Two components of maintaining developmental competence: microRNA-21 in the maturing oocyte and autophagy induction in the follicular stage ovary

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Iowa State University

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Two components of maintaining developmental competence: microRNA-21 in the maturing oocyte and autophagy induction in the follicular stage ovary

by

Benjamin J. Hale

A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Genetics

Program of Study Committee:
Jason W. Ross, Major Professor
Aileen F. Keating
Christopher K. Tuggle
Steven M. Lonergan
Bing Yang

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alternation after a degree is conferred

Iowa State University

Ames, Iowa

2017

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DEDICATION

I dedicate this dissertation to my parents, Robert and Laura Hale. Thank you for supporting me and giving me the situation where I could pursue exactly what I wanted.
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<td>3'UTR</td>
<td>3-prime untranslated region</td>
</tr>
<tr>
<td>AKT1</td>
<td>protein kinase B subunit 1</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>AP1</td>
<td>activation protein 1</td>
</tr>
<tr>
<td>ASCY3</td>
<td>adenylate cyclase 3</td>
</tr>
<tr>
<td>ATG</td>
<td>autophagy related protein</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>BAX</td>
<td>BCL2 associated X, apoptosis</td>
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<td>B-cell lymphoma 2 family membranes</td>
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<td>BCL2L1</td>
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<td>BECN1</td>
<td>Beclin 1</td>
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<td>cyclic adenosine monophosphate</td>
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<td>CDC25B</td>
<td>cell division cycle 25B</td>
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<td>CDK1</td>
<td>cyclin dependent kinase 1</td>
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<td>CL</td>
<td>corpora lutea</td>
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<td>CNOT</td>
<td>CCR4-NOT deadenylase</td>
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<td>aromatase</td>
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<tr>
<td>DCP1-DCP2</td>
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<td>DiGeorge syndrome critical region 8</td>
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<tr>
<td>endo-siRNA</td>
<td>endogenous siRNA</td>
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<tr>
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<td>exportin-5</td>
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<td>EXPO5</td>
<td>Exportin-5</td>
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<tr>
<td>FKBP12</td>
<td>FK506-biding protein</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle stimulating hormone</td>
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GC  granulosa cells
GPCR  G-protein coupled receptors
GV  germinal vesicle
GVBD  germinal vesicle breakdown
HS  heat stress
HSP  heat shock protein
IBMX  3-isobutyl-1-methylxanthine
INSR  insulin receptor
IVM  *in vitro* maturation
LC3  microtubule associated protein 1 light chain 3 alpha/beta
LH  luteinizing hormone
MAPK  mitogen activated protein kinase
MII  metaphase II
MIR21  microRNA-21
miRNA  microRNA
MPF  maturation promoting factor
mTOR  mechanistic target of rapamycin
mTORC1  mTOR complex 1
ncRNA  noncoding RNA
NF-κB  nuclear factor of kappa light polypeptide gene enhancer in B cells
OCT4  octamer-binding transcription factor 4
PABP  poly(A)-binding protein
PDCD4  programmed cell death 4
PDE3A  phosphodiesterase 3A
PIK3C3  phosphatidylinositol 3-kinase catalytic subunit type 3
piRNA  PIWI-interacting RNA
<table>
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<tr>
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<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>pri-miRNA)</td>
<td>primary miRNA</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homolog</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SCNT</td>
<td>somatic cell nuclear transfer</td>
</tr>
<tr>
<td>SI</td>
<td>seasonal infertility</td>
</tr>
<tr>
<td>STAT3</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TRBP</td>
<td>transactivating response element RNA-binding protein</td>
</tr>
<tr>
<td>ULK1</td>
<td>unc-51 like autophagy activating kinase 1</td>
</tr>
<tr>
<td>VMP1</td>
<td>vacuole membrane protein 1</td>
</tr>
<tr>
<td>WEE1</td>
<td>WEE1 G2 checkpoint kinase</td>
</tr>
<tr>
<td>ZP3</td>
<td>zona pellucida glycoprotein 3</td>
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I would like to thank my major professor, Dr. Jason Ross, for giving me this opportunity. You have been exceptionally patient as I have rambled on about ideas I am excited about. Thank you for giving me the skill set I need to chase whatever exciting thing comes next. Also, you decorated the greatest birthday cake of all time. I would like to thank Dr. Aileen Keating. The discussions that you and Jason led during Repro Discussion Group were monumentally helpful for me. Thank you for creating an environment where it was easy to share and develop ideas. I would also like to thank my other committee members, Dr. Christopher Tuggle, Dr. Steven Lonergan, and Dr. Bing Yang. Thank you for your continued support.

There are too many fellow graduate students that I have had the pleasure to meet and become friends with during my PhD to list here. I would like to thank everyone in the Ross, Keating and Baumgard labs. Without the collaboration between these three groups much of my work would not have been possible. A special thank you to past office mates, Dr. Jill Madden, Dr. Elane Wright, and Dr. Katrin Hollinger, as well as the people I get to work with now, Dr. Malavika Adur, Jake Seibert, Dr. Yunsheng Li, and especially Mackenzie Dickson.
This dissertation describes two processes that the oocyte and ovary potentially utilize to maintain reproductive competence: autophagy in response to heat stress and microRNA-21 function during meiotic maturation. Heat stress (HS) occurs when heat accumulation (from internal and external sources) exceeds heat dissipation. HS is associated with seasonal infertility and therefore is a production issue and profitability constraint in the swine industry. Autophagy is the process by which somatic cells recycle cellular components and it is activated by a variety of stressors. Therefore, characterizing autophagy in the ovary and oocyte is valuable because of the potential of autophagy to mitigate the detrimental effects of HS. Additionally, we also characterized the function of a specific microRNA (miRNA), microRNA-21 (MIR21), during oocyte maturation and the breakdown of the germinal vesicle (GVBD). The developmental competence of the oocyte is determined by molecular events that occur up to and during GVBD, though there is almost entirely no transcription occurring during GVBD. Thus, the maturing oocyte is reliant on post-transcriptional gene regulation (PTGR) and/or interactions with the surrounding cumulus cells to regulate the mRNA repertoire and proteome prior to fertilization. We hypothesized that miRNA is active within the oocyte and its biogenesis is dependent on GVBD.

To characterize the effect of HS on autophagy induction in the ovary and oocyte, we utilized both an in vivo model as well as in vitro model. Twelve gilts were synchronized and subjected to cyclical HS (n = 6) or thermal neutral (TN; n = 6) conditions for five days during the follicular phase. The abundance of autophagy-related proteins in total ovarian protein was compared between gilts that had either experienced TN conditions for 5 days or HS conditions
for 5 days. Ovarian tissue was fixed and sectioned to compare the localization of autophagy-related proteins in the ovary between gilts that had either experienced TN or HS conditions for 5 days. Based on the effects of HS on cell morphology within the follicle and changes in autophagy-related proteins, autophagy induction occurs in response to HS during the follicular phase. To further characterize the autophagy response directly in the pig oocyte, cumulus-oocyte-complexes were aspirated from 2-4 mm follicles and subjected to different temperature treatments during in vitro maturation (IVM). The oocytes experienced either TN conditions throughout the entire IVM, or HS during the first or second half of IVM. The abundance of autophagy-related proteins was compared between metaphase II (MII) arrested oocytes, and the cleavage of LC3 was compared at different time points during IVM. This data suggests autophagy as a potential mechanism activated that the oocyte could use during environmental stress to recycle damaged cellular components to maintain developmental competence.

IVM was also utilized to characterize the function of a specific miRNA, microRNA-21 (MIR21), during oocyte maturation and GVBD. MiRNA, a class of functional small RNA, interact with the 3’ untranslated region (UTR) of target mRNA to affect their abundance and translational efficiency. Of particular importance is miRNA-21 due to its role in regulating programmed cell death 4 (PDCD4), and ultimately inhibiting apoptosis. To characterize the function of MIR21 in relation to GVBD, pig oocytes collected from aspirated 2-4 mm follicles experienced IVM at normal conditions or in the presence of a chemical inhibitor of GVBD, 3-isobutyl-1-methylxanthine (IBMX). Oocytes were collected at different time points of IVM with or without IBMX. The state of GVBD was compared to the abundance of mature MIR21, abundance of PDCD4, and activation of nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB). The cumulative results of this study suggest that MIR21 is activated and
important during meiotic maturation of the oocyte, and that NF-κB is potentially driving MIR21 increase in the oocyte.
CHAPTER 1: INTRODUCTION

The main goal of reproduction is to produce a healthy offspring that has the genetic material from two separate gametes. For this to occur, a multitude of events are required to reach fruition. If the follicle does not release a healthy oocyte, embryonic development will not occur after fertilization, and a pregnancy will not be maintained. All of these events require the production of a healthy gamete. This is especially important for the female gamete, the oocyte, as the ovary contains a finite number of oocytes. Any stress that is detrimental to an organism’s oocytes has the potential to limit the lifetime reproductive efficiency of that organism.

The oocyte is a fascinating and dynamic cell. Derived from primordial germ cells, the oocyte grows and reaches a meiotic arrest until puberty and estrous cyclicity begins. The ability of the oocyte to reach meiotic maturation and undergo fertilization to produce an embryo is dependent on processing the required molecular machinery within the ooplasm, and this varies even within oocytes from the same organism. Understanding the molecular machinery that maintains meiotic competence is imperative to understanding how the ovary and the oocyte regulate the events preceding fertilization and maintain these events under environmental stress.

This dissertation describes two processes that the oocyte and ovary potentially utilize to maintain reproductive competence: autophagy and noncoding RNA regulating of mRNA abundance. Herein reproductive competence is defined as the ability to efficiently produce healthy offspring. Autophagy is the process by which the cell sequesters damaged cellular components so that they can be degraded and recycled. The first two studies
presented herein characterize the effect of heat stress on autophagy induction in the ovary and oocyte. By inducing autophagy, the oocyte and ovary potentially mitigate the deleterious effects of HS. Functional noncoding RNA is a mechanism by which the cell regulates protein abundance above the level of transcription. miRNA, a specific type of noncoding RNA, acts as a guide to direct protein complexes to target mRNA, and this interaction affects the abundance of subsequent translated protein. miRNA directed regulation is especially important in the oocyte because the oocyte is transcriptionally quiescent during the drastic changes in chromatin configurations and meiotic regulatory proteins. To understand the role of microRNA in the oocyte, abundance and function of a specific microRNA, miRNA-21, was characterized at different time points leading to the metaphase II arrest that precedes fertilization.

By filling the knowledge gap concerning how the specific molecular machinery in the oocyte regulates meiotic maturation and maintains developmental competence, we increase the likelihood that we will be able to identify and maintain healthy oocytes in domestic animals as well as in human health. Being able to identify healthy oocytes will expedite processes in assisted reproductive techniques, and understanding how the oocyte maintains developmental competence under environmental stress may allow for the development of mitigation techniques that maintain reproductive efficiency.
CHAPTER 2
LITERATURE REVIEW

Sections of this literature review come from the following previously published material:


**Utilization of Pigs in Agriculture**

Between December 2015 and February 2016 U.S. pig producers weaned an average of 10.3 pigs per litter and 29.6 million pigs were weaned on U.S. farms [1]. In 2014, meat animals earned the U.S. over $100 billion and pigs made up $26.5 billion of that [2]. Advances in breeding and technology have made remarkable increases in swine production efficiency, but there appears to be an increasing demand for pork production. In 2015 the current world population was estimated at 7.3 billion, and it was predicted that the world will contain 9.7 billion people by 2050 [3]. The Dietary Guidelines for Americans [4] suggest 5.5 ounces of meat per day for a balanced diet. If the world population is at 9.7 billion, it will take 3.3 billion pounds of meat per day to enable the growing population to meet their essential nutrient demands.

Obviously, there is a disparity at the global level, and many good insecure regions have insufficient amounts of animal protein in their diet. However, this does illustrate the necessity for continued advances in animal agriculture and pork production. This necessity
is further compounded by the steady increase in global temperatures. According to the National Oceanic and Atmosphere Administration, a majority of the world experienced above-average temperatures in 2015, and these incidences of above average temperature per year are occurring more frequently each year [5]. This increasing number of yearly periods of above average temperature has consequences on agricultural animals, as the warm summer months are associated with bouts of decreased reproductive efficiency in swine, termed seasonal infertility (SI).

**Seasonal Infertility and Heat Stress**

It is estimated that the U.S. swine industry loses $450 million annually due to the drop in reproductive competence associated with the warm summer months, or SI [6]. The drop in reproductive competence due to SI has been characterized in the US [7], Finland [8], Germany [9], and Thailand [10]. The manifestations of SI include delayed puberty onset, decreased signs of estrus after weaning, and a reduction in the proportion of sows conceiving and maintaining pregnancy [7, 8, 11-14]. Though some have suggested that photoperiod plays a role in SI [15], it appears that elevated temperatures during that time have a larger or at least cumulative effect on reproductive competence [16-18].

Heat stress (HS) is a physiological condition when an organism can no longer regulate their internal euthermic temperature. In mammals, metabolic heat, generated through processes such as muscle contractions, biochemical reactions, and digestion, can be offset by the body’s ability to dissipate heat through convection or conduction [19]. This heat dissipation mechanism only works when the ambient temperature is at a point below the body’s thermal regulatory zone. When the ambient temperature rises above this level,
mammals utilize evaporative cooling mechanisms, such as sweating and respiration [19]. Swine are especially susceptible to HS as they have no functional sweat glands, an increased metabolic rate due to breeding, and a substantial layer of subcutaneous adipose tissue [20-23].

When ambient temperatures are increased above the level that evaporative cooling is effective, the animal regulates physiological and metabolomic changes, such as redistribution of blood flow from the body core to the periphery and through reduced feed intake [24]. These changes, while successfully reducing the internal euthermic temperature, can be detrimental to other physiological processes [24, 25]. It is suggested that these physiological changes to reduce core temperature can cause disturbances in metabolism of water and protein, energy and mineral balance, enzymatic reactions, hormonal secretions, and blood metabolites [26].

Acclimation to HS involves changes in hormone abundance, such as epinephrine, leptin, prolactin, glucocorticoids, thyroid hormones and somatotropins, which could potentially cause dysregulation to physiological processes involved [27]. HS has been shown to increase insulin circulation in cows [28, 29], and pigs [30, 31]. This increase in insulin could be due to an activated cellular stress response [32], as heat is toxic to cells [33]. HS is associated with increased circulating pro-inflammatory cytokines, which lead to inflammation [34, 35], and has been shown to injure vascular endothelium [36]. Due to the perturbations caused by HS and the HS-induced decrease in reproductive efficiency, it is likely that HS is acting directly on the ovary.
Follicular Development

The mammalian ovary is composed of functional units, or follicles, which develop and grow to become responsive to gonadotropins from the anterior pituitary. These follicles then ovulate, releasing the oocyte, and form corpora lutea (CL). The mammalian ovary has two major roles: 1) production of the female gamete, the oocyte, prior to fertilization and 2) generation of hormones, such as progesterone, estradiol, inhibins, and relaxin involved in regulation of the estrus cycle [37, 38].

