regulation of oocyte maturation and early embryo development. Understanding molecular mechanisms of early embryo development will provide a more comprehensive
understanding of mammalian reproduction that can be fundamental in developing strategies to improve reproductive efficiency.

The expression of MIR21 in the maturing follicle and ovary has led us to develop the following mechanism for MIR21 function in the pig oocyte (Figure 3.10). MIR21 is expressed in primary follicles and the expression increased in the secondary follicle and includes MIR21 expression in both the oocyte and cumulus cells. In the tertiary follicle MIR21 expression is greatest in the oocyte albeit still expressed throughout the granulosa cells within the follicle. GV oocytes collected from tertiary follicles between 2-6 mm in diameter express abundant PDCD4 protein and MIR21 compared to MII arrested oocyte which displayed a decrease in PDCD4 protein and an increase in MIR21 expression. This relationship between MIR21 and PDCD4 in the oocyte is important to block PDCD4 induced cell apoptosis. Therefore the increase in MIR21 is important to suppress PDCD4 translation for oocyte maturation.
**Figure 3.10** Proposed mechanism of MIR21 function in the developing follicle and maturing oocytes. MIR21 is expressed in the primary follicle and this expression increases in the oocyte and the surrounding cumulus cells in the secondary follicle. Expression of MIR21 increases even more in the oocyte of the tertiary follicle and MIR21 is also expressed in the theca and granulosa cell layers within the follicle. A GV stage oocyte has increased PDCD4 and low MIR21 compared to the MII arrested oocyte which has an increase in MIR21 and a decrease in PDCD4. This mechanism suggests MIR21 blocks PDCD4 function to prevent cell apoptosis.
Figure 3.10

Mechanism of MIR21 in the Oocyte

Primary Follicle → Secondary Follicle → Tertiary Follicle

MIR21 → MIR21

GVBD

MII arrested oocyte

PDCD4

Cell Death

- Cell cycle inhibition
- Apoptosis
- Death

MIR21

PDCD4
CHAPTER IV

Development of an in vitro heat stress model for pig oocyte maturation


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Jason W. Ross – Principal Investigator, Experimental design
Abstract

Seasonal infertility related to elevated temperatures has a detrimental impact on the reproductive efficiency of sows throughout the United States. Heat stress prior to ovulation, at the time of breeding, has been demonstrated to decrease conception rates and litter size indicating the oocyte may be affected thus altering embryonic development in the sow. The objective of the present study was to identify differentially expressed heat stress markers in metaphase II arrested oocytes and 4-cell stage embryos that could be used to develop mitigation strategies to deter the detrimental effects of elevated temperatures during oocyte maturation. Coding and non-coding RNAs were selected as oocyte and embryo markers to detect a transcriptional response to heat stress. During in vitro maturation, cumulus oocyte complexes were subjected to four environmental conditions: in vitro maturation at 39°C for 42 h (control), in vitro maturation for 42 hours at 41.0°C, (HS1); in vitro maturation for 21 hours at 39.0°C followed by 21 hours at 41.0°C, treatment 4 (HS2); in vitro maturation for 21 hours at 41.0°C followed by 21 hours at 39.0°C (HS3). The timing and duration of heat stress was reflected in reduced maturation rates, decreased developmental competency of embryos and altered expression of heat stress and developmental competency markers. HS1 oocytes had the most significant decrease in developmental capacity and blastocyst development; which was reflected in transcriptional changes in expression of MIR21, PDCD4 and HSP90α at the 4-cell stage of development. These data demonstrate a temporal relationship between MIR21 and its mRNA target PDCD4 in 4-cell stage embryos produced from HS1 oocytes. Significant changes in MIR21 expression in 4-cell embryos suggests it may serve as a molecular marker of developmental competence following heat stress during
oocyte maturation. In addition to MIR21, HSP90A expression was greatest in the 4-cell embryos produced from HS1 oocytes while PDCD4 mRNA expression was the least in these embryos. This pattern could be used as a measure of the negative effects of heat stress to evaluate translational responses which could provide tools for improving survival or response to heat stress in oocytes during maturation. These gene expression patterns at the 4-cell stage provide evidence that the embryo is capable of responding to heat stress the oocyte experiences prior to ovulation and fertilization. This data provides clues to molecular markers and biological mechanisms which could be used to mitigate the effects of heat stress on female gamete production.
Introduction

Seasonal infertility associated with elevated temperature is a critical barrier to sustaining and improving production efficiency in the swine industry in the United States. Elevated ambient temperatures have been associated with a decrease in litter size, farrowing rates and decreased overall production (Edwards et al., 1968; Omtvedt et al., 1971).

Impaired oocyte or early embryo maturation due to thermal stress may be associated with changes in molecular markers which could provide insight into the mechanisms of seasonal reproductive loss due to elevated ambient temperatures. Identification of molecular markers that respond to elevated temperatures in the maturing oocyte that have an effect on early embryo development could provide tools to enhance reproductive efficiency during thermal stress.

Evidence that heat stress may impact not only early embryo development but possibly oocyte maturation has been demonstrated with previous in vitro models of heat stress during embryo development. A nine hour culture of pig embryos at 42°C following IVF significantly decreased blastocyst formation rates from 20.6% to 8.8% (Isom et al., 2007). Heat stress for only 4 hrs at 41.5°C also reduced the developmental capabilities of parthenogenetically activated oocytes (Tseng et al., 2006). Sows exposed to heat stress for 5 days beginning at day 0 of the estrous cycle also showed a significant reduction in the number of viable embryos after 27 days of gestation with only 6.8 (39.1%) viable embryos present compared with 11.0 (68.8%) in the controls (Tompkins et al., 1967). Breeding generally occurs around the time of ovulation which typically occurs in the mid to latter half of the estrus phase of the estrous cycle (Soede et al., 1992). Heat stress during days 0 to 5 of the estrous cycle likely had an effect on the oocyte prior to fertilization. Gilts exposed to
heat stress on either 0 to 8 or 8 to 16 days of gestation also had a reduced number of viable embryos per gilt compared with controls on days 30 to 36 of gestation and pregnancy rate was decreased to 57% in gilts exposed to heat stress on days 0 to 8 of gestation (Omtvedt et al., 1971).

Embryonic development following fertilization is largely dependent on the presence of maternal expression and storage of mRNAs and proteins in the oocyte until activation of the embryonic genome at the 4-cell stage (Schoenbeck et al., 1992; Tomanek et al., 1989). Approximately 3237 genes are detected at statistically different abundances between germinal vesicle oocytes and 4-cell stage embryos in the pig (Whitworth et al., 2005b). Depletion of the maternal stores of mRNAs is partially controlled via the 3'UTR of the expressed transcripts (Brevini et al., 2006; Tadros and Lipshitz, 2005). MiRNAs are 18-24 nt long non-coding RNAs which interact with the 3’UTR of a target mRNA to mediate post-transcriptional gene expression (Bartel, 2004). Many miRNA have been identified as mediators of cellular stress response via robust gene expression suppression (Wilmink et al., 2010). The interaction of the mature miRNA and target mRNA results in a post-transcriptional gene silencing (PTGS) via translation inhibition or degradation of the target mRNA and in some cases chromatin silencing via methylation (Bartel, 2004; Chen and Meister, 2005). Dicer, the enzyme required for miRNA production has been shown to be present in mice and zebrafish (Murchison et al., 2007; Reddy et al., 2008; Wienholds et al., 2005). Furthermore a knockout mouse model confirmed that dicer expression is required for early embryonic development (Bernstein et al., 2003). MiRNA have been predicted to confer PTGS to a few or more than 800 mRNAs based on recognition sites in the 3’UTR (Brennecke et al., 2005; Rajewsky, 2006). Therefore alterations of only a few miRNA during embryonic
development could result in significant changes in transcript and protein abundance which may impact embryo viability.