The primordial follicle pool forms the stock from which all follicles will emerge [39]. Approximately 500,000 primordial follicles are present in both ovaries by 10 days after birth in swine [40]. Initiation of follicle activation involves endocrine actions and regulatory effects between the granulosa cells (GC) and the oocyte [41, 42]. Early follicle growth is characterized by an increase in the number of layers of GC, forming secondary follicles that contain a growing oocyte surrounded by up to 20 layers of GC [43]. The layers of GC separate from each other and form a fluid filled antrum, the defining characteristic of a tertiary follicle [44]. Mature follicles require approximately 83 days to form in the pig ovary and contain an oocyte surrounded by 10-30 layers of GC [45]. Follicle growth is independent of gonadotropins until the tertiary stage, at which time follicular recruitment occurs [46].

Follicular recruitment refers to the formation of a pool of antral follicles from which the ovulatory follicles are selected [47], and follicular recruitment occurs due to rising levels of follicle stimulating hormone (FSH) released from the anterior pituitary [48, 49]. The selected follicles will continue to grow for an additional 5-7 weeks in pigs [45]. This follicular response to gonadotropins contributes to selection and ovulation rate [50]. All
follicles on both ovaries ovulate within a short time frame [51]. Atresia may occur at any time during development of antral follicles, though most follicles are lost during the transition from secondary to large dominant follicles [52]. Only a small amount of recruited follicles (30 – 40%) complete final maturation and ovulate [49, 53].

Ovulation occurs at the peak of the follicular growth in response to the surge of luteinizing hormone (LH) from the anterior pituitary [54]. The release of LH occurs as part of a positive feedback mechanism from the anterior pituitary due to increased concentration of 17β-estradiol [55]. In order for the oocyte to be released from the follicle, proteolytic enzymes and prostaglandins are activated, which lead to the digestion of the follicle wall collagen [56].

**Effects of Heat Stress on the Ovary**

HS can reduce the size of dominant follicles in the first third [57] or in the second half [58, 59] of the estrus cycle in cows. In goats, it has been shown that HS delays follicular recruitment as well as lowers blood 17β-estradiol concentration [60]. In rats, HS decreases the number of ovulated oocytes [61], and mRNA abundance of FSH and LH receptors in GC [62]. HS has also been shown to diminish steroid production in cultured mouse GC and induce apoptosis [63]. In the pig, HS affects steroidogenic signaling as characterized through increased mRNA abundance of insulin receptor (INSR), protein kinase B subunit 1 (AKT1), LH receptor, and aromatase (CYP19a) [64]. Since HS can affect follicular cells, it is also plausible that HS damages the oocyte.
Figure 2.1 Follicular growth and development in the ovary
The functional unit of the ovary is the follicle, containing an oocyte surrounded by one or more layers of ovarian somatic cells. The primordial follicles form the pool from which all follicles will emerge, and they remain dormant until puberty. The granulosa cells differentiate surrounding the oocyte, forming primary follicles. Secondary follicles are characterized by at least two layers of granulosa cells (shown in blue) and surrounded by theca cells (shown in purple). The layers of granulosa cells separate from each other and form a fluid filled antrum, the defining characteristic of a tertiary follicle. Tertiary follicles that become gonadotropin responsive are selected to become Graafian (or dominant) follicles, which ovulated in response to the surge in luteinizing hormone. The corpus luteum forms from the luteinized granulosa and theca cells.
**Oogenesis and Meiotic Maturation**

Mammals are born with a finite number of primordial follicles that originate from the germ cell pool. After specification in the embryo, primordial germ cells expand in number by division and migrate to the site of the developing ovary to form germline cysts [65]. Following recruitment and selection, the maturing oocyte undergoes germinal vesicle breakdown (GVBD) facilitating the progression through meiosis until arrest at metaphase II (MII). Following GVBD the oocyte is transcriptionally quiescent until after fertilization and the activation of the embryonic genome occurs around the 2- to 8-cell stage of development depending upon species.

**Oocyte Prophase I Arrest**

The meiotic cell cycle begins during fetal development, and the oocyte is arrested at prophase I until puberty [66]. The maintenance of this meiotic arrest is in part controlled through maintaining constant levels of cyclic adenosine monophosphate (cAMP) via transfer from the cumulus GC to the oocyte and synthesis of cAMP in the oocyte [67-69]. In the oocyte, activation of G-coupled protein receptors in the plasma membrane stimulate the Gs protein which interacts and activates adenylate cyclase, which in turn transforms adenosine triphosphate (ATP) into cAMP [70, 71]. This is demonstrated when adenylate cyclase 3 (ASCY3)-deficient mouse oocytes undergo spontaneous resumption of meiosis due to decreased levels of cAMP [72]. The use of chemical phosphodiesterase inhibitors during *in vitro* maturation (IVM) have been used to inhibit meiotic resumption, indicating that resumption will not occur if cAMP cannot be hydrolyzed back into ATP [73-75].
Cross-talk between the oocyte and surrounding GCs plays a major role in maintaining meiotic arrest at the diplotene stage of prophase I, as the gap junctions between the oocyte and cumulus cells act as a mechanism to transfer cAMP and potentially other GVBD inhibitory factors [76, 77]. The maintenance of elevated levels of cAMP via the gap junctions results in activation of protein kinase A (PKA), which in turn leads to the inhibition of the maturation promoting factor (MPF) complex [78]. The MPF complex is composed of cyclin dependent kinase 1 (CDK1) in complex with cyclin B, and is inactive when threonine\textsuperscript{14} and tyrosine\textsuperscript{15} of CDK1 are phosphorylated [79, 80]. The activation of cell division cycle 25B (CDC25B) phosphatase and inactivation of the WEE1 G2 checkpoint kinase (WEE1) leads to MPF activation and the exit from the prophase I arrest of the oocyte [81].

**Resumption of Meiosis in the Oocyte**

Stimulation of meiotic resumption occurs through LH indirectly from the GC, as the oocyte lacks the LH receptor. The disruption of the gap junctions stops the supply of cAMP from surrounding granulosa cells to the oocyte, which leads to decreased levels of cAMP in the oocyte [67, 82]. This reduction of cAMP inactivates PKA, leading to activation of CDC25B phosphatase and inactivation of WEE1 kinase, culminating in the dephosphorylation of CDK1, activation of the MPF complex, and resumption of meiosis [81, 83, 84].

After the resumption of meiosis, GVBD occurs, duplicated chromosomes are separated, and half the genetic material is extruded as the polar body [85, 86]. In the pig, GVBD can be separated into five stages based on chromatin configurations (GV0 - GV4),
where the final stage is characterized by condensed chromatin that is no longer surrounded by a nucleolus [86]. The oocyte is then arrested again at metaphase II until fertilization. Maintenance of MII arrest occurs via stabilization of the MPF complex and organization of the spindle. The stabilization of the MPF complex is maintained by the CSF and Mos-mediated mitogen activated protein kinase (MAPK) pathway [87-89].

In the pig oocyte, it has been shown that isoforms of MAPK, p44 ERK1 and p42 ERK2 are present in growing oocytes, but not active until MPF activation [90, 91], and that meiotic competence is directly correlated with MPF activation and MAPK [92]. Full oocyte competence is reached in follicles approximately 3 – 5 mm in diameter, as detected through the ability of the oocyte to complete maturation and develop normally after fertilization [93]. Activation of MPF occurs concomitant with the beginning of GVBD, and sharply rises as the oocyte reaches the MII stage [94].

**Effects of Heat Stress on the Oocyte**

HS has been associated with reduced oocyte developmental competence, and induces apoptosis in *in vitro* fertilized and parthenogenetically-activated porcine embryos [95-97]. While the heat shock protein (HSP) machinery is constitutively expressed in the somatic cells of the ovary, there is only a change in heat shock protein mRNA abundance in the oocyte in response to prolonged HS [97]. Our group has shown that HS decreases the maturation rate for *in vitro* matured oocytes, and the quality of subsequent embryos from heat-stressed oocytes [98]. This suggests that mitigating environmental stress within ovarian cells could partially alleviate the decrease in reproductive competency caused by HS. One potential inherent mechanism to decrease cellular stress is autophagy.
**Autophagy**

Autophagy is the process by which somatic cells recycle energy through the reutilization of cellular components and is activated in somatic cells by a variety of stressors. There are three major types of autophagy: chaperone-mediated autophagy, microautophagy, and macroautophagy. Macroautophagy accounts for the largest amount of energy reacquisition of the three different types [99]. Autophagy is the sequestration of cytoplasm into a double-membraned cytosolic vesicle, the autophagosome, that fuses with a lysosome to form an autolysosome for degradation by lysosomal hydrolases [100]. The steps of autophagy can be broken down into induction, autophagosome formation, autophagosome-lysosome fusion, and degradation [101]. These processes are marked by the formation of large protein complexes, and much of the regulation occurs at the post-translational level [102, 103].

The best characterized regulator of autophagy induction is the mechanistic target of rapamycin (mTOR) [104, 105], due to mTOR being a central regulator that is part of the cellular pathway that responds to amino acid starvation, reduction in ATP, and reactive oxygen species (ROS) [106-108]. The inhibition of mTOR, as part of the mTOR complex 1 (mTORC1), is directed in part through AKT or AMPK, and inhibition of mTORC1 leads to induction of autophagy [109-111]. Though, autophagy induction is also regulated through beclin 1 (BECN1) interacting proteins ULK1 and phosphatidylinositol 3-kinase catalytic subunit type 3 (PIK3C3, formerly VPS34) [112, 113], which interact with and activate BECN1.

Once autophagy is initiated, BECN1 associates with PIK3C3 to initiate nucleation of the autophagosome [114, 115]. The extension of the autophagosome involves two
ubiquitin-like conjugation pathways. The first of which includes the formation of autophagy related gene (ATG)-12 forming a complex with ATG5 and then ATG16 [116]. In the formation of this complex, ATG7 and ATG10 act as E1 and E2 enzymes that sequentially cause the conjugation of ATG12 to ATG5 at a lysine residue through an isopeptide bond [117]. This ATG12-ATG5 complex then incorporates ATG16, forming a complex that interacts with and lengthens the autophagosomal membrane [116, 118, 119]. This complex associates with the autophagosomal membrane during extension and then dissociates from the autophagosome upon completion of autophagy [116, 120, 121].

The second ubiquitin-like conjugation pathway involves microtubule associated protein 1 light chain 3 alpha/beta (LC3), which was the first discovered mammalian homologue of yeast ATG8 [122]. LC3 is cleaved by ATG4, which exposes a terminal glycine residue. The cleaved LC3 forms a thioester intermediate with ATG3, which acts as an E2 conjugating enzyme, and this complex formation is catalyzed by ATG7 acting as an E1-like activating enzyme [123]. LC3 is then transferred from the ATG3-LC3 complex to phosphatidylethanolamine (PE) through the ATG12-ATG5 complex, where LC3-PE interacts with the autophagosomal membrane and causes its extension [118].

Both basal and stress-induced autophagy have been observed in the embryo and oocyte. Deficiencies in autophagy-related genes negatively affect both early and late stage embryonic development [124-127]. Embryos also respond to external stressors by the induction of autophagy [128, 129]. In an oocyte-specific ATG5 knockout mouse model, the embryo fails to develop past the 4-cell stage after fertilization [130]. Furthermore, LC3-II is detectable during initial culture of pig oocytes [131], and the autophagy protein, Beclin1 (BECN1), has been observed in the mouse oocyte [132].
Autophagy is inducible in an AKT-dependent manner in rat GC culture [133, 134], and exposing mice to cigarette smoke activates the autophagy pathway in the ovary via AMP-activated protein kinase (AMPK) [135, 136]. Autophagy has also been suggested to be an important process in follicle depletion, working both with and independently of apoptosis [137, 138]. Autophagy is a potential mechanism that the ovary and oocyte could use under HS to recycle damaged cellular components.

**Pig as a Scientific Model**

Pigs have many anatomic and physiological similarities to humans which make them excellent model species. The pig genome is highly conserved with the human genome, allowing for the pig to serve as an excellent biomedical and scientific model [139]. Miniature breeds of pigs, such as Yucatan, Hanford, and Gottingen, can be utilized in cases where domestic agricultural pigs would grow too large for housing constraints [140]. Pigs have similar cardiovascular, digestive, and urinary systems to humans. Also, the heart, liver, pancreas, and kidney of pigs have long been considered ideal for xenographic procedures in place of human organs [141]. The capability to produce valuable transgenic pig models exists through somatic cell nuclear transfer (SCNT) [142-144]. As of 2011, transgenic pigs derived from SCNT have been made for 68 different genetic modifications for use in biomedical and agricultural research [145].

Due to similarity between species, pig oocytes have been used as a model to evaluate oocyte maturation for human oocytes [146-148]. The temporal regulation of oocyte maturation is more similar in the pig to human than rodent models, as human oocytes take 24 – 40 hours to mature, pig oocytes take 42 hours to mature, and mice oocytes
require only 14 hours to mature before fertilization [149, 150]. Species specific differences have been described during chromatin configuration at germinal vesicle stages, with pig and primates sharing more similarities in intermediary stages than mouse and primates [149]. Protein synthesis is required for GVBD in pigs [151] and humans [152] but not mice [153]. After fertilization and subsequent embryo development, the developmental stage of embryonic genome activation in humans is more similar to pigs than mice [154].

Due to the fact that the pig oocyte shares many similarities to the human oocyte, the pig oocyte is an excellent biomedical and agricultural model. Reduced oocyte developmental competence is a primary reason for the reduced potential of in vitro produced embryos [155-157]. The cytoplasmic changes occurring during oocyte maturation are essential for embryonic development [157], and understanding how these changes are regulated is a necessity for improving assisted reproductive techniques. MicroRNA are potentially an important regulator of protein abundance change in the oocyte during maturation because their mode of action occurs above the level of transcription.

**MicroRNA Biogenesis and RISC**

It is estimated that only about 1% of the human genome encodes for protein [158], but the ENCODE project revealed that about 75% of the human genome is transcriptionally active [159, 160]. In the last two decades it has become increasing apparent that this noncoding RNA (ncRNA) has an active role in regulating cellular processes.

Small regulatory ncRNA include microRNA (miRNA), endogenous siRNA (endo-siRNA) and PIWI-interacting RNA (piRNA), which differ in origin, length, and Argonaute
protein partners through which they elicit their biological function [161-165]. Both miRNA and endo-siRNA are processed step-wise from double-stranded precursors that eventually result in 20-22 nucleotide mature small RNA. Alternatively, piRNA are generated from long single-stranded precursors into 26-31 nucleotide functional molecules in a Dicer-independent manner [165-167].