*In vivo* and *in vitro* heat stress models have shown heat stress to be detrimental to embryonic development in the pig however the specific effects of heat stress during oocyte maturation on RNA expression of subsequently produced embryos developed in thermal neutral condition have not been defined. Our hypothesis is that despite appearing phenotypically similar, 4-cell embryos produced from oocytes exposed to HS during *in vitro* maturation (IVM) will demonstrate altered gene expression and have a reduced ability to develop to the blastocyst stage. The objective of this study is to define expression changes in coding and non-coding RNA molecules during oocyte maturation and embryonic development in response to elevated temperatures during oocyte maturation. The development of such a model of heat stress during oocyte maturation will be useful in identifying markers that can be utilized in characterization of heat stress in swine and the effectiveness of mitigation strategies.

**Materials and Methods**

**Oocyte Collection and *In vitro* Maturation**

Sow ovaries were obtained from an abattoir and transported to the laboratory in a thermos maintained at 30-35°C. Antral follicles (3-6 mm) were aspirated by using an 18-gauge needle attached to a 10 mL disposable syringe. The cumulus-oocyte complexes (COCs) with multiple layers of intact cumulus cells and uniform ooplasm were selected for GV oocyte collection or maturation. *In vitro* maturation was accomplished by culturing COC for 42 h in maturation media (TCM 199 medium (Gibco BRL, Grand Island, NY)
supplemented with 0.1% polyvinyl alcohol (PVA) (w/v), 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 1 μg/mL gentamicin, 0.57 mM cysteine, 0.5 μg/mL luteinizing hormone, 0.5 μg/mL follicle-stimulating hormone, and 10 ng/mL epidermal growth factor) in 5% CO₂. During *in vitro* maturation, cumulus oocyte complexes were subjected to four environmental conditions: *In vitro* maturation at 39°C for 42 hrs (control), *in vitro* maturation for 42 hrs at 41.0°C, (HS1); *in vitro* maturation for 21 hrs at 39.0°C followed by 21 hrs at 41.0°C, treatment 4 (HS2); *in vitro* maturation for 21 hrs at 41.0°C followed by 21 hrs at 39.0°C (HS3). Following *in vitro* maturation, matured COCs were separated from oocytes by vortexing the COCs in 0.1% hyaluronidase in Hepes-buffered Tyrode medium containing 0.01% PVA.

**In vitro fertilization and Culture**

*In vitro* fertilization and embryo culture was performed as previously described (Zhao et al., 2009). Briefly, oocytes with a polar body following *in vitro* maturation were rinsed and transferred into equilibrated 50 μL droplets of mTBM medium (modified Tris-buffered medium) plus 2mM caffeine and 2mg/ml BSA (bovine serum albumin) with around 35 oocytes per drop. Fresh Duroc boar semen was rinsed twice using Dulbecco phosphate buffered saline (DPBS) plus 1 mg/ml BSA and diluted to a concentration of 1 × 10⁶ cells/ml using mTBM medium plus caffeine and BSA. Fifty microliters of the sperm sample was added to the droplets with oocytes to give a final sperm concentration of 0.5 × 10⁶ cells/ml followed by incubation for approximately 5 hours at 39°C in 5% CO₂. Following fertilization, oocytes were washed and cultured in 500 μl of porcine zygote maturation media 3 (PZM3) in four-well Nunclon dishes (Nunc) at 39.0°C in 5% CO₂.
Quantitative RT-PCR

Quantitative RT-PCR was used to analyze the expression of specific putative markers of oocyte and embryo quality and response to heat stress. MIR21 and programmed cell death 4 (PDCD4) were measured to determine effects on cell cycle regulation, viability and apoptosis. Heat shock factor 1 (HSF1) and heat shock protein 90A (HSP90A) mRNA was measured to determine response to heat stress by the oocyte. Small pools of 25 MII oocytes or embryos (n=4) were collected from each treatment and all markers were measured within the same group and time point. Statistical differences in gene expression between treatments in MII arrested oocytes and 4-cell stage embryos were determined using the MIXED procedure in SAS.

Statistical Analysis

PROC MIXED of the Statistical Analysis System was used to determine statistical differences of all data including percentage maturation and differences in C\textsubscript{T} value for RT-PCR data. Significance (\( P < 0.05 \)) was determined for the model and least-square means was used to determine significant differences between treatment samples (control, HS1, HS2, and HS3). The effect of heat stress treatment on MIR21, PDCD4, HSF1, and HSP90A was determined using C\textsubscript{T} value. Replication was included as a covariate. Data are displayed as relative expression means ± SEM (n=4). Percentage graphs are set to reflect the GV stage as 100% expression and all other treatments are relative to GV.
Results

Oocyte maturation and blastocyst rate are influenced by heat stress.

A total of 667, 698, 697 and 716 cumulus oocyte complexes were in vitro matured for control, HS1, HS2 and HS3 treatment conditions, respectively. Control oocytes demonstrated the highest maturation rate (71.2 ± 3.7%). This was not different from HS2 (70.2 ± 0.7 %) but was significantly greater than HS1 (55.1 ± 6.3 %) and HS3 (54.0 ± 6.2 %) (Figure 4.1) suggesting that oocytes experiencing HS during the early stages of oocyte maturation are more tolerant of heat stress than those exposed to heat stress during later stages of oocyte maturation or for the duration of in vitro maturation.

Following in vitro fertilization and development, the percentage of MII arrested oocytes producing embryos capable of development to the four cell stage within 60 hrs was not different between treatments despite being numerically lower in oocytes from the HS1 (Control, 51.3 ± 6.2%; HS1, 38.3 ± 4.4%; HS2, 48.2 ± 6.3%; HS3, 52.0 ± 8.6%) (Table 4.1, Figure 4.2). However, of the embryos capable of developing to the 4-cell stage, those capable of continued development to the blastocyst stage was significantly affected by exposure to heat stress during in vitro maturation (Table 4.1, Figure 4.3). Control embryos produced the greatest number of blastocysts on Day 6 as a percentage of 4-cell embryos at 60 hours post fertilization (29.4 ± 4.5%) compared to other treatments (HS1, 1.6 ± 1.1%, HS2, 13.3 ± 1.0%; HS3, 21.6 ± 3.8%). Heat stress during the entire 42 hour period of in vitro maturation significantly impaired the ability of the embryo to develop to the blastocyst stage. However, the negative impact of the heat stress appears to primarily take effect during the first 21 hours of in vitro maturation as developmental ability, as measured by blastocyst formation rate, is significantly lower in HS2 compared to HS3 (P < 0.001).
**Figure 4.1** Maturation rates for MII development of oocytes heat stressed during IVM. Heat stress during oocyte maturation has a negative effect on HS1 and HS3 groups. This data suggests that heat stress during the first 21 hrs of oocyte maturation has a greater effect than heat stress during the last 21 hrs of maturation. Control and HS2 had similar maturation rates (approximately 70%) while HS1 and HS3 were significantly lower ($P = 0.01$).
Figure 4.1

Oocyte Maturation to MI arrest

Heat Stress Treatments

- Control
- HS1
- HS2
- HS3

Oocyte Maturation

0% 20% 40% 60% 80% 100%
Figure 4.2 Percentage of IVF embryos produced from oocytes heat stressed during IVM, which developed to >4-cell stage at 60 hrs post fertilization. MII arrested oocytes from each heat stress treatment were used for IVF to determine the developmental ability of heat stress oocytes. Embryos with 4 or more blastomeres of equal size were counted at 60 hrs post fertilization to determine the effect of heat stress during IVM on developmental competency. All heat stress treatment groups were able to develop to the 4-cell stage by 60 hrs at a similar rate ($P= 0.33$).
Figure 4.2