MicroRNA are transcribed from their own genes or the introns or exons of host genes [168, 169]. Studies have used bioinformatics to predict the connection between miRNA, transcription factors, host genes, and the targets of miRNA in renal cell carcinomas and pancreatic cancer [170, 171]. There are examples of specific miRNA that are dependent on the expression of the host gene or specific transcription factors (reviewed in [172]).

The miRNA class can be further divided into two groups distinguished by their biogenic pathway: canonical and non-canonical. Both canonical and non-canonical miRNA are first transcribed as primary miRNA (pri-miRNA) by RNA polymerase II [173]. A secondary RNA structure is formed based on sequence complementation within the RNA molecule, which produces 60-75 nucleotide (nt) hairpins that can occur as clusters within a single pri-miRNA [169, 173]. The canonical miRNA hairpins are recognized by DiGeorge syndrome critical region 8 (DGCR8), which directs the RNase III enzyme Drosha to cleave the base of the hairpin resulting in a pre-miRNA [174-176]. Recent studies suggest that Drosha is responsible for both recognition and cleavage of the pri-miRNA while DGCR8 increases the precision of this cut [177]. This pre-miRNA structure, composed of a hairpin with a 3′ overhang is exported out of the nucleus via Exportin-5 [178, 179]. MicroRNA derived from the non-canonical pathway do not undergo processing
by the DGCR8/Drosha microprocessing complex, but rather are cleaved by other endonucleases or transcribed directly as a short hairpin [180, 181]. Once canonical and non-canonical miRNA reach the cytosol, both are cleaved by the RNase III enzyme, Dicer in complex with transactivating response element RNA-binding protein (TRBP), resulting in a functionally mature miRNA [173, 182-185].

Once miRNA and endo-siRNA are in their mature forms following Dicer cleavage, they can interact with the family of Argonaute proteins (AGO 1-4; also known as EIF2C-4) to cause association of the RISC machinery [186]. An AGO protein associated with either a miRNA or endo-siRNA interacts with target mRNA by using the complementarity between the small single-stranded RNA molecule and the 3’ untranslated region (UTR) of the target mRNA. If there is complete complementarity between the small RNA molecule and target mRNA 3’ UTR, then the target mRNA is cleaved. If the complementarity is incomplete, translation is down-regulated through mRNA destabilization or interaction with the translation machinery [187-189].

The association between AGO2 and GW182 (also known as TNRC6A), a marker for GW/P bodies (RNA processing bodies), has been shown to be incorporated in miRNA mediated mRNA degradation [190, 191]. GW182 interacts with AGO2 through the Argonaute characteristic PIWI domain [192-194]. GW182 also interacts with several proteins related to RNA degradation, such as CCR4-NOT deadenylase (CNOT), decapping mRNA 1 and 2 (DCP1-DCP2) complexes, and poly(A)-binding protein (PABP) [194, 195].
MicroRNA biogenesis is initiated via the activity of RNA polymerase II resulting in synthesis of a primary miRNA transcript (Pri-miRNA) that is both capped and polyadenylated. The appropriate spatial complementation of specific nucleotides within the transcript results in the formation of hairpin secondary structures that are recognized by the RNA processing complex consisting of DROSHA and DGCR8. The enzymatic activity of this protein complex results in cleavage and removal of the hairpin structure (now considered a pre-miRNA) from the primary transcript. Exportin 5 facilitates the transport of pre-miRNA from the nucleus into the cytoplasm of the cell where it is recognized by DICER and the loop is cleaved leaving a short duplex mature miRNA molecule. Upon dissociation, either strand from the duplex miRNA structure can be utilized by the RNA induced silencing complex to contribute to post transcriptional gene regulation though impacting mRNA stability and/or translation efficiency in addition to contributing to chromatin modifications to control gene expression.
MicroRNA in the Oocyte

The inability to transcribe mRNA after GVBD and the potential for PTGR has led numerous research groups to pursue the characterization of the ncRNA expressed in the maturing oocyte. MiRNA, endo-siRNA and piRNA have all been demonstrated to be expressed in oocytes of multiple species at various stages of development [196-202].

Though miRNA are expressed abundantly in the oocyte, it has been shown that *Dgcr8* is not required for the maturing mouse oocyte, and loss of DGCR8 has no noticeable effect on mRNA regulation [203]. Loss of both Dicer and AGO2 does have an observable effect on oocytes and both are required for healthy oogenesis in mice [203-206]. This has led to the hypothesis that endo-siRNA have a more critical role in oocyte development and maturation rather than miRNA. It is surprising, though, that everything required for miRNA regulation is present in the oocyte, but miRNA would have no function [207].

In pigs, we have sequenced the small RNA population of both the oocyte and cumulus cells during IVM and demonstrate the portfolio of endo-siRNA, miRNA and piRNA [196]. During IVM of the oocyte and meiotic progression, few changes were evident except the abundance of miR-21 and miR-574-3p which were significantly up-regulated and down-regulated during IVM, respectively [196].

Deciphering the specific roles and contributions that siRNA and miRNA offer to the maturing oocyte and developing embryo is being illuminated in conditional knockout mice. Mice with a zona pellucida glycoprotein 3 (ZP3)-driven conditional loss of DGCR8 are fertile, but have reduced fecundity [203, 208]. This would suggest that in mice, siRNA and miRNA produced through non-canonical biogenesis are more likely necessary for maturation of oocytes capable of yielding developmentally competent embryos [206].
However, the reduced fecundity with litter size being substantially reduced in ZP3-cre driven DGCR8 knockout suggesting that some miRNA may contribute to developmental competency of the subsequently produced embryo even if not active in the oocyte during maturation [203]. Additionally, this observed suppressive effect of miRNA in the maturing mouse oocyte has yet to be demonstrated in other mammalian species.

**Role of MicroRNA in Fertilization and Embryo Development**

Sperm, in addition to the oocyte, also possess a diverse portfolio of ncRNA [209, 210]. The abundance of miRNA may be related to the biogenesis and expression of small RNA contributing to gametogenesis in males [211]. Presumably, the ability of sperm derived miRNA have a limited impact on the maternal mRNA profile following fertilization [210]. However, some exceptions may exist, as miR-34c, a miRNA associated with the differentiation of male germ cells [212], has been shown to influence development in mice following fertilization [213]. Liu et al. (2012) identified six miRNA, including miR-34c, present in mice sperm and zygotes but absent in mature oocytes. The impact on development occurs via the ability of miR-34c to interact with BCL2 and subsequently contribute to the regulation of the first embryonic cleavage following oocyte activation [213].

After fertilization and during cleavage, the embryo begins expressing RNA transcripts from the embryonic genome [214]. Transcription of pri-miRNA takes place in the mouse two-cell embryo, but mature transcripts are not seen until the four-cell stage [206, 215]. Dicer and DGCR8 activity have been shown to be necessary for the formation of the epiblast [216-219], and in some organisms it has been postulated that miRNA play
an important role in maternal mRNA clearance before zygotic gene activation (ZGA) [220-223].

Following fertilization, successful embryonic development requires broad transcriptional arrest and mRNA clearance to deplete maternally stored mRNA transcripts in coordination with ZGA and subsequent mRNA and protein production. Maternal mRNA depletion is, in part, controlled via the 3'UTR of the expressed transcripts [224, 225]. Some small RNA, including miRNA are abundantly expressed during oocyte maturation and early embryonic development in *Xenopus laevis* [226], *Drosophila* [227, 228], zebrafish [223, 229], mice [206] and pigs [196] suggesting the opportunity for small RNA to influence the posttranscriptional outcome of the ensuing embryo.

**MicroRNA in Embryonic Stem Cells**

The machinery required for the biogenesis of miRNA has been shown to be required for stem cell differentiation and the maintenance of pluripotency [230]. The disruption of Dicer and DGCR8 leads to defects in different mouse models as well as human cell culture [176, 216, 217, 231]. *Dicer* deletion in mice results in embryonic lethality [231] while *Dicer* null embryonic stem (ES) cells are unable to differentiate properly [216]. *Dgcr8* knock-out mice ES cells show a phenotype similar to Dicer-deficient ES cells, with reduced proliferation and defects in differentiation [218]. Collectively, suggesting that canonical miRNA biogenesis is needed for ES cell differentiation and proliferation.

MicroRNA expression clusters exist that appear to play a role in ES cell regulation including the let-7 family, the miR-290 cluster, and the miR-17-92 cluster. Members of the let-7 miRNA family are highly expressed in differentiating cells [232, 233] suggesting that
the let-7/LIN28 feedback-loop contributes to the maintenance of pluripotency. Specifically, let-7g can be regulated by LIN28, which is highly expressed in pluripotent cells [234]. The 3'UTR of LIN28 mRNA is also targeted by let-7, forming a potential feedback-loop [234-237]. The relationship to pluripotency is that pluripotent factors such as Oct4, Sox2, Nanog, and Tcf4 may activate the expression of the LIN28 gene, which in turn inhibits differentiation, partially a result via let-7g inhibition [230].

The miR-290 cluster is composed of six miRNA (miR-290 through miR-295) of which all are expressed in undifferentiated mouse ES cells, and their abundance decreases after differentiation [238]. This cluster is transcribed in single polycistronic transcripts which are regulated by a common promoter [239] and mice with a homozygous deletion of all six members of the miR-290 cluster results in embryonic lethality [240]. Further demonstrating the importance of the miR-290 cluster is that exogenously delivered miRNA of the miR-290 cluster can partially rescue the self-renewal capacity of Dicer-null cells [221, 241].

The miR-17-92 cluster is strongly expressed in undifferentiated ES cells, and forms a polycistronic transcript which generates miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92a-1 [233, 238, 242]. The association with the miR-17-92 cluster with pluripotency is that the transcription of this cluster is activated by the oncogene, c-Myc [243]. Because c-Myc in combination with pluripotency factors Oct4, Sox2 and Klf4 can induce pluripotency (iPS cells), it is suggested that the miR-17-92 cluster also plays a role in pluripotency as well as stem cell renewal [244].
**MicroRNA in Embryo Implantation**

During the peri-implantation period of pregnancy, uterine epithelial cells and conceptus trophectoderm develop adhesion competence in unison to initiate an adhesion cascade within a restricted period of the uterine cycle; termed the window of receptivity or implantation. One example of examining differential expression of miRNA in mouse endometrium during the window of implantation found 32 miRNA to be upregulated. Of these 32 miRNA, miR-101, miR-144, and miR-199a* were of particular interest due to their predicted interaction with cyclooxygenase-2 (Cox2) mRNA [245]. Proinflammatory immune signaling is associated with uterine receptivity in multiple species [246]. Several miRNA have been identified to influence the expression of inflammatory and immune response mediators such as let7, miR-17-5p, miR-20a, miR-106a, miR-125b, miR-146, and miR-155 [247-251]. Both miR-125b and miR-155 are involved in the development of T, B and dendritic cells, and the transcription of these miRNA are activated by nuclear factor of kappa light polypeptide gene enhancer in B cells (NF-κB) [252]. Utilizing the SOLiD sequencing system, we have identified the presence of numerous small RNA in pig uterine endometrium during the implantation window [253]. How exactly specific small RNA contribute to uterine function and ultimately facilitate embryo implantation is largely unknown. However, conditional deletion of *Dicer* in mice driven by progesterone receptor or anti-Müllerian hormone receptor type 2 promoters resulted in sterility, abnormal development and altered signaling pathways in the uterus suggesting important biological contributions of small RNA to the function of the uterine endometrium [254, 255]. With respect to embryonic implantation, we have also queried the small RNA profile of the rapidly elongating pig conceptus during the establishment of the fetal:maternal interface.
Multiple classes of small RNA are present, and some miRNA, such as miR-23b, miR-21 and miR-10a significantly increase in abundance during rapid trophoblastic elongation on days 12 to 14 of gestation [256]. What remains unknown whether these changes facilitate interactions with the uterine endometrium or contributing to regulating cell differentiation associated with changes occurring in the inner cell mass and/or placenta.

**Placenta-Specific miRNA**

Following successful implantation, the survival of the growing fetus is entirely dependent on the placenta to coordinate gas exchange, supply of nutrients, removal of waste products, and immunological protection. The expression of miRNA is abundant in the human placenta, and there are distinctive expression profiles [257-259]. The miRNA biogenesis protein machinery also seems to be essential for placenta development and function. [260].

Of particular importance to placental specific miRNA expression are the pregnancy associated miRNA clusters chromosome 19 miRNA cluster (C19MC) and miR-371-3 cluster. Expression of miRNA from these clusters change throughout pregnancy [261] and differs among humans with preterm labor compared to normal term pregnancies [262]. These clusters are also located within imprinted genes that are known to be involved with embryonic development and cellular differentiation [263]. Interestingly, C19MC is one of the largest miRNA gene clusters in the human genome [264, 265] and is only expressed from the paternally inherited chromosome and is controlled by the methylation of the upstream promoter region [266]. While little is known regarding the regulation of C19MC, the expression appears to be restricted to the reproductive system and the placenta [259,
though expression of miR-498, a component of the C19MC cluster, has been reported in the fetal brain [267]. Interestingly, no homologues of this cluster have been found in rat, mouse, or dog [268]. The miR-371-3 cluster consists mainly of 3 miRNA (miR-371a-3p, miR-372, and miR-373-3p) sharing the same seed sequence, AAGUGC [269] and is predominantly expressed in the placenta [264].

**Additional RNA Classes**

With the utilization of high-throughput sequencing and the collection of deeper representations of the transcriptome, the regulatory roles of additional ncRNA classes are becoming better understood. Two types of ncRNA that are recently being given more attention are long ncRNA (lncRNA) and circular RNA (circRNA). One of the first lncRNA to be discovered in mammals was X-inactive-specific transcript (*Xist*), known for its role in turning off transcription of one of the copies of the X-chromosome via changing chromatin structure [270-272]. Since then, further characterization of other RNA molecules have demonstrated the regulatory abilities of lncRNA with respect to reproductive tissues. The overexpression of the lncRNA increased pluripotency associated transcription factors OCT4, NANOG, and SOX2. This potentially occurs through the mechanism of lncRNA decreasing the interaction between the transcription factors and associated miRNA [273]. It has also been proposed that lncRNA play a regulatory role in the testis as the expression level of lncRNA is much higher in the mammalian testis compared with other organs [274].

Additionally, newly discovered circular RNA (circRNA) are rapidly becoming an area of interest as a result of their apparent ability to act as a miRNA sequestering sponge
The human circRNA, antisense to Cerebellar Degeneration-Related protein 1 (CDR1as), undergoes non-linear splicing and circulation [276]. CDR1as is known to be targeted by miR-671, and contains 74 repeats of the miR-7 seed matches [276, 277]. Another interesting candidate circRNA that acts to sequester miRNA is the testis specific sex-determining region on the Y chromosome (Sry) [278]. The Sry gene is flanked by inverted repeats that induce circRNA in vitro [279] and are thought to be essential for the Sry circular structure [278, 280]. Hansen et al. (2013) found 16 putative target sites for miR-138, and showed that miR-138 inhibits luciferase activity when using a Sry-luciferase reporter.

**MicroRNA-21**

MicroRNA-21 (MIR21) is an anti-apoptotic factor that is ubiquitously found in different types of cancer cells and a variety of diseases [281]. This is in part due to its ability to target and suppresses pro-apoptotic proteins, including programmed cell death 4 (PDCD4) and phosphatase and tensin homolog (PTEN) [282, 283]. Predictions using TargetScan v6.2 suggest there are currently 382 predicted MIR21 targets identified in the human genome [284]. Outside of apoptotic regulating targets, MIR21 also has the potential to regulate pathways associated with cell cycle arrest, suppression of tumor growth, and chromosome assembly [281, 285].

A variety of profiling studies have demonstrated a dramatic change of miRNA expression in cancer cells compared to normal tissues [286-288]. In these cancer profiles, up regulation of miRNA was associated with reduced apoptotic signaling and increased cell cycle progression [289]. The potential role of MIR21 in malignancy was first reported
by Chan et al. [290], and this has been expounded upon as MIR21 has been characterized as a central component in pathways regulating cell growth [282, 283, 291].

The pri-MIR21 is transcribed from an evolutionary conserved promoter that resides in the tenth intron of an overlapping coding gene, vacuole membrane protein 1 (VMP1, previously TMEM49), though transcription of MIR21 appears to be regulated independently [292]. Several conserved enhancer elements are in the MIR21-specific promoter, including binding sites for activation protein 1 (AP1), epithelium specific transcription factor (ETS), CCAAT/enhancer binding protein-α (C/EBPα), nuclear factor-1 (NFI), serum response factor (SRF), p53, and signal transducer and activator of transcription (STAT3) [292]. The complexity of this promoter illustrates the potential for MIR21 to have a role in a wide variety of cellular processes.

Interestingly, expression of MIR21 is also affected by estrogens and androgens, and a highly conserved androgen response element (ARE) has been identified within the MIR21 promoter [293]. Androgen appears to act as a positive regulator of MIR21 in prostate cancer cells [293], while estradiol might downregulate MIR21 [294]. In MCF-17 breast cancer cells, MIR21 is downregulated in response to estradiol induction [294].

MIR21 is a component of at least two feedback loops containing well characterized transcription factors, AP1 and NF-κB [282, 295]. In one regulatory loop, MIR21 abundance is increased by AP1 activation, and subsequently interacts with the PDCD4 mRNA, causing its degradation [282]. Similarly, NF-κB has been shown to drive MIR21 expression directly [295], culminating in MIR21-targeted inhibition of PDCD4 or PTEN [296, 297].
The pro-apoptotic tumor suppressor protein, PDCD4, is known to be downregulated or absent in a variety of cancer cell types [298, 299]. Activation of PDCD4 results in reduced tumor formation, inhibition of cellular invasion, promotion of apoptosis [300, 301]. The PDCD4 protein is localized to the nucleus and is constitutively expressed in normal tissues [302]. Activation of PDCD4 results in inhibition of Jun proto-oncogene, AP1 transcription factor (JUN) activation of mitogen-activated protein kinase kinase kinase kinase 1 (MAP4K1), which is an upstream activator of MAPK8, (previously JNK), culminating in inducing apoptosis [303, 304].

Conclusions

Overall, high ambient temperature causes detrimental effects on agricultural animal reproductive efficiency. HS potentially affects reproductive efficiency by acting directly on the ovary, during follicular development, and the oocyte, during meiotic maturation. If HS is detrimental to ovarian somatic cells and the oocyte, then autophagy is a mechanism by which to mitigate this stress by recycling cellular components.

The pig offers excellent potential as a scientific model to decipher the cytoplasmic changes that occur in the oocyte to bring about meiotic maturation. The oocyte is transcriptionally quiescent after GVBD, and must rely on post-transcriptional gene regulation. MicroRNA is a potential regulator of these key processes as its mechanism of action occurs through RNA-RNA interactions.
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CHAPTER 3: HEAT STRESS INDUCES AUTOPHAGY IN PIG OVARES DURING FOLLICULAR DEVELOPMENT

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Abstract

Hyperthermia or heat stress (HS) occurs when heat dissipation mechanisms are overwhelmed by external and internal heat production. Hyperthermia negatively affects reproduction and potentially compromises oocyte integrity and reduces developmental competence of ensuing embryos. Autophagy is the process by which cells recycle energy through the reutilization of cellular components and is activated by a variety of stressors. Study objectives were to characterize autophagy-related proteins in the ovary following cyclical HS during the follicular phase. Twelve gilts were synchronized and subjected to cyclical HS (n = 6) or thermal neutral (TN; n = 6) conditions for five days during the follicular phase. Ovarian protein abundance of BECN1 and LC3B-II were each elevated as a result of HS (P = 0.001 and 0.003, respectively). The abundance of the ATG12-ATG5 complex was decreased as a result of HS (P = 0.002). Regulation of autophagy and apoptosis occurs in tight coordination, and BCL2 and BCL2L1 are involved in regulating both processes. BCL2L1 protein abundance, as detected via immunofluorescence, was increased in both the oocyte (~1.6-fold; P < 0.01) and granulosa cells of primary follicles (~1.4-fold P < 0.05) of HS ovaries. These results suggest that ovarian autophagy induction occurs in response to HS during the follicular phase, and that HS increases anti-apoptotic signaling in oocytes and early follicles. These data contribute to the biological understanding of how HS acts as an environmental stress to affect follicular development and negatively impact reproduction.
Introduction

Heat stress (HS) occurs when an animal’s core temperature rises above biologically imprinted set points because inherent thermoregulatory mechanisms can no longer sufficiently cope with elevated environmental temperature [1]. Attempts to maintain euthermaia during HS can lead to perturbations in physiological processes, such as redistribution of blood flow, losses in feed efficiency, growth performance, reproductive ability, and altered body composition [2, 3]. These detrimental effects of HS create an annual financial burden on animal agriculture. St. Pierre et al. [4] predicted that the combined effects of HS accounted for an approximately $2 billion loss in United States livestock industries, and the economic burden created by HS on the United States swine industry alone was estimated to be $900 million per year [5].

Though HS is well-known to compromise follicular and early embryo development in a variety of animal models and agriculturally important species [6-10], little is known about how HS affects the integrity of follicular cells. Heat stress impairs gonadotropin receptor function and steroidogenesis in the granulosa cells (GC) [11-13]. Our group has shown that HS decreases the maturation rate for in vitro matured oocytes, and the quality of subsequent embryos from heat-stressed oocytes [14]. This suggests that mitigating stress within ovarian cells could partially alleviate the decrease in reproductive competency caused by HS. One potential inherent mechanism to decrease cellular stress is autophagy.

Autophagy is a key regulator of cellular homeostasis [15], which includes three major types of autophagy: chaperone-mediated autophagy, microautophagy, and macroautophagy. Macroautophagy accounts for the highest turnover of cellular components of the three different types [16]. Macroautophagy (hereafter called autophagy)
is the sequestration of cytoplasm into a double-membraned cytosolic vesicle, the autophagosome, which fuses with a lysosome to form an autolysosome for degradation by lysosomal hydrolases [17]. The steps of autophagy are induction, autophagosome formation, autophagosome-lysosome fusion, and degradation [18]. These processes are marked by the formation of large protein complexes, and regulation can occur at the post-translational level via protein-protein interactions [19, 20].

Beclin 1 (BECN1) plays an important role in the nucleation of autophagosomes via an association with phosphatidylinositol 3-kinase catalytic subunit type 3 (PIK3C3) [21, 22]. During autophagy there are two ubiquitin-like conjugation pathways involved with the extension of the autophagosomal membrane. One pathway includes the formation of the Autophagy Related (ATG)12-ATG5-ATG16 complex, where ATG7 acts like an E1-activating enzyme to conjugate ATG12 to ATG5 [23, 24]. The second ubiquitin-like conjugation pathway results in the cleavage of microtubule associated protein light chain 3 (LC3) alpha/beta, exposing a glycine residue at the C-terminal end. This process results in the conjugation of LC3 with phosphatidylethanolamine (PE), ultimately forming LC3-II [25]. Generation of LC3-II is a well described marker of mammalian autophagy [26, 27].

Autophagy is a mechanism by which dysfunctional intracellular machinery is disposed of, thus autophagy represents a potential molecular mechanism by which the ovary and oocyte attempt to mitigate the detrimental effects of HS. Our working hypothesis is that HS upregulates the autophagy pathway and the objective of this study was to characterize specific autophagy proteins in response to HS. To test this hypothesis, sexually mature female pigs were subjected to either TN or HS conditions during the follicular phase
of the estrous cycle, and autophagy-related proteins were characterized in the collected ovaries.

Materials and Methods

Animals and Experimental Design

All animal procedures were approved by the Iowa State Institutional Animal Care and Use Committee. Twelve gilts were reproductively synchronized using 15 mg altrenogest (Matrix®; Merck Animal Health, Madison, NJ), a progesterone analog, which was administered orally for 14 days to ensure gilts were entering the follicular phase immediately following withdrawal. Beginning immediately after altrenogest withdrawal and concurrent with synchronized follicular development, gilts were subjected to cyclical HS (n = 6) or TN (n = 6) conditions for five days. During the 5 days of temperature treatment the 6 gilts in the TN room experienced a constant temperature of approximately 20°C ± 0.5°C. The gilts in the HS room experienced steadily increasing temperature at approximately 0.5°C per hour to a maximum ambient temperature of approximately 31°C at 2000 hours. The HS room was then allowed to cool off during the night. This diurnal pattern of cyclical HS treatment was used to mimic environmental HS experienced at a commercial level. Rectal temperatures were taken on all 12 gilts at 0800 hours and then every hour between 1200 and 2000 hours for all 5 days up until sacrifice. At 124 hours following altrenogest withdrawal, gilts were sacrificed and ovaries were harvested and protein extracted for western blot analysis. Due to an effective synchronization program, ovaries were collected at the late follicular stage of the estrous cycle preceding expected ovulation.
**Ovary Collection**

After each gilt was sacrificed and ovaries were excised, the number of dominant follicles were counted on both ovaries and the diameter of each dominant follicle was measured with digital calipers (KD Tools®). Dominant follicles were defined based on morphology and a diameter over 6 mm [28]. One ovary was then flash frozen in liquid nitrogen and the other was fixed in 4% formaldehyde, alternating left and right between gilts. Frozen ovary samples were broken into pieces under liquid nitrogen using a mortar and pestle and then mechanically homogenized in 10 mM phosphate buffer containing 2% SDS. Protein concentrations were estimated using the Pierce® bicinchoninic acid assay (BCA) Protein Assay Kit (Thermo Scientific), and each whole ovary protein sample per animal was diluted to 5 mg/mL. Fixed ovaries were processed, paraffin embedded, sectioned to 5 µm, and mounted onto slides at the University of Iowa Comparative and Histology Lab.

**Transmission Electron Microscopy**

Sections of ovary previously fixed in 4% paraformaldehyde in phosphate buffered solution (PBS) were rehydrated, cut to approximately 2 mm cubes, then and fixed in cacodylate buffer containing 2% paraformaldehyde, 2% glutaraldehyde, and 0.1 M sodium for 24 h at 4°C. The sections were then washed 3 times for 15 min in 0.1 M cacodylate buffer, and post-fixed in 1% osmium tetroxide in 0.1M cacodylate buffer for 1 h. The sections were washed again 3 times for 10 min each in deionized water, en bloc stained with 2% uranyl acetate for 2 h, washed once for 10 min in deionized water, and dehydrated in 50% graded ethanol for 30 min. Sections were then dehydrated through graded ethanol series (70%, 85%, 95%, 100%) and transitioned into 100% acetone. Samples were
infiltrated and embedded into EPON epoxy resin (Electron Microscopy Sciences, Hatfield, PA). Thick and thin sections were made using a Leica UC6 ultramicrotome (Mager Scientific, Dexter, MI). Light microscope images were taken after staining with 1% toluidine blue-o with an Olympus BX-40 compound microscope. Electron microscope images were taken using a JEOL 200kV 2100 scanning and transmission electron microscope (Japan Electron Optics Laboratories, USA, Peabody, MA). Sections of ovarian cortex from 3 gilts that experienced TN conditions and 3 gilts that experienced HS conditions (n = 6) were used to visualize the effect of temperature treatment on follicular cell morphology via transmission electron microscopy (TEM).

Western blot analysis

Extracted protein samples were loaded into a 4-20% Tris glycine gel (Lonza PAGEr® Gold Precast Gels) using 50 µg per lane, where 12 lanes contained extracted whole ovarian protein from each animal (6 TN and 6 HS). The BioRad Mini PROTEAN Tetra System was used to run the gel at 60 volts (V) for 30 min followed by 120 V for 90 min. Following separation, the proteins were transferred to a nitrocellulose membrane using the iBlot® 2 Dry Blotting System (Life Technologies), with 20 V for 1 min, 23 V for 4 min, and then 25 V for 2 min. Membrane blocking was conducted using 5% milk in PBS for 1 hr at room temperature. Antibodies procured from Cell Signaling Technology™ were used: rabbit anti-BECN1 (C3495), rabbit anti-LC3B (3868), rabbit anti-ATG5 (12994), rabbit anti-ATG12 (4181), rabbit anti-phosphorylated BCL-2 (Thr56; 2875), rabbit anti-BCL2 (2970), rabbit anti-BCL2 like 1 (BCL2L1; 2764), or rabbit anti-caspase 3 (CASP3, 9665) was added (1:1000 dilution) to the membrane in 0.5% milk in PBS overnight at 4°C. A membrane using normal rabbit IgG in place of the primary antibody was used as a
negative control. Following primary antibody incubation, the membranes were washed with PBST (PBS with 0.1% Tween) three times at room temperature for 10 min each. Donkey anti-Rabbit IgG (Amersham™ ECL™ NA934) was incubated (1:2000) with the membrane for 1 hr at room temperature. The membrane was then washed three times for 10 min each at room temperature. Horseradish peroxidase substrate (Millipore, Billerica, MA) was added to the membrane for 1 min in the dark, and it was then exposed to x-ray film and developed for visualization. Average pixel intensity for the band corresponding to the target of each primary antibody was compared for each blot using Image Studio™ Lite (Li-Core®). Loading discrepancies between samples of every blot were corrected for each protein by normalization to β-actin, and all graphs presenting Western blot data are after normalization to β-Actin. For every blot done a negative control of rabbit IgG instead of primary antibody was done, and no signal was detected in these negative controls.