Developmental Competency

Heat Stress Treatments

Control  HS1  HS2  HS3

Development

0%  10%  20%  30%  40%  50%  60%  70%
Figure 4.3 Percent blastocyst development from oocytes heat stressed during IVM. Heat stress during the last half of oocyte maturation causes a significant decrease in blastocyst development. Less than 2% of HS1 oocytes were able to develop to the blastocyst stage, indicating heat stress throughout oocyte maturation had the most significant effect on early embryo development. HS2 blastocyst rate (15%) was half that of the control (30%) which demonstrates that heat stress in the last half of oocyte maturation had a negative effect on the ability of those oocytes to develop to the blastocyst stage.
Figure 4.3

Blastocyst development

Percent Blastocysts

Heat stress treatment

Control HS1 HS2 HS3
Characterization of gene expression in MII arrested oocytes and 4-cell stage embryos following *in vitro* maturation

MicroRNA-21 (MIR21) and *programmed cell death 4 (PDCD4)* mRNA

The expression of MIR21 and its mRNA target PDCD4 had an inverse relationship in MII arrested oocytes and 4-cell embryos after heat stress during IVM. Mature MIR21 expression was increased in MII-arrested oocytes in HS2 and HS3 treatments compared to HS4 (*P* < 0.05) but not significantly different from HS1 (*P* > 0.05, Figure 4A). *PDCD4* mRNA expression was not significantly different in MII-arrested oocytes between the heat stress treatments (*P* = 0.35, Figure 4B).

In 4-cell stage embryos produced from oocytes for each treatment, mature MIR21 expression was similar between control, HS2 and HS3 (*P* = 0.53) and significantly greater in embryos from HS1 oocytes (*P* = 0.02, Figure 4.5A). However, *PDCD4* mRNA expression in 4-cell embryos was increased in those embryos produced from HS1 oocytes compared to embryos produced from control, HS2 and HS3 oocytes (*P* = 0.03, Figure 4.5B).
Figure 4.4 MIR21 and *PDCD4* mRNA expression in heat stressed MII arrested oocytes.  (A) MIR21 expression in MII arrested oocytes was greater in HS1 and HS2 compared to HS3 (*P* < 0.05) but not significantly different from control (*P* < 0.05).  (B) *PDCD4* mRNA expression was increased (*P* < 0.05) in the control oocytes relative to the heat stress groups. *PDCD4* mRNA was not significantly different among the heat stress treatments (*P* = 0.35).
Figure 4.4

(A)

**MIR21 expression in MII arrested oocytes exposed to heat stress during IVM**

<table>
<thead>
<tr>
<th>Heat Stress Treatments</th>
<th>Relative Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.0</td>
</tr>
<tr>
<td>HS1</td>
<td>4.0</td>
</tr>
<tr>
<td>HS2</td>
<td>1.0</td>
</tr>
<tr>
<td>HS3</td>
<td>5.0</td>
</tr>
</tbody>
</table>

(B)

**PDCD4 mRNA expression in MII arrested oocytes after heat stress during IVM**

<table>
<thead>
<tr>
<th>Heat Stress Treatment</th>
<th>Relative Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.0</td>
</tr>
<tr>
<td>HS1</td>
<td>6.0</td>
</tr>
<tr>
<td>HS2</td>
<td>2.0</td>
</tr>
<tr>
<td>HS3</td>
<td>2.0</td>
</tr>
</tbody>
</table>
**Figure 4.5** MIR21 and *PDCD4* mRNA expression in 4-cell embryos derived from oocytes heat stressed during IVM. (A) MIR21 expression in 4-cell stage embryos was similar for control, HS2 and HS3 (*P > 0.10*) although expression was significantly decreased in 4-cell embryos derived from HS1 oocytes (*P = 0.02*). (B) *PDCD4* expression was increased (*P < 0.05*) in 4-cell embryos produced from HS1 oocytes compared to HS2 and HS3 oocytes.
Figure 4.5

(A) MIR21 expression in 4-cell embryos produced from heat stressed oocytes

(B) PDCD4 mRNA expression in 4-cell embryos produced from heat stressed oocytes
**Heat Shock Markers**

To measure a direct effect of heat stress on the oocytes during *in vitro* maturation, heat shock factor 1 (HSF1) and heat shock protein 90A (HSP90A) mRNA were measured in all treatments. *HSF1* was not significantly different (*P = 0.18*) in MII stage oocytes for all heat stress treatments (Figure 4.6A). *HSF1* was also not different (*P = 0.50*) between treatments among embryos at the 4-8 cell stage (Figure 4.6B). Similarly, *HSP90A* mRNA was not different (*P = 0.41*) in MII oocytes for all heat stress treatments (Figure 4.7A), however, in 4-8 cell stage embryos, *HSP90A* mRNA expression was affected by treatment (*P < 0.05*) with lowest expression in control embryos and greatest expression in embryos produced from HS1 oocytes (Figure 4.7B).
Figure 4.6 Heat Shock Factor 1 mRNA expression was not different in heat stressed oocytes or IVF embryos produced from heat stressed oocytes. (A) Heat shock factor 1 was not significantly different in MII stage oocytes for all heat stress treatments ($P = 0.18$). (B) Heat shock factor 1 was also similar between all treatments among embryos at the 4-cell stage ($P = 0.50$).
Figure 4.6

(A)

**HSF1 mRNA expression in MII arrested oocytes**

![Graph showing HSF1 mRNA expression in MII arrested oocytes]

(B)

**HSF1 mRNA expression in 4-cell embryos derived from heat stressed oocytes**

![Graph showing HSF1 mRNA expression in 4-cell embryos derived from heat stressed oocytes]
**Figure 4.7** Heat Shock Protein 90α mRNA expression in MII arrested oocytes and 4-cell embryos following heat stress during IVM. (A) Heat shock protein 90α was not significantly different in MII oocytes for all heat stress treatments ($P = 0.41$). (B) Heat shock protein 90α mRNA expression was lowest in embryos produced from control IVM conditions and greatest in the HS1 oocyte maturation treatment ($P < 0.05$).
Figure 4.7

(A) 

*HSP90A* mRNA expression in MII arrested oocytes exposed to heat stress during IVM

![Graph showing mRNA expression](image)

(B) 

*HSP90A* mRNA expression in 4-cell embryos derived from heat stressed oocytes

![Graph showing mRNA expression](image)
Discussion

Heat stress has been shown to have detrimental effects on early embryo development prior to activation of the zygote genome and suppresses embryo viability (Alliston et al., 1965; Tseng et al., 2006). Heat stress at the time of breeding or shortly after fertilization reduces conception rates, blastocyst development and litter size (Edwards et al., 1968; Isom et al., 2007; Omtvedt et al., 1971). Developing a fundamental knowledge of the effects of heat stress on the female gamete is essential to fully characterizing the negative impact heat stress has on reproductive efficiency in pigs.

Our *in vitro* model of heat stress during oocyte maturation enabled the quantitative measurement of response to heat stress and demonstrated the effects on molecular factors that are associated with developmental competency. All oocytes subjected to heat stress in our model were able to develop to MII although heat stress significantly reduced maturation for HS1 and HS3 groups. Gene expression differences between control and heat stress treatments were observed although MII oocytes were phenotypically similar for all treatments.

Transcription of mRNA is halted at germinal vesicle breakdown and does not resume until activation of the zygote genome at the 4-cell stage in pigs. *HSP90A* mRNA was greatest in 4-cell stage embryos from HS1 oocytes indicating that a transcriptional response to heat stress may have been initiated prior to GVBD and transcriptional silencing. MIR21 abundance was also highest in HS1 group at the 4-cell stage of embryo development and may partially explain *PDCD4* mRNA expression differences, being the lowest in 4-cell embryos derived from HS1 oocytes. The regulation of PDCD4 by MIR21 has been well documented in many cell lines (Asangani et al., 2007; Fujita et al., 2008b; Lu et al., 2008; Talotta et al.,
2009a) including the pig oocyte (see chapter 3.). The MIR21:PDCD4 interaction previously described in the oocyte suggests that MIR21 is suppressing translation of *PDCD4* as PDCD4 protein abundance decreased although no difference in mRNA abundance was observed. In this study, MIR21 expression is least abundant at the 4-cell stage in HS1 while *PDCD4* mRNA is concomitantly elevated. The lack of MIR21 abundance suggests a decreased ability for the embryo to regulate PDCD4. *PDCD4* mRNA, if translated, may contribute to a higher incidence of apoptosis of the developing embryo resulting in diminished blastocyst development. The relationship observed in this study suggests that during heat stress MIR21 expression in HS2 and HS3 may be up regulated to mediate survival of the embryos by suppressing PDCD4.

During oocyte maturation MIR21 increases from GV to MII arrest while PDCD4 protein translation is repressed resulting in decreased PDCD4 protein in MII arrested oocytes (Chapter 3). Data in the mouse ovary suggests that inhibiting MIR21 has no effect on PDCD4 mRNA or protein abundance but does cause a decrease in the number of oocytes ovulated and induces apoptosis in granulosa cells in culture (Carletti et al., 2010).

The ovarian hormone profile was affected by heat stress in an *in vitro* study which demonstrated that progesterone and estradiol release increased in swine ovaries cultured at 41.5°C compared to 37.5°C (Sirotkin and Kacaniova, 2010). Leptin synthesis and activity was decreased in pig ovaries resulting in decreased IGF-1 and progesterone in an *in vitro* heat stressed model (Narayansingh et al., 2004). Hormone receptor abundance for both progesterone and estrogen are decreased in the rat ovary when heat shock protein 70 is over expressed in cystic rat follicles (Salvetti et al., 2009). These studies taken together suggest
that heat stress in the ovary effects hormone profiles that could impact gene expression and miRNA such as MIR21.

HSF1 is a primary regulator of several genes that encode heat shock proteins, plays a role in mediating cell homeostasis, and is highly expressed in the oocyte (Christians et al., 2000; Metchat et al., 2009). HSP90A has been shown to be regulated by HSF1 in the mouse oocyte and this regulation is necessary for the normal progression of meiosis (Metchat et al., 2009). Changes to normal HSF1 expression in the oocyte may cause post fertilization abnormalities (Christians et al., 2000). Heat stress during oocyte maturation did not significantly affect HSF1 mRNA in our samples; however, HSP90A, known to be regulated by HSF1, was greater in 4-cell embryos from HS1 oocytes. This data suggests that HSF1 and HSP90A may not be major responders to heat stress in the maturing oocyte. Alternatively, expression of these markers and others may be partially regulated by post transcriptional gene regulation in the oocyte during maturation.

Elevated body temperatures at the time of breeding and the following days up to implantation causes a reduction in conception rates and viable embryos (Edwards et al., 1968; Omtvedt et al., 1971). The model for heat stress used in this study has demonstrated that heat stress during oocyte maturation alters the gene expression of 4-cell stage embryos. The genes we studied are important regulators of developmental competence, cell proliferation and viability (Bierkamp et al., 2010; Carletti et al., 2010; Chan et al., 2005; Metchat et al., 2009). The differences in expression of specific molecules in the oocyte and early embryo demonstrated in this study may be useful markers for demonstrating the effectiveness of strategies to mitigate the effects of heat stress on swine reproduction.
This heat stress model can be used to identify the normal markers for oocyte maturation and early embryo development as well as those affected by heat stress during IVM. Evaluating molecular markers in MII arrested oocyte and 4-cell embryos that are differentially expressed between heat stressed and control treatments will provide insight into gene expression that is required for normal maturation compared to impaired maturation. The similar phenotype of the 4-cell stage embryos obtained at 60 hours demonstrated that appearance of the developing early embryo is not indicative of the embryo’s ability to develop to the blastocyst stage. Therefore this model could be used to identify markers within the normally developing embryo which could be used as markers of viability.
CHAPTER V

MiRNA Expression during Rapid Trophoblastic Elongation of the Porcine Conceptus

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Abstract

During the peri-implantation stage of pregnancy, porcine conceptuses undergo a rapid morphological rearrangement of their trophectoderm and initiate contact with the uterine endometrium to establish an initial interface necessary in the formation of a diffuse epithelialchorial placenta. Numerous mRNA molecules are differentially expressed during this dynamic period of conceptus growth. To better understand mechanisms of post transcriptional gene regulation during this process we conducted massively parallel deep sequencing of small RNA during conceptus elongation and the initial phase of uterine attachment. Total RNA from day 12 spherical (8-10 mm; D12S), day 12 filamentous (>100 mm; D12F) and day 14 filamentous (D14F) conceptuses was isolated and subjected to small RNA library construction using the Small RNA Expression Kit. The libraries were then subjected to deep sequencing analysis using the SOLiD sequencing system. Sequence reads were mapped to the pig genome to determine the number of miRNA reads for each stage of development. Total reads for each library and frequency of each miRNA within libraries was determined. Based on the frequency within a library, we identified differentially expressed miRNA utilizing RT-PCR. During the transition from D12S to D12F, MIR301a, and MIR-200a were increased in abundance while MIR-23b, MIR-342, MIR-30a and MIR-10a were among those most abundantly expressed in D14F compared to earlier stages of development. These data demonstrate expression of numerous non-coding RNA markers of rapid trophoblastic elongation in the pig.
Introduction

During the peri-implantation stage of pregnancy, porcine conceptuses undergo a rapid morphological rearrangement of their trophectoderm and initiate contact with the uterine endometrium to establish an initial interface necessary in the formation of a diffuse epithelialchorial placenta. Numerous mRNA molecules are differentially expressed during this dynamic period of conceptus growth (Blomberg et al., 2005; Ross et al., 2009). One potential mechanism of regulating transcript turnover and protein expression during this stage of embryonic development is non-coding RNA. MiRNA, one class of non-coding RNA, are significant regulators of gene expression via posttranscriptional gene regulation (PTGR) (Bartel et al., 2004). MicroRNA interact with the 3' UTR of mRNA and impact protein expression through translation inhibition or mRNA degradation (Zamore et al., 2005; Bartel et al., 2004; Bracken et al, 2011;Friedman et al, 2009). Early fetal development may involve more than 10,000 genes (Niemann and Wrenzycki, 2000) for which the temporal and spatial regulation must be tightly controlled for normal development. The majority of prenatal embryonic loss in the pig is thought to occur between 12 and 18 days of gestation during conceptus elongation and implantation (Anderson, 1978).

The pig blastocyst is unique in the speed and extent to which it elongates from a spherical to ovoid to tubular and finally to a filamentous stage before implantation (Geisert et al., 1982). The trophoblast expands to increase placental surface to maximize maternal:conceptus nutrient exchange during gestation (Stroband and Van der Lende, 1990). The pig conceptus morphologically changes within hours whereas other domestic livestock species such as the cow or ewe take days for the conceptus to elongate and establish its space in the uterus. The pig conceptus elongates from ovoid (<10 mm) to filamentous (>150 mm).
in about 4 hrs between day 11 and 12 of gestation (Anderson, 1978; Geisert et al., 1982). The developing embryo must signal its presence by the synthesis of estrogen on or around day 12 of gestation therefore steroidogenic genes must be activated and regulated (Guthrie, 1975; Perry et al., 1973). Interleukin 1-β (IL1B), an inflammatory response molecule is also up regulated in the conceptus (Ross et al., 2003). Both steroidogenesis and immune response require activation of many pathways which could be regulated by miRNA. Targets could include mRNAs for estrogen receptors and mRNAs within the steroid synthesis pathways. The estrogen driven network which integrates with the IL1B network and others during ovoid to tubular transition indicates a vast web of genes that are essential for normal conceptus elongation (Blomberg et al., 2008).