**Immunofluorescence staining**

Ovarian tissues were paraffin embedded, sectioned 5 µm thick, and mounted on microscope slides. Slides underwent two 5 min washes in CitriSolv™, were hydrated by two incubations of 100% ethanol for 3 min, a 1 min incubation in 95% ethanol followed by a 1 min incubation in 80% ethanol. Antigen retrieval was accomplished by a 40 min incubation at 95°C in Tris-EDTA buffer (10 mM Tris Base, 1 mM EDTA solution, 0.05% Tween 20, pH 9). Tissue sections were blocked for 30 min in bovine serum albumin (BSA; Sigma-Aldrich™), followed by an incubation in primary antibody in 5% BSA (1:100) overnight at 4°C. The following day, slides were washed for 10 min in PBS three times, incubated in fluorescein linked goat anti-rabbit IgG secondary antibody (Life Technologies) in 5% BSA (1:500) for 1 h, and then subjected to three additional 10 min
PBS washes. Images of ovarian sections were captured with an inverted microscope at 200× and 400× magnification. Negative controls included exclusion of primary antibody, as well as replacing the primary antibody with normal rabbit IgG. ImageJ software (NCBI) was used to quantify signal difference between treatments for fluorescence microscopy [29]. Two ovarian sections per animal were used to quantify oocyte-specific BCL2L1, BECN1, or LC3B abundance, where at least three oocytes of either secondary or early tertiary follicles per gilt, or ten primary follicles per gilt, were quantified. The intensity of fluorescence signal presented is oocyte specific signal intensity per area of individual oocytes or the primary follicle specific signal intensity per area of primary follicles per image.

**Statistical Analysis**

Statistical analysis of rectal temperature differences, ovary follicle number, ovary follicle type, western blot data, and immunofluorescence signal was conducted using PROC MIXED in SAS® with random effects, where a standard student t-test was used to compare statistical differences. Statistical significance was determined when $P$ values were less than or equal to 0.05.

**Results**

**HS had no effect on ovarian follicle size or number of follicles**

The average room temperature for each day was $20.3 \pm 0.5^\circ C$ for TN conditions, and the HS room temperature ranged from 26-32°C to mimic a diurnal pattern. During the maximal heat load for each day, the HS pigs had increased ($P = 0.001$) average rectal temperatures ($39.8 \pm 0.2^\circ C$) compared to the TN pigs ($38.8 \pm 0.2^\circ C$; Fig. 3.1A). There was
no treatment effect detected between TN and HS animals on the number of tertiary follicles per ovary ($P = 0.20$) or follicle diameter ($P = 0.96$; Fig. 3.1C and D). Large, dominant follicles were apparent in all ovaries regardless of group.

**HS increases vacuolization of the oocyte and surrounding granulosa cells**

To determine the effects of HS on cellular morphology of the oocyte and the surrounding granulosa cells, TEM was performed on ovarian sections (n=3/treatment). HS qualitatively increased the incidence of vacuolization of the oocytes and granulosa cells (GC), observed as spherical white spaces, compared to TN oocytes and granulosa cells (Fig. 3.2A - C). The GC surrounding oocytes from HS ovaries had increased incidence of sequestered cytoplasm of smaller diameter (Fig. 3.2B) while the oocytes from HS ovaries contained large vacuole-like structures (Fig. 3.2C). These results indicate that HS may not alter the number of tertiary follicles, though HS does affect the cellular morphology of oocytes and surrounding GC.

**Markers of autophagy are increased in the ovary due to HS**

Western blots using total ovarian protein were conducted to characterize the protein abundance of autophagy-induction markers BECN1, LC3B-II, and the abundance of the ATG5-ATG12 complex. HS increased ovarian protein abundance of BECN1 ($P = 0.001$) compared to ovaries from TN gilts (Fig. 3.3A and B). The abundance of LC3B-II was increased in HS ovaries ($P = 0.003$), as well as the ratio of LC3B-II to LC3B-I ($P = 0.006$; Fig. 3.3B) demonstrating an increase in the induction of autophagosome formation. Cyclical HS did not affect ATG5 or ATG16 abundance in whole ovarian protein. The abundance of ATG12 in complex with ATG5 decreased in the HS ovaries compared to ovaries from animals in TN conditions ($P = 0.002$; Fig. 3.3A and B). Though the reduction
of ATG12 in complex with ATG5 due to HS was unexpected, the increase of both BECN1 and cleaved LC3B-II support that HS induces components of autophagy signaling in the ovary.

Immunohistochemical (IHC) staining of ovarian sections was conducted for both BECN1 and LC3B. BECN1 was detected mainly in interstitial tissues (Fig. 3.4A – D) and the granulosa cells of tertiary follicles (Fig. 3.4G – J). LC3B was also detected mainly in interstitial tissues (Fig 3.5A – D), as well as oocytes of growing follicles, (Fig. 3.5G – J). The abundance of oocyte specific signal pertaining to either BECN1 and LC3B was quantified and a difference between TN and Hs groups was not detected ($P > 0.05$; Data not shown). These immunostaining results indicate that autophagy related proteins were present in ovaries from either TN or HS gilts.

**Anti-apoptotic signaling is increased in the ovary due to HS**

B-cell lymphoma 2 (BCL2) family members, BCL2 and BCL2 like 1 (BCL2L1; formerly BCL-xL), can regulate both autophagy and apoptosis through interaction with BECN1 or BCL2 associated X, apoptosis regulator (BAX), respectively [30-32]. The abundance of total BCL2 was not affected by HS, but HS increased the abundance of phosphorylated BCL2 at Thr$^{56}$ (Fig. 3.5A and B). The abundance of BCL2L1 was also increased due to HS (Fig. 3.6A and B), and BCL2L1 was detected in the interstitial tissues (Fig. 3.7 A – D) and GC and theca cells of tertiary follicles (Fig. 3.7G – J) from both TN and HS ovaries (Fig. 3.7C). There was an increase in BCL2L1 abundance in prophase I arrested oocytes (Fig. 3.8A and C) and the somatic cells of primordial follicles of ovaries from gilts subjected to HS, visualized via immunofluorescence (Fig. 3.8B and D; $P < 0.05$). The phosphorylation of BCL2 at the Thr$^{56}$ amino acid is known to decrease apoptotic
signaling [33]. HS increased the phosphorylation of BCL2 at Thr\(^{56}\) as well as BCL2L1 abundance in the ovary. Collectively, five days of cyclical HS altered apoptotic signaling in the ovary via BCL2 and BCL2L1 in the ovary, and specifically BCL2L1 in the oocyte, as quantified through IHC (Fig. 3.7A and B). Also, there was little to no detection of caspase 3 (CASP3) cleavage in either the TN or HS ovaries (data not shown), suggesting that apoptosis was not induced in the ovary at the level of HS the gilts experienced [34].

**Discussion**

Seasonal infertility is described as the drop in reproductive competence during the summer months [35]. Though seasonal infertility is due to a multitude of factors, such as photoperiod [36], associated elevated temperatures during the summer months have a large or at least cumulative effect on reproductive competence [37, 38]. Collectively, HS costs the global animal agriculture industries billions of dollars per year. There are numerous mechanisms through which HS compromises animal production, which include substantial reductions in reproductive competence [3]. Specifically, HS compromises female reproduction through a variety of mechanisms including altering ovarian function [2].

Here we utilize an *in vivo* HS model to characterize the effect of HS on the ovary. This model was based on previous studies [13, 39]. As in previous studies, gilts expressed HS associated behavioral symptoms: lethargy, decreased feed-intake, and increased respiration rate (data not shown). The gilts appeared to acclimate to HS by day 5, as observed through rectal temperatures. This was expected due to previous work characterizing the response to HS in pigs [40, 41], and it has been suggested that physiological acclimation to HS can itself be harmful to the animal [42, 43]. Follicular
development appeared to occur normally under HS, yet qualitatively HS increased the amount of unhealthy primary follicles, visualized via TEM. The data presented here suggest that HS can increase cellular stress within the ovary, and that this induces autophagy.

Autophagy is a mediator of the cellular stress response and has previously been shown to occur in the ovary [44-46]. Autophagy induction can be manifested in response to several environmental stressors such as cigarette smoke, which increases ovarian autophagosome formation in mice [45]. Heat stress compromises pig oocyte maturation and competency in in vitro studies [14, 47, 48], although little is known regarding the stress response within the follicle during in vivo exposure to HS.

The five day in vivo exposure to cyclical HS during the follicular phase increased vacuole-like structures in the oocyte and GC. The formation of areas of cytoplasmic sequestration was present in both the oocyte and surrounding GC in ovaries from heat-stressed gilts providing visual evidence of autophagosome formation. Interestingly, these vacuole-like structures appeared larger in the oocyte compared to the surrounding GC, which had areas of sequestered cytoplasm more similar to the size of mammalian autophagosomes (0.5 to 1.5 μm;[49]). The majority of autophagosome visualization in follicular cells has been in GC [45, 50, 51], and whether autophagy actually occurs in the oocyte is poorly understood. Oocytes have been classified as having autophagosome-like features [52, 53], but autophagosome formation has not definitively been demonstrated in oocytes. Autophagy also appears to be necessary for oocyte maintenance, as the loss of BECN1 or ATG7 results in premature loss of oocytes [54]. Therefore, HS causes
morphological changes in ovarian cells and qualitatively increased autophagosome-like features.

Mammalian oocytes have previously been shown to undergo necrosis in ovarian culture [53], developing a morphology similar to neural cells undergoing necrosis [55, 56]. Autophagy is indispensable for neural cells, which do not divide after differentiation [57]. We speculate that oocytes could be utilizing autophagy in the same manner as neural cells, as neural cells and oocytes both must maintain longevity through mechanisms other than cell division. Beclin 1 complexes are central to membrane trafficking and control of autophagy in neural cells [58]. It is likely that the large areas of sequestered ooplasm seen here arise via BECN1 complexes and lead to the degradation of protein aggregates or damaged organelles.

In addition to the detection of autophagosome-like structures in the oocyte and GC, HS increased the abundance of proteins associated with autophagy induction in the pig ovary. The abundance of BECN1 and the ratio of LC3B-II to LC3B-I was increased, in comparison with ovaries from TN gilts. Whole ovary abundance of ATG5 in complex with ATG12 was decreased by HS, which was unexpected as the ATG12-ATG5 complex is part of the ubiquitin-like conjugation pathway for autophagosome formation [59]. This result could potentially be due to the difference in the dynamics between the two ubiquitin-like conjugation pathways that extend the autophagosomal membrane [23]. Additionally, this study demonstrated substantial formation of autophagosome-like structures at the single time-point of ovary collection, which suggests that ATG12-ATG5 complex formation may not have been detected at its most abundant level.
Both BECN1 and LC3B were localized to the ovarian interstitial cells and follicular cells in immunostained sectioned ovarian tissue. The presence of these proteins in ovarian cells suggests that autophagy could be utilized in response to HS. The signal from antibody detected BECN1 and LC3B IHC was not quantified due to the high degree of different cell types in the ovary and variability in signal within a single ovarian tissue section and between animals. Even within the same cell-type in the same ovarian tissue section there appeared to be spatial differences in signal intensity for both BECN1 and LC3B. Though localization of BECN1 and LC3B to a specific cell type was consistent between different gilts, signal intensity appeared to be variable.

The fact that differences in BECN1 and LC3B abundance between TN and HS treatments were detected through western blotting but differences were not seen through IHC could potentially be due to changes in circulating immune cells in the ovary due to HS. In the mouse, immune cell function has been linked to estrous cyclicity [60], and feed restriction in rats reduces the number of macrophages surrounding preovulatory follicles [61]. This study does not explicitly characterize the effect of HS on autophagy in circulating immune cells. Though, it is the author’s opinion that treatment differences in signal intensity for BECN1 and LC3B were not detected through IHC because of the complexity and degree of different cell types within the ovary. The IHC data presented here indicate that future studies should be done to characterize changes in the localization of autophagy-related proteins in the ovary due to HS.

The biological importance of autophagosome formation in the ovary in response to environmental stress hinges on the role of the autophagosome after HS-induced formation. One potential feature of autophagosome formation in the ovary could be for the purpose of
mitophagy, or the selective engulfment of damaged mitochondria by autophagosomes and the subsequent degradation via lysosomes [68]. Mitophagy is of interest, as oocytes of developing follicles greatly increase the mitochondrial population [69, 70], which is subsequently parsed out during holoblastic cleavage of developing embryos prior to zygotic genome activation [71-73].

Evidence for the importance of autophagy is that murine models with loss of ATG7 function, critical for both the cleavage of LC3 and the formation of the ATG5-ATG12 complex, results in the accumulation of defective mitochondria in multiple tissue types [25, 74-76]. The observed increase of BECN1 in this study is congruent with others demonstrating that mitochondrial damage is associated with increased puncta of BECN1 in oocytes [77].

This study investigated a single time point after five days of HS thereby making a detailed characterization of autophagic flux not possible. However, the results suggest that HS increased autophagosome formation in the oocyte and surrounding follicular cells, providing impetus to investigate specific upstream regulators of autophagy, such as protein kinase B subunit 1 (AKT1) and mechanistic target of rapamycin (mTOR; formerly mammalian target of rapamycin) [78, 79], in future studies. It has also been previously shown that HS increases the phosphorylation AKT1 in the ovary [13], which could potentially inhibit mTOR, thereby inducing autophagy [80]. Understanding these mechanisms, which are important for further elucidating what cellular structures are being targeted by autophagosomes, could further lead to the development of strategies to mitigate the effects of HS on reproduction.
One such feasible approach to mitigate HS-induced deleterious effects on livestock reproduction is to manipulate autophagy through diet. This is feasible because affecting autophagy through dietary supplementation has been shown to decrease memory loss [81], increase immunity to viral infections [82], and alleviate fatty-liver disease [83]. As such, it seems plausible that increased autophagy through dietary supplementation could increase livestock animal reproductive efficiency during abiotic or cellular stress. Speculatively, because autophagy is a mechanism by which the oocyte mitigates environmental stress, the addition of autophagy inducers in *in vitro* maturation media could increase oocyte quality in techniques such as *in vitro* fertilization and embryo transfer. There is also a myriad of chemicals that increase autophagy, which could be valuable to include in assisted reproductive techniques.

Autophagy and apoptosis are regulated in tight coordination, partly through BCL2 family member proteins, which are important regulators of apoptosis during mammalian ovary development [62-66]. The dual role of BCL2 family members to regulate both autophagy and apoptosis is mediated by the ability of BCL2 and BCL2L1 to prevent apoptosis by inhibiting the formation of mitochondrial pores that release cytochrome C [67], while BCL2 and BCL2L1 can also interact with BECN1 to regulate autophagy [31, 32].

Phosphorylation status of BCL2 mediates its regulatory capacity, as phosphorylation at the threonine 56 (T56) position is associated with anti-apoptotic signaling, and the deletion of T56 eliminated its ability to inhibit mitochondrial depolarization [33]. Our study demonstrates increased ovarian T56 phosphorylation of BCL2 due to HS. We also observed a five-fold increase in the abundance of BCL2L1 in the
HS oocyte compared to the ovaries of TN gilts, suggesting that anti-apoptotic signaling is increased in the ovary due to HS concomitant with markers of autophagy induction.

The abundance of BCL2L1 was increased in ovaries from gilts subjected to HS, specifically in the oocytes, compared to TN. As BCL2L1 regulates both autophagy and apoptosis through its interaction with BECN1 or BAX, respectively [84], not only does this shed some light on how autophagy could be regulated in the oocyte, but provides rationale to further characterize autophagy specifically in the oocyte undergoing HS. Wright et al. (2012) have previously shown that HS not only reduced the maturation rate of oocytes, but also lowered the developmental competency of oocytes that entered metaphase II.

Not only does HS affect the oocyte specifically, but HS is also detrimental to the surrounding follicular cells. The space between the oocyte and GC appeared increased and GC were morphologically less healthy in the follicles from ovaries of gilts that underwent HS compared to the ovaries of TN gilts. If HS affects primordial or primary follicles in the ovary, then this could decrease the amount of healthy follicles in the follicular reserve, thereby decreasing the lifetime reproductive capacity of an animal [85].

In conclusion, markers of autophagy induction are elevated in the pig ovary due to HS adding to our understanding of intra-ovarian signaling contributing to folliculogenesis and oocyte development during HS. Subsequent experiments are warranted to test whether the competency of produced oocytes can be increased by taking advantage of artificial induction of autophagy, either in vitro or in vivo, thereby creating mitigation strategies to maintain reproductive integrity under environmental stress.
Acknowledgement

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Figure 3.1. Heat Stress Did Not Affect Follicle Size or Number of Follicles.
Figure 1. Heat Stress Did Not Affect Follicle Size or Number of Follicles. During the maximal HS load for each day, the pigs undergoing HS had increased \( P < 0.01 \) average rectal temperatures compared to pigs in TN conditions (A) due to increased environment temperature (B). HS had no effect on the number of tertiary follicles per ovary (C) nor the follicle diameter (D). This indicates that while the gilts undergoing the HS treatment had elevated body temperatures, tertiary follicles where still able to develop under HS conditions mimicking a diurnal pattern.
Figure 3.2. Environmental Heat Stress Increases Vacuolization in Follicles.

A

Thermal Neutral

Heat Stress

B

C

Oocyte

GC
Figure 3.2. Environmental Heat Stress Increases Vacuolization in Follicles. Ovaries from TN (n = 3) or HS (n = 3) gilts were examined by TEM to characterize cellular morphology of follicles. Representative images of follicles from either a TN or HS ovary (A). The follicles of gilts exposed to HS conditions had increased space between the oocyte and surrounding granulosa cells (GC). The HS GC had increased vacuole-like structures (B). The oocytes of gilts objected to HS also had increased vacuole-like structures. The arrows denote vacuole-like structures where cytoplasm is being sequestered (C).
Figure 3.3. Heat Stress Alters Autophagy-Related Protein Abundance in the Ovary.

A

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![Bar graph showing relative signal for different proteins under thermal neutral (TN) and heat stress (HS) conditions.]

* Denotes significant differences.
Figure 3.3. Heat Stress Alters Autophagy-Related Protein Abundance in the Ovary.

Gilts either underwent five days of cyclical HS or thermal neutral (TN) conditions after synchronization into the follicular phase. Western blotting of whole ovary protein for each gilt (n = 12) with antibodies directed towards autophagy-related proteins (A) showed an increase in protein abundance due to HS for BECN1, LC3B-II alone, and the ratio of LC3B-II/LC3B-I (B). Asterisk denotes $P < 0.05$. 
Figure 3.4. Localization of BECN1 in the Ovary
**Figure 3.4. Localization of BECN1 in the Ovary.** BECN1 was localized to the ovarian interstitial tissues (A – D) and tertiary follicles (G – J). Representative greyscale images of the fluorescein signal alone (A, C, D, G, I, K) and representative composite images of antibody labeled BECN1 in green and DAPI stained chromatin in blue (B, D, F, H, J, L) are presented. Negative controls using rabbit IgG instead of rabbit anti-BECN1 primary antibody were used (E, F, K, L), and each image was captured at the same exposure where no signal was detected in the negative controls. White arrows point out areas of detected signal. White bar represents 100 microns.
Figure 3.5. Localization of LC3B in the Ovary.
Figure 3.5. Localization of LC3B in the Ovary. LC3B was localized to the ovarian interstitial tissues (A – D) and oocytes of growing follicles (G – J). Representative greyscale images of the fluorescein signal alone (A, C, D, G, I, K) and representative composite images of antibody labeled LC3B in green and DAPI stained chromatin in blue (B, D, F, H, J, L) are presented. Negative controls using rabbit IgG instead of rabbit anti-LC3B primary antibody were used (E, F, K, L), and each image was captured at the same exposure where no signal was detected in the negative controls. White arrows point out areas of detected signal. White bar represents 100 microns.
Figure 3.6. Heat Stress Impacts Anti-Apoptotic Protein Abundance in the Ovary.

Anti-apoptotic signaling is increased in the ovary due to HS. Western blotting of whole ovary protein for each gilt (n = 12) with antibodies directed towards either total BCL2, phosphor-BCL2 Thr56, or BCL2L1 (A) showed an increase in phosphorylation of BCL2 at Thr56, but not total BCL2, and in increase in the abundance of BCL2L1 (B). Immunohistochemistry detected signal for BCL2L1 in the interstitial tissues and tertiary follicles for animals that underwent either TN or HS conditions, albeit not different between treatments (C). Asterisk denotes $P < 0.05$. White bar represents 100 microns.
Figure 3.7. Localization of BCL2L1 in the Ovary.
Figure 3.7. Localization of BCL2L1 in the Ovary. BCL2L1 was localized to the ovarian interstitial tissues (A – D) and tertiary follicles (G – J). Representative greyscale images of the fluorescein signal alone (A, C, D, G, I, K) and representative composite images of antibody labeled BCL2L1 in green and DAPI stained chromatin in blue (B, D, F, H, J, L) are presented. Negative controls using rabbit IgG instead of rabbit anti-BCL2L1 primary antibody were used (E, F, K, L), and each image was captured at the same exposure where no signal was detected in the negative controls. White arrows point out areas of detected signal. White bar represents 100 microns.
Figure 3.8. Heat Stress Increases BCL2L1 Abundance in the Oocyte and primary follicles.
Figure 3.8. Heat Stress Increases BCL2L1 Abundance in the Oocyte and primary follicles. BCL2L1 abundance increased in oocytes and primordial/primary follicles due to HS. Immunohistochemistry using a primary antibody against BCL2L1 and a fluorophore-linked secondary antibody was done for ovary sections from gilts that either underwent five days of TN or cyclical HS conditions. BCL2L1 protein abundance was significantly increased in oocytes (A and C) and in primary follicles (B and D). ImageJ was used to quantify the fluorescent signal in oocytes. Asterisk denotes $P < 0.05$. White bar represents 100 microns.
CHAPTER 4: CHARACTERIZATION OF THE EFFECTS OF HEAT STRESS ON AUTOPHAGY INDUCTION IN THE OOCYTE

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Abstract

Heat stress (HS) occurs when heat accumulation exceeds heat dissipation and is associated with swine seasonal infertility. HS contributes to compromised oocyte integrity and reduced embryo development. Autophagy is a potential mechanism for the oocyte to mitigate the detrimental effects of HS by recycling damaged cellular components. To characterize the effect of HS on autophagy in oocyte maturation, oocytes underwent IVM in either thermal neutral (38.5°C; TN) conditions throughout the entire 42- hour maturation period (TN/TN), HS conditions (41.0°C) during the second half of IVM (TN/HS), or HS conditions during the first half of IVM (HS/TN). Oocytes were collected midway or after IVM to assess the effect of HS on autophagy-related proteins. The abundance of beclin1 (BECN1) or BCL2 like 1 (BCL2L1) was not affected by temperature ($P > 0.05$), though HS/TN increased the abundance of autophagy related gene 12 (ATG12) in complex with ATG5 after 42 hours of IVM compared to TN/TN controls ($P < 0.01$). The cleaved form of microtubule-associated protein 1 light chain 3 beta (LC3B), LC3B-II, was decreased in TN/TN oocytes ($P < 0.01$), TN/HS oocytes ($P < 0.01$) and HS/TN oocytes ($P < 0.01$) compared to oocytes collected at 0 hours of IVM, suggesting autophagosome utilization under HS. Colocalization BECN1 and BCL2L1 was not detected in oocytes. Rapamycin (1.0 nM) treatment increased oocyte maturation rate above vehicle control ($P < 0.03$) during HS/TN IVM, thereby supporting autophagy as a potential mechanism activated in the oocyte during HS to recycle damaged cellular components and maintain developmental competence.
Introduction

Heat stress (HS) occurs when heat dissipation methods are exceeded by both internal and external heat accumulation (Fuquay 1981; John 2006). Attempts to maintain euthermia during HS lead to perturbations in physiological processes, such as reduced feed intake, blood flow redistribution to the periphery, and endocrine changes. Collectively these adaptations reduce growth performance, reproductive ability, and alter body composition (Hansen 2009; Ross et al. 2015). These detrimental effects of HS create a financial burden on swine producers and animal agriculture in general. Almost two decades ago, St. Pierre et al. (St-Pierre et al. 2003) predicted that the combined effects of HS accounted for an approximately $2 billion loss in United States livestock industries. In addition, the economic burden of HS on the United States swine industry alone has been estimated to be $900 million per year (Pollman 2010).

A large portion of the financial burden caused by HS can be explained by a decrease in reproductive efficiency (Ross et al. 2015). HS reduces oocyte developmental competence, and induces apoptosis in in vitro fertilized and parthogenetically activated porcine embryos (Isom et al. 2007; Bertoldo et al. 2010; Pennarossa et al. 2012). Furthermore, while the heat shock protein (HSP) machinery is constitutively expressed in ovarian somatic cells, there is only a change in HSP mRNA abundance in the oocyte in response to prolonged HS (Pennarossa et al. 2012). This evidence suggests that the oocyte possesses at least some ability to respond to hyperthermia.

Autophagy is a potential stress mitigation response, as it is the process by which damaged cellular components are recycled. There are three major types of autophagy: chaperone-mediated autophagy, microautophagy, and macroautophagy. Macroautophagy
(referred to hereafter as autophagy) accounts for the largest amount of cellular resource recycling of the three different types (Klionsky 2005). Autophagy is the sequestration of cytoplasm into a double-membraned cytosolic vesicle, the autophagosome, that fuses with a lysosome to form an autolysosome for degradation by lysosomal hydrolases (Klionsky and Emr 2000). The steps of autophagy can be broken down into induction, autophagosome formation, autophagosome-lysosome fusion, and degradation (Pyo et al. 2012). These processes are marked by the formation of large protein complexes, and much of the regulation occurs post-translationally (Mizushima 2010; Mizushima et al. 2011).

Beclin 1 (BECN1) plays an important role in autophagosome nucleation via an association with phosphatidylinositol 3-kinase catalytic subunit type 3 (PIK3C3) (Kihara et al. 2001; Kang et al. 2011). During autophagy there are two ubiquitin-like conjugation pathways involved with the extension of the autophagosomal membrane. One pathway includes the formation of the Autophagy related (ATG)12-ATG5-ATG16 complex, where ATG7 acts like an E1-activating enzyme to conjugate ATG12 to ATG5 (Mizushima et al. 1998; Geng and Klionsky 2008). The second ubiquitin-like conjugation pathway results in the cleavage of microtubule-associated proteins 1 light chain 3 alpha/beta (LC3A/B), exposing a glycine residue at the C-terminal end. This process results in the conjugation of LC3 with phosphatidylethanolamine (PE), ultimately forming LC3-II (Taherbhoy et al. 2011), which is a well described marker of mammalian autophagy (Tanida et al. 2005; Martinet et al. 2006).

Autophagy and apoptosis are regulated in tight coordination, partly through B-cell lymphoma 2 (BCL2) family member proteins, important regulators of apoptosis during mammalian ovary development (Ratts et al. 1995; Hsu et al. 1996; Perez et al. 1999;
Tanner et al. 2011; Basavarajappa et al. 2012). The dual role of BCL2 family members to regulate both autophagy and apoptosis is mediated by the ability of BCL2 and BCL2 like 1 (BCL2L1; also known as BCL-XL) to prevent apoptosis by inhibiting the formation of mitochondrial pores that release cytochrome C, via interaction with BCL2 associated X, apoptosis regulator (BAX) (Moldoveanu et al. 2014), while BCL2 and BCL2L1 can also interact with BECN1 to regulate autophagy (Pattingre et al. 2005; Maiuri et al. 2007).

Both basal and stress-induced autophagy have been observed in the embryo and oocyte. Deficiencies in autophagy-related genes negatively affect both early and late stage embryonic development (Zeng et al. 2006; Fimia et al. 2007; Qu et al. 2007; Cecconi et al. 2008). Embryos can also respond to external stressors by inducing autophagy (Adastra et al. 2011; Xu et al. 2011). In the oocyte, autophagy related gene 5 (Atg5) knock-out mice fail to develop past the 4-cell embryonic stage (Tsukamoto et al. 2008). Furthermore, LC3-II is detectable during initial culture of pig oocytes (Lee et al. 2013), and BECN1 has been observed in the mouse oocyte (De Felici et al. 2008).

Autophagy represents a potential molecular mechanism by which the oocyte could mitigate the detrimental effects of HS. We have previously utilized an in vivo model to demonstrate that HS affects autophagy-related proteins in the pig ovary, increases the abundance of autophagosome-like structures in follicles, as well as increases BCL2L1 in the ovary (Hale et al. In Review). Our working hypothesis is that HS upregulates the autophagy pathway in the oocyte, and thus our study objective was to characterize the induction of autophagy in response to HS.
Materials and Methods

In Vitro Maturation

Pig ovaries were obtained from a local abattoir for isolation of cumulus-oocyte-complexes (COCs) to be subjected to in vitro maturation (IVM) (Zhao et al. 2009; Yang et al. 2012a). Briefly, follicles (2-4 mm) were aspirated and COCs were collected and washed in TL-Hepes with 0.1% polyvinyl alcohol (PVA). 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 approximately 42 hours at 38.5°C in 5% CO₂. Prior to IVM, an aliquot of germinal vesicle-intact (GV) stage oocytes for each replication were randomly selected from the COC pool. Oocytes used for analysis were stripped of cumulus cells via gentle vortex (6 to 8 min) in 1% hyaluronidase in TL-Hepes-PVA. To observe the effect of HS on IVM of oocytes, oocytes underwent three different IVM temperature treatments: 1) TN conditions (38.5°C) for the entirety of 42 hours (TN/TN), 2) TN conditions for the first 21 hours of IVM followed by HS (41°C) conditions for the following 21 hours (TN/HS), or 3) HS conditions for the first 21 hours of IVM followed by TN conditions for the following 21 hours (HS/TN). Following IVM oocytes were stripped of cumulus cells by vortexing 5-6 minutes in TL-Hepes-PVA supplemented with 1% hyaluronidase.