Conceptus elongation in the pig is asynchronous and embryos that develop slowly have a greater probability of dying (Bazer et al., 1993). This indicates that the temporal and spatial regulation of mRNAs and protein essential to cell differentiation, migration and transformation is complex and it is possible that several miRNA play vital roles in the regulation of conceptus elongation. We hypothesize that numerous miRNA are differentially expressed during conceptus elongation in the pig.

Identification and analysis of miRNA expression during rapid trophoblastic elongation is important for developing a biological understanding of PTGR in the developing pig conceptus during a critical period of pregnancy establishment. We conducted massively parallel deep sequencing of small RNAs during conceptus elongation and the initial phase of uterine attachment to identify small RNA expression patterns during this stage of development.
Materials and Methods

Research was approved by the Institutional Animal Care and Use Committee at Iowa State University.

Conceptus Collection

Forty-five cyclic multiparous sows were checked for estrus behavior daily and artificially inseminated at the onset of estrus and again 24 hours later. Sows were hysterectomized on day 12 and 14 of gestation. Uteri of pregnant sows were flushed with 20 mL sterile saline into a sterile petri dish and conceptuses were counted, collected and sorted by morphology on day 12 (spherical (8-10 mm), tubular (20-80 mm) and filamentous (> 100 mm)). All conceptuses from D14 were filamentous and the number of conceptuses was estimated based on the size of the conceptus mass and number of corpora lutea. Each litter was divided among cryogenic vials according to morphology, and frozen in liquid nitrogen and stored at -80°C until analysis.

RNA Extraction

RNA was extracted using miRVana™ miRNA Isolation Kit (Ambion) for each sample per manufacturer’s recommendations. Briefly, RNA extraction was performed as follows: 600 µL of lysis buffer was added to each sample mixed by vortex, 60 µL of homogenate solution was then added and the sample was vortexed again and placed on ice for 10 minutes. Following 10 minutes on ice, 600 µL acid phenol:chloroform was added and the sample was vortexed for 60 sec before centrifugation at 11000 RPM for 5 min. The upper phase containing the RNA was moved to a new tube and 750 µL of 100% ethanol was
added and mixed by pipetting. Then 700 µL was added to a filter and centrifuged at 10,000 RPM for 15 sec, remaining solution was added to the filter and centrifugation was repeated and flow through was discarded after each step. The filter, containing bound RNA, was then washed with 700 µL miRNA wash solution and centrifuged 15 sec followed by 2 washes with 500 µL of wash solution. The filter was dried by centrifugation at 10,000 RPM for 1 minute. RNA was then eluted in 25 µL and 75 µL of 95°C nuclease free water. RNA quality and quantity were determined using Agilent 2100 Bioanalyzer (Agilent, Waldbronn, Germany) and Nanodrop 1000 Spectrophotometer (Thermo Scientific, MA, USA). Samples were diluted to 2 µg/µL each and a pool of each morphological phenotype was created for a total of 8 µg/µL of each conceptus stage of elongation and 1µg was used per library.

**Library Construction for SOLiD Sequencing**

The three stages of conceptus development D12S, D12F and D14F were used to construct barcoded small RNA libraries (SOLiD™ Small RNA Expression Kit, Ambion) according to the manufacturer’s protocol with minor modifications (Figure 5.1., Yang et al., 2012). Samples were ligated with adaptor mix A, to yield the template for the SOLiD sequencing from the 5' end of the sense strand. Ligation consisted of Adaptor A mix (provided in kit; 2 µL), hybridization solution (3 µL) RNA sample (2 µL) and nuclease free water (1 µL). Samples were then placed in a thermal cycler for 10 min at 65°C followed by 5 min at 16°C. Next samples were put on ice and ligation buffer (10 µL) and ligation enzyme mix (2 µL) were added. Samples were then mixed, centrifuged briefly and incubated at 16°C for 16 h. Reverse transcription was used to synthesize cDNA at 42°C for 30 minutes followed by RNase H treatment. Each library was amplified with 18-22 cycles of PCR using the supplied
barcoded primer set and the recommended parameters. Amplified PCR products from each library were separated on 6% polyacrylamide gels. Gels were stained with SYBR gold dye, and the 105 to 150 bp region which included the miRNA with the adaptors was excised, and purified with MinElute PCR Purification Kit (Qiagen) to remove remaining primers, enzyme and salt. The quality and quantity of cDNA libraries were determined using Agilent 2100 Bioanalyzer (Agilent, Waldbronn, Germany) and Nanodrop 1000 Spectrophotometer (Thermo Scientific, MA, USA). Three small RNA cDNA libraries were subjected to massively parallel deep sequencing using the SOLiD sequencing system (Applied Biosystems) at the University of Iowa DNA Core Facility following the vendor’s recommendations.

Analysis and Classification of Short Reads

Bowtie and Blastn programs were used to map the generated short reads back to the pig genome (Sscr9.62). Bowtie was used directly to generate a colorspace index and perform the colorspace alignment of the short DNA sequences to the pig genome (Langmead et al., 2009). The seed length was set at 14 nt with one mismatch by trimming one to two bases from the left and right ends of each read. Blastn was used to align the transformed raw reads in fasta format with 17 base perfect matches. Custom scripts were used to transform the raw reads and quality control was performed (adaptor sequences trimmed off) and low-quality (>3 Ns) and low-complexity reads (>10 consecutive nucleotides) were discarded. The coordinates of short reads mapping to the pig genome at least 3 times were extracted to obtain genomic positions of potential small RNA and used for further analyses.
The mapped short reads were assigned their corresponding annotation by comparing their coordinates to those of non-coding RNA, protein-coding genes and repetitive elements, with at least 15 nt overlapping as determined with a custom Perl script. Ensembl Biomart was used to download the genomic coordinates of non-coding RNA and protein-coding genes (Scrr9.62). RepeatMasker was used to obtain coordinates of repetitive elements (LINE, SINE, etc.) on the genome sequences.
**Figure 5.1.** Flow chart of small RNA library preparation for SOLiD sequencing.
Figure 5.1

Less than 200 nt RNA extraction using the mirVana miRNA Isolation Kit

Adaptor ligation

Reverse transcription

18-22 cycles of PCR amplification

105-150 bp PCR product purification

cDNA library quality control

SOLiD sample preparation and sequencing
Quantitative RT-PCR

Quantitative RT-PCR was utilized to determine expression changes for MIR301a, MIR23b, MIR10a, MIR21, MIR200a, MIR574-3p, and MIR467a using commercially available TaqMan MicroRNA Assays (Applied Biosystems). Individual RNA samples (50 ng/µL) from spherical (n=3), Day 12 Filamentous (n=4) and Day 14 Filamentous (n=4) were used for quantitative RT-PCR. TaqMan® Reverse Transcription kit (Applied Biosystems) was used to construct cDNA. The RT mix was incubated at 16°C for 30 min, 42°C for 30 min and 85°C for 5 min. For the PCR reaction 1.33µL of the cDNA was used in a 20 µL reaction mixture. PCR parameters were 95°C for 10 min, followed by 50 cycles of 95°C for 15 sec and 60°C for 60 sec. All reactions were performed in duplicate to determine an average cycle threshold value. Samples were normalized to RNU43 as an endogenous small RNA control.