To characterize the temporal change in abundance of LC3B-II, oocytes were matured under either the TN/TN or HS/TN treatments. A pool of oocytes was collected before introduction into maturation media (0-hour). Oocytes were collected after 21 hours of either TN (21-hour TN) or HS conditions (21-hour HS). The remainder of the oocytes
that had experienced 21 hours of TN conditions were allowed to continue maturation under TN conditions for a total of 42 hours (42-hour TN/TN), and the remainder of the oocytes that had experienced 21 hours of HS conditions continued maturation under a subsequent 21 hours of TN conditions, for a total of 42 hours (42-hour HS/TN).

**Western Blot Analysis**

Pools of 50 denuded oocytes per replication were collected as described above after 21 or 42 hours of IVM. Oocyte pools were lysed in 5 µL of Laemmli sodium dodecyl sulfate buffer at 95°C for 4 minutes followed by 1 minute on ice and then centrifugation at 1000 rpm for 1 minute at room temperature. Lysates from fifty oocytes per lane were loaded onto a 4-20% Tris glycine gel (Lonza PAGE® Gold Precast Gels). The BioRad Mini PROTEAN Tetra System was used separate protein homogenates at 60 volts for 30 minutes followed by 120 volts for 90 minutes. The protein was transferred to a nitrocellulose membrane for 1 hour at 100 volts at 4°C. Membrane blocking was conducted using 5% milk in phosphate buffered solution with 0.5% Tween 20 (PBST) for 1 hour at room temperature. A rabbit anti-BECN1 (Cell Signaling Technology™, 3495), rabbit anti-LC3B (Cell Signaling Technology™, 3868), rabbit anti-ATG12 (Cell Signaling Technology™, 4181), rabbit anti-BCL2L1 (Cell Signaling Technology™, 2764), or normal rabbit IgG (Cell Signaling Technology™, 2729) as a negative control were added (1:1000 dilution) to the membrane in 0.5% milk in PBS overnight at 4°C. Following primary antibody incubation, the membranes were washed with PBST (PBS with 0.1% Tween) three times at room temperature for 10 minutes each. Donkey anti-Rabbit IgG (Amersham™ ECL™ NA934) was incubated (1:1000) with the membrane for 1 hour at room temperature. The membrane was then washed three times for 10 minutes each at
room temperature. Horseradish peroxidase substrate (Millipore, Billerica, MA) was added to the membrane for 1 minute in the dark, and was exposed to x-ray film and developed for visualization. Average pixel intensity for the band corresponding to the primary antibody was compared for each blot using Image Studio™ Lite (Li-Core ®). Signal from detection of each protein of interest was normalized to α-tubulin.

Rapamycin Oocyte IVM and Activation

Cumulus-oocyte-complexes were collected and subjected to IVM under the HS/TN treatment as mentioned above, with the addition of either DMSO vehicle control, 1.0 nM rapamycin, 10 nM rapamycin, or 100 nM rapamycin. In addition to rapamycin, IVM media for this study contained 10 ng/mL leukemia inhibitory factor (LIF; Sigma-Aldrich™ L5283), 40 ng/mL basic fibroblast growth factor (BFGF; Sigma-Aldrich™ F0291), and 20 ng/mL insulin-like growth factor 1 (IGF1; Sigma-Aldrich™ I36769). After 21 hours of HS IVM followed by a subsequent 21 hours of TN IVM, oocytes were denuded and healthy metaphase II oocytes containing extruded polar bodies were counted as a percent of total oocytes per treatment. Pools of 50 metaphase II arrested oocytes were then flash frozen in liquid nitrogen to be used for downstream analysis.

Oocyte Fixation and Immunohistochemistry

Oocytes were collected and denuded above from different time points during IVM and then fixed and mounted to slides as previously described (Martin et al. 2006; Yang et al. 2012b). Briefly, oocytes were fixed in 4% paraformaldehyde in PBS overnight at 4°C, and then transferred to 70% ethanol in PBS at 4°C. Oocytes were permeabilized in 0.5% Triton X-100™ (Sigma-Aldrich™, St. Louis, MO) for 30 minutes at room temperature. Next, oocytes were blocked in 5% bovine serum albumin (Sigma-Aldrich™, St. Louis,
MO) for 45 minutes at room temperature, then incubated with primary antibody overnight at 4°C. After approximately 24 hours, oocytes were washed twice in PBS for 30 minutes, and incubated with secondary antibody for 1 hour at room temperature. The oocytes were then washed twice in PBS for 30 minutes, and mounted to microscope slides using SlowFade® Gold Mountant containing DAPI (S36939, ThermoFisher Scientific™, Pittsburgh, Pennsylvania).

**Identification of Colocalization of Autophagy Markers**

To label proteins of interest for colocalization, rabbit anti-BCL2L1 (Cell Signaling Technology™, 2764S), rabbit anti-BECN1 (Cell Signaling Technology™, 3495S), mouse anti-BCL2L1 (Novus™, 46569), and mouse anti-BAX (Novus™, 28566) primary antibodies were used at a 1:200 dilution. Secondary antibodies used were anti-mouse IgG AlexaFluor 647 (Cell Signaling Technology™, 4410S) and anti-rabbit IgG FITC (Life Technologies™, F2765) at a 1:250 dilution. To control for nonspecific binding of primary antibodies, a pool of oocytes was incubated in normal rabbit IgG (Cell Signaling Technology™, 2729) and normal mouse IgG (Cell Signaling Technology™, 5415) at a 1:200 dilution instead of primary antibodies. A Leica SP5X MP confocal microscope (Exton, PA) was used to image antibody labeled proteins of interest at the High Resolution Microscopy Facility of the Iowa State University Office of Biotechnology.

Primary antibodies derived from different species were used so that secondary antibodies with different fluorophores would recognize and bind to the separate primary antibodies within the same fixed oocyte. This made it possible to view two different fluorophores conjugated to secondary antibodies at different excitation points. Three oocytes per temperature treatment per time point of IVM were viewed using a confocal
microscope under three different channels to detect fluorescence of DAPI stained chromatin, labeled rabbit anti-BCL2L1 and mouse anti-BAX, or labeled rabbit anti-BECN1 and mouse anti-BCL2L1.

**Statistical Analysis**

Statistical analysis of maturation rate, western blot data, and co-fluorescence data was conducted using PROC MIXED in SAS® Enterprise Miner Workstation version 14.1 (Carry, NC), where a standard student t-test was used to compare statistical differences. Statistical significance was determined when \( P \) values were less than or equal to 0.05. The PDC colocalization plugin (French et al. 2008) in the ImageJ processing program (Schneider et al. 2012) was used to calculate Pearson’s and Spearman’s correlation coefficient and scatter plots representing colocalization of signal intensity collected from confocal microscopy of individual oocytes.

**Results**

Heat Stress Decreases Oocyte Maturation Rate

To characterize the effect of HS on maturation, oocytes underwent IVM during one of three temperature treatments: TN conditions throughout the entire 42-hour maturation period (TN/TN), HS conditions during the second half of IVM (TN/HS), or HS conditions during the first half of IVM (HS/TN). Both HS treatments influenced maturation compared to TN/TN control, where the maturation rate of TN/TN oocytes was 66.9 ± 5.0\%, the maturation rate of TN/HS oocytes was decreased to 44.9% ± 5.0\% \((P < 0.01)\), and the maturation rate of HS/TN oocytes was decreased to 33.2 ± 1.6\% \((P < 0.01; \text{Table 4.1})\). The
maturation rate of HS/TN oocytes decreased compared to the maturation rate of TN/HS ($P \leq 0.05$; Table 4.1). These are comparable to previous results from our group (Wright 2012).  

**Heat Stress Affects Autophagy-Related Proteins**

The abundance of BECN1, the formation of the ATG12-ATG5 complex, and the cleavage of LC3B was measured via western blotting to characterize the effect of HS on autophagy induction in the oocyte. The abundance of BECN1 and BCL2 like 1 (BCL2L1) were unaffected by temperature treatment (Fig 1A and B). There was increased abundance of ATG12 in complex with ATG5 after 42 hours of IVM under the HS/TN temperature treatment compared to the TN/TN ($P < 0.01$) and TN/HS ($P < 0.01$; Fig 4.1A and B). Approximately 1.4-fold greater ATG12 in complex with ATG5 was observed in oocytes after HS/TN temperature treatment compared with either TN/TN or TN/HS treatmented oocytes (Fig 4.1A and 1B).

There was no effect of temperature treatment on the abundance of LC3B II in oocytes after IVM with any of the three temperature treatments ($P > 0.05$). Although, there was decreased LC3B-II in TN/TN ($P < 0.01$), TN/HS ($P < 0.01$) and HS/TN oocytes ($P < 0.01$) compared to oocytes collected at 0 hours of IVM (Fig 4.1A and B). Compared to oocytes collected at 0-hour, there was an approximately 24-fold lower abundance of LC3B-II in oocytes collected after TN/TN IVM, approximately 17-fold lower abundance in oocytes collected after TN/HS IVM, and approximately 73-fold lower abundance of LC3B-II in oocytes collected after HS/TN IVM (Fig 4.1A and B).

The observed sharp decrease in LC3B-II due to temperature treatment provided rationale for an IVM experiment in which oocytes were collected after 21 hours to determine the temporal effects of HS on LC3B-II. Although the abundance of LC3B-II did
not differ between TN/TN oocytes and HS/TN oocytes at 42 hours of IVM ($P = 0.92$), there
was decreased abundance of LC3B-II after 21 hours of HS (0.21 ± 0.6 relative band
intensity) compared to oocytes collected after 21 hours of TN (0.9 ± 0.26; $P < 0.01$; Fig
4.2A and B). These data suggest that HS affects the utilization of autophagy-related
proteins in the oocyte.

**BCL2L1, BAX, and BECN1 Interactions**

Oocytes that underwent IVM in either TN/TN or HS/TN conditions were fixed for
immunohistochemistry (IHC) to determine BCL2L1, BECN1, or BAX co-localization.
Based on the Pearson’s ($r_P$) or Spearman’s ($r_S$) correlation coefficients, where a $r_P$ or $r_S$
closer to 1 is indicative of co-localization (Table 2) and overlap of fluorescent signal (Fig
4.3A and B), neither the time point nor the temperature treatment affected colocalization
of BCL2L1 and BAX. Based on level of fluorescence, there was a qualitative increase in
BAX at 21 hours of HS compared to TN conditions (Fig 4.3A and B). There were no effects
of time or temperature treatment on the abundance of BCL2L1 based on level of
fluorescence (Fig 4.3A and B).

Based on the $r_P$ and $r_S$ correlation coefficients (Table 3) and overlap of fluorescent
signal (Fig 4.4A and B), there was little colocalization of anti-BECN1 and anti-BCL2L1
antibodies regardless of time point during IVM or temperature treatment (Table 4.3).
BECN1 staining appeared punctate all time points and temperature treatment with no
overlap with BCL2L1 staining (Fig 4.4A and B). There appeared to be more intense
punctate BECN1 foal staining in oocytes (Fig 4.4A and B). This data suggests that BCL2L1
is participating in the regulation of apoptosis in IVM oocytes under TN or HS conditions,
while not appearing to inhibit autophagy.
**Low Concentration of Rapamycin Increases Maturation Rate**

To determine if inducing autophagy in oocytes during HS increases the oocyte’s ability to mature to MII arrest, oocytes underwent IVM while experiencing HS during the first 21 hours and TN conditions the following 21 hours in the presence of vehicle control, 100 nM, 10 nM, or 1 nM rapamycin. Oocytes treated with all four concentrations of rapamycin were subjected to the HS/TN IVM treatment, based upon the effect of this temperature treatment on ATG12-ATG5 complex and LC3B-II abundance. The maturation rate of oocytes in the presence of DMSO vehicle control was 50.0 ± 4.1 in 100 nM rapamycin was 43.6 ± 5.6%, 47.8 ± 5.5% for 10 nM rapamycin, and 65.8 ± 5.0% for 1 nM rapamycin (Fig 4.5). Including 100 nM or 10 nM rapamycin in the IVM media had no effect on maturation rate after HS/TN compared to vehicle control (\(P = 0.31\) and \(P = 0.72\), respectively), though oocytes matured in the presence of 1 nM rapamycin had an approximately 1.3-fold increase in maturation rate compared to vehicle control (\(P = 0.03\); Fig 4.5). This data suggest that low concentrations of rapamycin provides the oocyte some resistance to HS and improves their ability to reach MII arrest, potentially through induction of autophagy.

**Discussion**

Autophagy is the process by which cellular components are recycled, and autophagy is activated by a variety of stressors (Zhang 2015). In autophagy, the autophagosome forms around and sequesters damaged organelles or misfolded proteins, and proceeds to degrade its contents after interaction with a lysosome. BECN1 associates with PIK3C3 to increase nucleation of autophagosomes (Kihara *et al.* 2001; Kang *et al.*...
2011), after which the autophagosome membrane is extended via two ubiquitin-like conjugation pathways including ATG12 or LC3. ATG12 forms a complex with ATG5 via an isopeptide bond, and this complex formation is indicative of increased autophagosome formation (Geng and Klionsky 2008). The cleavage of LC3-I to form LC3-II is indication of autophagy occurrence, as cleavage of LC3-I and the conjugation of PE allows for LC3-II to interact with the autophagosomal membrane (Kabeya et al. 2000; Martinet et al. 2006).

Whereas HS during IVM did not affect the abundance of BECN1 or BCL2L1 in MII oocytes, there was increased abundance of ATG12 in complex with ATG5 in MII oocytes after undergoing HS/TN IVM compared to other temperature treatments. This increase of ATG12-ATG5 complex formation suggests a rise in autophagosome formation (Kuma et al. 2002), and this increase specifically as a result of HS during the first half of IVM could be due to HS being applied before transcription inactivation within the oocyte. After the breakdown of the germinal vesicle (GVBD), the oocyte is almost entirely transcriptionally quiescent (De La Fuente et al. 2004), but oocytes that underwent the HS/TN temperature treatment during IVM experienced HS before GVBD at a time when a transcriptional response could have occurred.

We have previously characterized the blastocyst development rate of MII oocytes collected after either TN/HS or HS/TN IVM, used for in vitro fertilization (IVF). Decreased blastocyst development rate was observed in fertilized TN/HS oocytes compared to controls, yet blastocyst development rate from fertilized HS/TN oocytes did not differ from control treated oocytes (Wright 2012). Therefore we used the HS/TN temperature treatment for IVM to further characterize autophagy in oocytes under HS conditions
because this treatment decreases maturation rate, but a subset of oocytes can reach MII arrest and maintain the ability to produce blastocysts.