Relative quantification of expression levels was evaluated with the comparative C_T method as previously described (Ross et al., 2009). The ΔC_T value was determined by subtracting the target C_T of each sample from its RNU 43 C_T value. Following normalization, the ΔΔC_T was calculated using the single greatest sample ΔC_T value (the sample with the lowest expression) to subtract from all other sample ΔC_T value. Assuming that each cycle difference is equivalent to 2-fold difference, relative fold change for each sample was calculated by applying the equation 2^{ΔΔC_T}.

MiRNA pathways and gene targets

DIANA-mirPath was used to determine the pathways of the miRNA selected for validation (Table 5.1) (Papadopoulos et al., 2009). MiRNA were selected using human and
DIANA-microT-4.0 (beta version) with the exception of MIR467a which was only available in the mouse. The first 5 pathways listed for the KEGG pathway which could be validated in the literature to play a role in porcine conceptus elongation were selected and recorded in Table 5.1 along with number of target genes and –log (P-value).

Statistical Analysis

Statistical analyses of the differential expression of small RNA, repeats and proteins based on the number of short reads were done by using the MA-plot-based method and correction for false discovery rate in DEGseq (Wang et al., 2010b). Quantitative RT-PCR ΔC_T values collected for each stage of conceptus were analyzed with PROC MIXED of the Statistical Analysis System (SAS). Results are reported as least square means ± SEM.

Results

SOLiD Sequence Results

Total reads for all small RNA was 7,319K, 13,831K, and 30,618K for D12S, D12F and D14F respectively (Figure 5.2). PiwiRNA reads comprised the bulk of the small RNA reads for all conceptus stages with the greatest number of reads in the D12F at 98.12% of total reads, followed by D14F at 97.77% and D12S at 95.4%. MiRNA were the second greatest number of reads for all conceptus stages with 1.49% for D12S, 1.06% for D12F and 1.29% for D14F. A total of 368 miRNA identified by Ensembl in the pig genome including 242 for D12S, 254 for D12F and 256 for D14F were identified (Supplemental Table 5.1).
**Figure 5.2** Distribution of small RNA library as derived from the pig genome excluding piRNA which consisted of > 95% for each library. MicroRNA were the most abundant small RNA and retroRNA was the least abundant in all stages of conceptus elongation (A) Small RNA distribution for D12S conceptus excluding piRNA. (B) Small RNA distribution or D12F conceptus excluding piRNA. (C) Small RNA distribution of D14F conceptus. For each chart, each small RNA class is represented by a different color.
MiRNA Chromosome Distribution

Chromosome distribution was mapped for all reads within each conceptus sample. Reads per chromosome were similar \((P = 0.97)\) between conceptus stages. Chromosome read distribution was different \((P < 0.01)\) between chromosomes for all conceptus stages (Figure 5.3). Chromosome 1 had the greatest number of reads \((11.4\) to \(11.6\%)\) for all conceptus stages while chromosome 17 and 9 \(< 1\%)\) had the least reads for all conceptus stages (Figure 5.3).

Quantitative RT-PCR via stem-loop miRNA assay

To verify expression during conceptus elongation, quantitative RT-PCR was utilized to quantify expression differences of MIR4057, MIR467, MIR10a, MIR574, MIR23b, MIR301 and MIR21 during conceptus elongation. MIR4057 and MIR467 had numerically higher expression in D12F conceptus but it was not significantly different from D12S or D14F \((P = 0.27\) and \(P = 0.22\), respectively, Figure 5.4A). MIR10a was increased 7-fold in D14 \((P < 0.05)\) and there was no significant difference in MIR10a between D12S and D12F \((P = 0.67,\) Figure 5.4A). MiR23b was increased 7-fold in D14 \((P < 0.05)\) and there was no significant difference in MIR23b within D12S and D12F \((P = 0.42,\) Figure 5.4A). MIR574 expression was similar between D12S and D12F \((P = 0.94)\), however D14F had approximately 12-fold greater expression compared to D12S and D12F \((P < 0.05,\) Figure 5.4B). MIR301a expression was lowest in D12S conceptus \((P < 0.05)\) compared to D12F which was increase 23-fold and D14F which was increased 12-fold (Figure 5.3B). There was no significant difference in MIR301 within D12F and D14F \((P = 0.13,\) Figure 5.4B). MIR21 expression was lowest in D12S \((P < 0.05)\), increased 11-fold in D12F \((P < 0.01)\) and was
Figure 5.3 MiRNA distribution of reads across the pig genome. All miRNA reads were mapped to each chromosome in the pig genome to characterize origin of expression. A) Distribution of miRNA for D12S conceptus reads across the pig genome by chromosome. B) Distribution of miRNA for D12F conceptus reads across the pig genome by chromosome. C) Distribution of miRNA for D14F conceptus reads across the pig genome by chromosome.
Figure 5.3

[Bar chart showing the ratio of reads by chromosome.]
Figure 5.4 Quantitative RT-PCR analysis of miRNA expression during rapid trophoblast elongation of the pig conceptus. A) Expression of MIR4057 and MIR467 were not significantly different ($P = 0.27$ and $P = 0.22$, respectively) between conceptus stage of development but had numerically greater expression in D12F than D12S or D14F. MIR10a expression was greatest in D14F conceptus ($P = 0.02$) and was similar ($P = 0.99$) for D12S and D12F. B) MIR574 and MIR23b had similar expression in D12S and D12F and D14F conceptus had 10 fold greater expression ($P < 0.05$). MIR301 had the least expression in D12S ($P = 0.07$) although D12F and D14F had similar expression ($P = 0.20$). MIR21 was significantly different for all three conceptus stages with D12S being the lowest and D14F having the greatest expression with a 20 fold increase over D12S ($P < 0.01$). MIR200a expression was greatest in D14F ($P < 0.05$) and the lowest in D12S ($P < 0.05$).
Figure 5.4

(A)

MiRNA library validation

Relative Expression

MIR4057  MIR467  MIR10a  MIR23b

MIR4057  MIR467  MIR10a  MIR23b

(D1S  D12F  D14F)

(B)

MiRNA Library Validation

Relative Expression

MIR574  MIR301  MIR21  MIR200a

MIR574  MIR301  MIR21  MIR200a

(D1S  D12F  D14F)
most abundant in D14F with a 21-fold increase compared to D12S ($P < 0.001$, Figure 5.4B). MIR200a had the lowest expression in D12S and was increased 26-fold in D14F ($P < 0.05$, Figure 5.4B).

**MiRNA Pathway prediction with DIANA-mirPath**

Based on DIANA-mirPath predictions all miRNA evaluated appear to have the capacity to regulate pathways related to early embryo development (Table 5.1). Pathways which could be targeted by 3 or more of the validated miRNA were axon guidance, MAPK signaling pathway, and Wnt signaling. MIR301a is predicted to target as many as 13 genes in the TGF-beta signaling pathway and 14 genes in the calcium signaling pathway. Other pathways of interest include focal adhesion, tight junction, hedgehog signaling pathway, cytokine-cytokine receptor interaction, p53 signaling pathway, apoptosis, cell cycle and GnRH signaling pathway, all of which are known to play roles in early embryo development.
Table 5.1 MIRNA function predicted by DIANA miR-PATH.

<table>
<thead>
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<th>miRNA(^1)</th>
<th>KEGG Pathway(^1)</th>
<th>Target Genes(^3)</th>
<th>-log(P-value)(^4)</th>
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<td>Axon guidance</td>
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<td>Focal adhesion</td>
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<td></td>
<td>Tight junction</td>
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<td>Jak-STAT signaling pathway</td>
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</table>

\(^1\)MiRNA identification
KEGG pathways that have been identified as targets for the miRNA selected. The first five pathways that have been identified in the literature to play a role in conceptus elongation were selected for this table.

Genes within the KEGG pathway that are predicted to be targets of the miRNA.

Significance value.
Discussion

Embryo elongation in the pig is a dynamic morphological process which is highly regulated to ensure normal development and implantation. MiRNA are temporally regulated during embryo development and elongation. The small RNA library described in this chapter provides an array of miRNA that may interact with many biological pathways potentially affecting cell differentiation, migration, transformation, steroidogenesis and development of the porcine conceptus.

The number of reads per conceptus stage and total number of unique reads increased during conceptus elongated from D12 to D14. DNA and protein also increase during this period however the protein to DNA ratio decreases (Pusateri et al., 1990) suggesting the potential for translation suppression and regulation. Chromosomal distribution of miRNA reads were similar for all stages of conceptus elongation. However earlier development indicates a similar chromosomal distribution pattern with miRNA reads distributed across all chromosomes during oocyte maturation from GV to MII as well as early embryo development up to the blastocyst stage (Yang et al., 2012). The distribution of miRNA reads is widely spread throughout the genome likely due to the unique biological processes associated with this stage of conceptus development which include fetal development, elongation, and implantation.

PiRNA constitute the largest small RNA class in the cumulus oocyte complex and early embryo; which we also observed during conceptus elongation. PiRNA protect genome integrity by interacting with the Argonaute superfamily via piwi proteins (Kim et al., 2010). Similar to miRNA, piRNAs confer gene silencing and are also associated with epigenetics and transposon silencing (Halic and Moazed, 2009; Kim et al., 2010).
The library validation confirmed dynamic changes in miRNA expression during conceptus elongation. DIANA-mirPath identified pathways that may be affected by miRNA expressed during conceptus elongation in the pig. MIR301a was highest in filamentous conceptus and this miRNA is predicted to interact with transforming growth factor beta (TGF\(\beta\)). TGF\(\beta\) expression is associated with porcine implantation which begins at day 13 (Gupta et al., 1996; Massuto et al., 2010). The embryonic disc located near the center of the elongating trophectoderm continues to develop and signaling such as axon guidance, Wnt signaling, and p53 signaling pathways guide the conceptus development during this period. Several of the miRNA validated in this study including MIR23b, MIR10a, MIR200a, and MIR467a interact with these pathways.

The miRNA we validated tended to have greater expression in filamentous embryos compared to spherical. This may be due to changes in morphology and not cell type. The elongating conceptus increases in length however the ratio of protein:DNA decreases throughout this stage of development suggesting the structure changed is not due to an increase in cells (Geisert et al., 1982; Pusateri et al., 1990). Other studies have also found a limited number (>100) of changes in mRNA expression during the transition from spherical to filamentous (Ross et al., 2009). This could be due to signals for the uterine environment controlling or initiating the elongation progress rather than the conceptus.

Although none of the miRNA we validated are predicted to play a direct role in steroidogenesis, MIR200a is predicted to target seven different genes in the gonadotropin releasing hormone signaling pathway. MIR301a is predicted to target up to 14 genes in the calcium signaling pathway. Calcium is known to be important in the uterus during pregnancy establishment and is thought to be involved in estrogen signaling (Choi et al.,
2009). However little is known about calcium regulation in the developing conceptus or uterine environment. MIR301a and MIR200a expression have also been reported during embryonic development of the mouse (Ahn et al., 2010; Diez-Roux et al., 2011). MIR23b, MIR200a and MIR467a all target mRNA involved in tight junction and focal adhesion pathways which play a role in regulating the morphological transformation. MIR23b is also reported to interact with argonaute 2 which regulates miRNA-induced RNA silencing (Kim et al., 2011). MIR23b is most abundant in D14F conceptus which also have the most abundant miRNA reads. MIR23b may be important in regulating the influx of miRNA during conceptus elongation through its relationship with argonaute 2.

Cytokine-cytokine interactions are important for conceptus:maternal interactions during pregnancy establishment (Ziecik et al., 2011). MIR21 and MIR574-3p may play roles in regulating the balance of cytokines in the conceptus for successful implantation. MiRNA in the elongating conceptus likely regulate not only the changes of the conceptus itself but may also interact with the maternal uterine environment. MIR21 has been characterized in many cancer cells lines as well as the ovary and oocyte (Asangani et al., 2007; Carletti et al., 2010; Chan et al., 2005; Dillhoff et al., 2008; Lu et al., 2008; Zhu et al., 2008; Wright et al., in prep.). MIR21 expression increased significantly at each stage of development. MIR21 regulates apoptosis by interacting with PDCD4 thereby promoting cell survival and cell cycle progression (Dillhoff et al., 2008; Lu et al., 2008).

In summary this elongating conceptus library provides data which will lead to a better understanding of the conceptus elongation process and the potential contribution of PTGR. Conceptus elongation is a complicated process of morphological transformation, steroidogenesis and embryo development. Further investigation will be needed to determine
the role of individual miRNA within the multiple biological processes that occur during conceptus elongation.
CHAPTER 6

Summary and Conclusion

This dissertation describes the role of MIR21 in oocyte maturation, effects of heat stress on genetic markers including MIR21 during oocyte maturation and a library of miRNA expression during conceptus elongation. MiRNA are important in regulating biological processes including oocyte maturation and embryo development. MIR21 in the oocyte is needed to regulate \( PDCD4 \) mRNA translation so that the oocyte is able to mature properly and develop to the blastocyst stage. Heat stress during oocyte maturation can alter MIR21 expression along with other genetic markers which are essential to normal oocyte maturation and development. MiRNA are also expressed during embryo elongation in the pig. The small RNA library of elongating pig conceptus identified several hundred miRNA which are expressed and likely play biological roles regulating this process. Additionally, several miRNA from this library were validated to demonstrate the dynamic changes in expression of miRNA during conceptus elongation. This data may be utilized to develop a better understanding of oocyte maturation and embryo development and ultimately provide tools for improving reproduction in the pig.

This work may lead to several opportunities for discovery in the future. Further elucidation of the function of MIR21 in the oocyte and developing embryo is needed. Additional targets of MIR21 need to be investigated in both the oocyte and developing embryo. Our work has shown that MIR21 affects oocyte maturation. Over-expression of MIR21 during maturation should be evaluated to determine the ability to improve oocyte maturation.
The heat stress model we developed provided further insight into MIR21 function in addition to evidence that stress to the oocyte is reflected in the subsequent embryo. Heat stress caused damage to either MIR21 stores or transcription therefore supplementing heat stressed oocytes or developing embryos with MIR21 in the culture media could rescue the developing embryos and allow normal maturation. MIR21 and PDCD4 could also be used as markers to determine if strategies to mitigate the effects of heat stress are effective. The heat stress model could be expanded to include additional time points in development to determine when transcriptional changes are occurring. Blastocysts developed from this model could be transferred into a surrogate to evaluate the effects of heat stress during oocyte maturation on the fetus. In vivo studies should also be performed to determine if heat stress during oocyte maturation and early embryo development has the same effect on coding and non-coding RNA that we observed in the in vitro model. By validating the in vitro model with an in vivo study we can further investigate ways to mitigate the effects of heat stress on the oocyte thereby improving reproductive performance during heat stress in swine.

The small RNA library created for conceptus elongation is a valuable biological resource. MiRNA identified in this library can be matched to mRNA expression data from the same period in development and provide information about PTGR during this process. Understanding embryo elongation in the pig could provide tools for improving litter size, optimizing available uterine space and reducing conceptus loss during early gestation.
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APPENDIX I

MiRNA expression identified by Ensemble by read greatest to least for each stage of conceptus development: D12 spherical, D12 filamentous, and D14 filamentous.
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APPENDIX II.

Protocol for In Vitro Production of Porcine Embryos

Prepare in vitro maturation medium

1.) Place 5 mL TCM -199 (basic medium) into a 15 mL and add 0.007 g cysteine (5x stock)

2.) Add 1mL from step (1) to 19 mL of TCM -199 medium
   a. Add 20 µL of EGF (epidermal growth factor) final concentration 10 ng/mL
   b. Add 5 µL of FSH and LH to each well – final concentration 0.5 μg/mL (stock 100x)
   c. Maturation plates only – add enough mineral oil to cover top surface of media

3.) Place plates in incubator at least 3 h prior to balance the atmosphere

4.) Ovaries should arrive in a container at 37º C
   a. Rinse ovaries 3 x in 0.9% saline with antibiotics (gentamicin 250 mg/L)
   b. 18 gauge needle and 10 mL syringe
      i. Place open end of needle below the follicle (3 to 6 mm antral follicle) put vacuum pressure on syringe until follicle deflates
      ii. Place collected follicular fluid in 50 mL tube
      iii. After all ovaries have been aspirated allow fluid to settle in incubator ~ 10 min
      iv. Pour off excess fluid and rinse oocytes with PVA-TL-Hepes 2 x waiting 10 min between so oocytes can settle in the bottom of the tube
      v. Divide pellet at the base of the tube between several plates for searching
      vi. Optimum oocytes will have several layers of cumulus cells surrounding the entire oocyte and be dark in color (cumulus-oocyte complex - COC)
      vii. Transfer all COC to each well of the wash plate
      viii. Transfer 50 to 70 COC to each well of the maturation plate
ix. Incubate 42 to 44 h at 39 °C. 5% CO₂ in air.

5.) Oocyte denuding
   a. Place all oocytes in 1 mL tube of hyaluronidase (1 mg/mL) to remove cumulus cell layers.
   b. Vortex at medium speed (3-5) for 4 min.
   c. Repeat as needed.

6.) MII oocyte collection
   a. Maturing oocytes will develop a polar body on one side of the cytoplasm.
   b. Collect these oocytes for fertilization or parthenogenesis.
APPENDIX III.

Western Blot

1.) **Sample preparation**

2.) Add SDS 5x =2.5 µL to each sample add water until total volume of sample is 13µL and mix by pipet

3.) Heat samples to 95°C for 4 minutes then ice for 1 minute

4.) Centrifuge 10,000 rpm for 1 min at RT

5.)

6.) Assemble gel apparatus and add running buffer between plates to check for leakage. If only one gel use spacer wrapped multiple times (3x) with parafilm.

7.) **Load gel (premade)**

8.) Ladder 10 µL first well then 5 µL of chemi ladder + 5µ L SDS buffer to second well followed by samples 13 µL each

9.) Run 30 minutes at 60 volts

10.) Run up to 90 minutes at 120 volts watch for color to reach the base of the gel.

11.) Transfer- wet all materials with transfer buffer before assembling and roll out bubbles at each step. Cut filter paper and membrane to size of gel and cut top right corner of membrane to indicate direction of gel.

12.) Sponge

13.) Whatman filter paper

14.) Membrane

15.) Gel

16.) Whatman filter paper

17.) Sponge

18.) Black holder

19.) Add buffer to bucket until half full insert loading apparatus into holder and place ice block behind loading block. Fill to top place lid on top securely.

20.) Run at 4°C 100 volts for 1 hour

21.) Wash PBS if needed

22.) Block for 1 hr with 5% milk at RT or 4°C overnight

23.) Wash with PBST

24.) Add first antibody PBST 0.5% milk on shaker at 4°C overnight

25.) Wash 3x PBST for 10 minutes each

26.) Add 2nd Antibody in PBST 0.5% milk + 1µL S-Protein for chemi ladder RT for 1 hr

27.) Wash 3 x PBST 10 minutes each

28.) Wash ECL (made fresh 1:1 of each chemical) 1 to 1.5 minute in the dark. drain off excess but do not wipe off. Wrap in cling wrap and image immediately.
APPENDIX IV.

Tunnel Assay

Four to eight cell embryos collected at 60 hrs post activation were used to determine the influence of miR21 on viable blastomeres within an early developing embryo. In Situ Cell Death Detection Kit, Fluorescein by Roche was used to detect and quantify apoptosis (programmed cell death) within each blastomere of the early embryos. Embryos (n=5) were obtained at 60 hrs of development for all treatments (control, negative control, and MiR 21 inhibitor 1.0 nM) and selected for uniform holoblastic cleavage. Embryos were placed in 4% paraformaldehyde, freshly prepared overnight, washed in PBS 30 minutes followed by incubation in 0.1% Triton X-100, 0.1% sodium citrate for 2 minutes on ice. TUNEL reaction was prepared as instructed by Roche. Embryos were washed twice in PBS and resuspended in TUNEL reaction mixture at 37°C for 60 minutes in a dark, humidified incubation chamber. Embryos were then washed twice with PBS and plated on slides with DAPI for nuclear staining. Wavelength was detected at 515-565 nm (green) for TUNEL and 340-380 nm (blue) for DAPI. Images were overlaid and nuclei were counted to determine total cell count within an embryo and total cell death within an embryo.
APPENDIX V.

Immunostaining for oocytes and early embryos

1. 4% paraformaldehyde overnight at 4°C
2. 0.5% triton X-100 in DPBS 30 minutes at RT
3. 3% BSA in DPBS 45 minutes at RT
4. First antibody in 3% BSA at 4°C overnight
5. 0.05% PBST wash 2x 3 minutes
6. Second antibody 3% BSA Alexa 674 Anti-rabbit 1:250 RT for 1 hour
7. 0.05% PBST wash 2x 30 minutes
8. 2% paraformaldehyde for 10 minutes at RT
9. 0.05% PBST wash
10. Mount with Dapi anti-fade

Use 16 well plate and wash as follows Steps 8 and 9 will need to be in a new plate (4 well is fine)
APPENDIX VI.

In situ hybridization of ovary sections

Deparaffinized

1. 2x wash xylene or citroso, 5 min each
2. Hydrate in 2 changes of 100% ethanol for 3 minutes
3. 95% and 80% ethanol for 1 minute
4. Rinse in distilled water

Preheat citrate buffer at 95°C

5. Immerse slides in buffer for 20-40 min
6. Allow to cool to room temp
7. Block for 30 min 5% BSA (for hybridization skip to Hybridization section below)
8. Incubate sections in primary antibody at 4°C overnight
9. Wash 3x PBS, 10 min each
10. Incubate 1 hr in dark with secondary antibody in 5% BSA
11. Wash 3x PBS for 10 min each
12. Mount with DAPI anti-fade and seal cover slip with nail polish

Hybridization

1. Warm hybrid solution to 50-70 °C probe specific, place slide in for 1 hr
2. Add probe overnight at hybrid temp
3. Washes: 50% hybridization solution/2x SSC at 60°C for 10 min 2x
4. 2x SSC for 10 min 3x
5. 0.2x SSC/25% PBST at RT for 10 min 3x
6. 0.2x SSC/50% PBST at RT for 10 min 3x
7. 0.2x SSC/75% PBST at RT for 10 min 3x
8. PBST at RT for 10 min 3x
9. Add DAPI and place slide cover over
10. Seal edge with nail polish

Hybridization solution (Hyb+)

- 50-65% Formamide
- 5x SSC
- 0.1% Tween 20
- Adjust the pH to 6.0 with citric acid
- 50 µg/mL Heparin
- 1 mg/mL tRNA

Prepare the hybridization mix with the appropriate stringency (20mL)

<table>
<thead>
<tr>
<th></th>
<th>50%</th>
<th>60%</th>
<th>65%</th>
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<tbody>
<tr>
<td>Formamide (freshly de-ionized)</td>
<td>10mL</td>
<td>12mL</td>
<td>13mL</td>
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<tr>
<td>20x SSC</td>
<td>5mL</td>
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<tr>
<td>20% Tween 20</td>
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<tr>
<td>1M citric acid</td>
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<tr>
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