At 21 hours of IVM, there was decreased abundance of LC3B-II in oocytes that had undergone HS compared to oocytes that underwent TN IVM, suggesting that HS exacerbates the decrease in LC3B-II. A decrease in LC3B-II abundance during IVM could be interpreted as an increase in autophagosome utilization, since at least in some cell types, LC3B-II is degraded when autophagosomes interact with the lysosome (Tanida et al. 2005). This inverse relationship between decreased LC3B-II abundance and increased ATG12-ATG5 complex formation could be explained by the fact that the ATG12-ATG5 complex detaches from the autophagosomal membrane after autophagosome formation is complete (Mizushima et al. 2001).

Autophagy and apoptosis are regulated in tight coordination, partly through BCL-2 family member proteins, which are important regulators of apoptosis during mammalian ovary development (Ratts et al. 1995; Hsu et al. 1996; Perez et al. 1999; Tanner et al. 2011; Basavarajappa et al. 2012). The dual role of BCL-2 family members to regulate both autophagy and apoptosis is mediated by the ability of BCL-2 and BCL2L1 to prevent apoptosis by inhibiting the formation of mitochondrial pores that release cytochrome C (Moldoveanu et al. 2014), or BCL-2 and BCL2L1 can interact with BECN1 to inhibit autophagy (Pattingre et al. 2005; Maiuri et al. 2007).

We have previously detected an increase in BCL2L1 protein abundance in the pig ovary due to HS, and immunostaining of sectioned ovarian tissue detected increased BCL2L1 localization in prophase I-arrested oocytes and primary follicles of ovaries from post-pubertal gilts subjected to HS (Hale et al. In Review). In this study the abundance of
BCL2L1 in MII arrested oocytes after IVM was not affected by HS, suggesting increased BCL2L1 abundance in the oocyte is dependent on other biological contributors. The mechanisms regulated by BCL2L1 in an oocyte during maturation is potentially still affected by HS, as BCL2L1 regulates autophagy or apoptosis through protein-protein interactions (Maiuri et al. 2007; Moldoveanu et al. 2014). We therefore characterized colocalization of BCL2L1 with either BAX or BECN1 to determine if HS can affect BCL2L1 protein interactions.

Colocalization of BCL2L1 with BAX remained constant in oocytes regardless of time point of IVM or temperature treatment, although, based on relative fluorescence signal, there did appear to be a higher abundance of BAX in oocytes collected after 21 hours of HS compared to TN oocytes. There was little to no colocalization of BECN1 with BCL2L1 in oocytes regardless of time point of IVM or temperature treatment, though HS appeared to induce more BECN1 protein. Based on the high degree of colocalization between BCL2L1 and BAX, as well as the fact this protein-protein interaction is well characterized in somatic cells (Mukhopadhyay et al. 2014), BCL2L1 may be inhibiting the release of cytochrome C in oocytes during IVM regardless of TN or HS conditions. The distinct lack of colocalization between BCL2L1 and BECN1 indicates that BCL2L1 is not inhibiting autophagy under the IVM conditions used in this study. This finding, coupled with the fact that HS affects abundance of autophagy-related proteins, suggests that HS is inducing autophagy in oocytes during maturation.

If autophagy has protective effects in terms of developmental competence on the oocyte, then artificially activating autophagy in oocytes undergoing HS should have a positive effect on meiotic maturation rate. Rapamycin induces autophagy in yeast (Noda
and Ohsumi, 1998) and mammalian cells (Blommaart et al., 1995), via the inhibition of the mechanistic target of rapamycin (mTOR; formerly mammalian target of rapamycin) complex 1 (mTOC1). It is well characterized that mTOR is a central regulator of cellular metabolism and cell fate (Shimobayashi and Hall, 2014). The mechanism of rapamycin inhibition of mTORC1 involves rapamycin forming a complex with FK506-binding protein (FKBP12), which binds directly to mTORC1 (Brown et al., 1994; Sabatini et al., 1994). While this mechanism is not completely understood, modeling of the rapamycin-FKBP12 complex bound to mTOR suggests rapamycin displaces the alignment of some mTORC1 substrates to the catalytic cleft (Yang et al., 2013; Shimobayashi and Hall, 2014).

Autophagosome-like structures have been previously detected in oocytes and granulosa cells (Lobascio et al., 2007; De Felici et al., 2008; Gannon et al., 2012), and rapamycin inclusion in IVM media has beneficial effects in pig (Song et al., 2014; Lee et al., 2015) and bovine (Song et al., 2012) oocytes. In this experiment, oocytes underwent IVM subjected to HS in the presence of rapamycin in the maturation media. The maturation rate of the oocytes in 10 or 100 nM rapamycin was not different from oocytes that matured in the presence of vehicle control, however, the maturation rate of oocytes in 1 nM rapamycin was increased compared to control. These results are similar to other experiments that characterize pig oocytes undergoing IVM in normal conditions in the presence of rapamycin (Song et al., 2014). The higher concentrations of rapamycin may have had no overt effect on maturation rate because too much inhibition of mTOC1 could be toxic, or at least not beneficial, to the oocyte. Since mTOR is a major regulator of nutrient sensing and cell fate decision (Shimobayashi and Hall, 2014), there could be a threshold that exists where the negative effects of inhibition of mTORC1 outweigh the
beneficial effects of autophagy induction. There could also be a threshold at which excessive induction of autophagy is detrimental to the oocyte, as autophagy and apoptosis regulation are tightly linked (Mukhopadhyay et al. 2014).

Embryonic development is dependent on oocyte competence, which is determined by the cytoplasmic contents of the oocyte (Krisher and Bavister 1998; Gosden 2002). HS is associated with reduced oocyte developmental competence and embryonic development (Isom et al. 2007; Bertoldo et al. 2010; Pennarossa et al. 2012). Autophagy is a potential mechanism that the oocyte could utilize during HS to recycle damaged cellular components. Herein we demonstrate that HS affected autophagy-related proteins in maturing oocytes, and that pharmacological induction of autophagy increased oocyte maturation during HS. These data add to the understanding of the components of oocyte viability, which is necessary for improving the efficiency of assisted reproductive techniques and developing environmental stress mitigation strategies to improve reproduction (Jurema and Nogueira 2006; Whyte and Prather 2011).

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Figure 4.1. Heat stress during the first half of IVM increases the abundance of ATG12 in complex with ATG5.
Figure 4.1. Heat stress during the first half of IVM increases the abundance of ATG12 in complex with ATG5.

Oocytes collected from aspirated pig follicular fluid underwent *in vitro* maturation (IVM) in either TN conditions throughout the entire 42-hour maturation period (TN/TN), HS conditions during the second half of IVM (TN/HS), or HS conditions during the first half of IVM (HS/TN). Representative western blots for BECN1, ATG12-ATG5 complex, BCL2L1, LC3B, and α-tubulin (A) from oocytes collected before IVM (GV) and after 42 hours of IVM. There was increased abundance of ATG12 in complex with ATG5 after 42 hours of IVM under the HS/TN temperature treatment compared to the TN/TN and TN/HS (B). Different superscripts within the same protein denotes significant difference ($P < 0.05$).
Figure 4.2. Heat stress exasperates the decrease of LC3-II in the oocyte.
Figure 4.2. Heat stress exasperates the decrease of LC3-II in the oocyte.

Oocytes collected from aspirated pig follicular fluid underwent *in vitro* maturation (IVM) in either TN conditions throughout the entire 42 hour maturation period or HS conditions during the first half of IVM (HS/TN). Representative western blot for LC3B from oocytes having undergone TN or HS conditions in IVM after 21 or 42 hours or collected before IVM (A). There was decreased abundance of cleaved LC3B-II at 21 hours of HS IVM compared with 21 hours of TN IVM (B). Asterisks denotes significant difference ($P < 0.05$) from control.
Figure 4.3. Colocalization of BAX and BCL2L1.
Figure 4.3. Colocalization of BAX and BCL2L1.

Oocytes that underwent either TN/TN IVM or HS/TN IVM were fixed and used for IHC to determine colocalization. Neither the time point during IVM nor the temperature treatment affected colocalization of antibody-labeled BCL2L1 and BAX in the oocyte. Based on level of fluorescence, there appeared to be a qualitative increase in BAX (red) at 21 hours of IVM under HS compared to 21 hours of IVM at TN conditions, though there appeared to be no effect on the abundance of BCL2L1 (green; A). The comparison between 21-hour TN (B), 21-hour HS (C), or negative control (D) shows that there was punctate yellow signal representing colocalization in both TN and HS oocytes. For A, the white bar in images in the left and middle columns represent 50 micrometers, and the white bar in images in the right column represents 20 micrometers. The white arrows point to areas of colocalization. The images in the right column are magnified 5× images from the same oocyte from the middle column.
Figure 4.4. Co-localization of BECN1 and BCL2L1.
Figure 4.4. Co-localization of BECN1 and BCL2L1.

Oocytes that underwent either TN/TN IVM or HS/TN IVM were fixed and used for IHC to determine co-localization. Colocalization of BECN1 (green) and BCL2L1 (red) was low regardless of time point during IVM or temperature treatment. There appeared to be more punctate intense spots of BECN1 staining in oocytes having undergone 21 hours of IVM under HS compared to the oocytes that underwent 21 hours of IVM at TN conditions (A). The comparison between 21-hour TN (B), 21-hour HS (C), or negative control (D) shows that there was punctate green signal representing BECN1 but no colocalization in both TN and HS oocytes. For A, the white bar in images in the left and middle columns represent 50 micrometers, and the white bar in images in the right column represents 20 micrometers. The white arrows point to areas of punctate BECN1 signal but no colocalization. The images in the right column are magnified 5× images from the same oocyte from the middle column.
Oocytes underwent IVM with the HS/TN temperature treatment in the presence of vehicle control, 100 nM, 10 nM, or 1 nM rapamycin. The inclusion of 100 nM or 10 nM rapamycin in the IVM media had no effect on maturation rate after HS/TN IVM compared to vehicle control, though oocytes matured in the presence of 1 nM rapamycin had increased maturation rate compared to vehicle control.
### Table 4.1. Oocyte maturation to MII arrest under different temperature treatments.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Total Oocytes Matured</th>
<th>Percentage MII Arrested Oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>TN/TN</td>
<td>269</td>
<td>67% ± 5.0³</td>
</tr>
<tr>
<td>HS/TN</td>
<td>242</td>
<td>33% ± 1.6²</td>
</tr>
<tr>
<td>TN/HS</td>
<td>315</td>
<td>44% ± 5.0³</td>
</tr>
</tbody>
</table>

¹Temperature treatment oocytes experienced during IVM: TN throughout the entire IVM (TN/TN) or HS during the first half (HS/TN) or second half (TN/HS) of IVM.  
²Total number of GV oocytes matured for each treatment from four replications.  
³Percentage of MII arrested oocytes from each treatment. Mean ± SEM.  
⁴Values with different superscripts in the same column are significantly different (P < 0.05)

### Table 4.2. Correlation coefficients indicative of BCL2L1 and BAX colocalization.

<table>
<thead>
<tr>
<th>Hour of IVM</th>
<th>Treatment</th>
<th>Pearson’s R³</th>
<th>Spearman’s R³</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>TN</td>
<td>0.75</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>TN</td>
<td>0.81</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>TN</td>
<td>0.76</td>
<td>0.76</td>
</tr>
<tr>
<td></td>
<td>HS</td>
<td>0.76</td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td>HS</td>
<td>0.74</td>
<td>0.76</td>
</tr>
<tr>
<td></td>
<td>HS</td>
<td>0.76</td>
<td>0.76</td>
</tr>
<tr>
<td>42</td>
<td>TN/TN</td>
<td>0.72</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>TN/TN</td>
<td>0.76</td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td>TN/TN</td>
<td>0.74</td>
<td>0.76</td>
</tr>
<tr>
<td></td>
<td>HS/TN</td>
<td>0.76</td>
<td>0.77</td>
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<td></td>
<td>HS/TN</td>
<td>0.74</td>
<td>0.76</td>
</tr>
<tr>
<td></td>
<td>HS/TN</td>
<td>0.76</td>
<td>0.76</td>
</tr>
</tbody>
</table>

¹Pools of at least 3 oocytes were collected after 0, 21, or 42 hours of IVM.  
²Oocyte experienced either TN throughout the entire IVM or HS during the first half of IVM (HS/TN).  
³The PDC colocalization plugin in the ImageJ processing program was used to calculate Pearson’s and Spearman’s correlation coefficient representing colocalization of BCL2L1 and BAX in individual oocytes. As per French et al. (2008), a Pearson’s R of 0.72 and a Spearman’s R of 0.63 is considered a high degree of colocalization.
**Table 4.3.** Correlation coefficients indicative of BECN1 and BCL2L1 colocalization.

<table>
<thead>
<tr>
<th>Hour of IVM&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Treatment&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Pearson's R&lt;sup&gt;3&lt;/sup&gt;</th>
<th>Spearman's R&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>0.31</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.16</td>
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<tr>
<td></td>
<td></td>
<td>0.29</td>
<td>0.33</td>
</tr>
<tr>
<td>21</td>
<td>TN</td>
<td>0.28</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>TN</td>
<td>0.35</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>TN</td>
<td>0.16</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>HS</td>
<td>0.29</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>HS</td>
<td>0.21</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>HS</td>
<td>0.47</td>
<td>0.53</td>
</tr>
<tr>
<td>42</td>
<td>TN/TN</td>
<td>0.31</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>TN/TN</td>
<td>0.30</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>TN/TN</td>
<td>0.36</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td>HS/TN</td>
<td>0.23</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>HS/TN</td>
<td>0.38</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>HS/TN</td>
<td>0.21</td>
<td>0.25</td>
</tr>
</tbody>
</table>

<sup>1</sup>Pools of at least 3 oocytes were collected after 0, 21, or 42 hours of IVM.

<sup>2</sup>Oocytes experienced either TN throughout the entire IVM or HS during the first half of IVM (HS/TN).

<sup>3</sup>The PDC co-localization plugin in the ImageJ processing program was used to calculate Pearson's and Spearman's correlation coefficient representing co-localization of BCL2L1 and BECN1 in individual oocytes. As per French et al. (2008), a Pearson’s R of 0.09 and a Spearman’s R of 0.15 is considered a low degree of colocalization.
CHAPTER 5: INHIBITION OF GERMINAL VESICLE BREAKDOWN USING IBMX INCREASES MICRORNA-21 IN THE PORCINE OOCYTE

A paper formatted for The Journal of the Society for Reproduction and Fertility

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Abstract

Germinal vesicle breakdown (GVBD) occurs during oocyte meiotic maturation, and precedes a period where transcriptional processes are virtually inactive until activation of the zygotic genome. Thus, the maturing oocyte is reliant on post-transcriptional gene regulation (PTGR) and/or interactions with the surrounding cumulus cells to regulate the mRNA repertoire and proteome prior to fertilization. MicroRNA (miRNA), a class of functional small RNA, interact with the 3’ untranslated region (UTR) of target mRNA to affect their abundance and translational efficiency. Of particular importance is miRNA-21 (MIR21) due to its role in regulating programmed cell death 4 (PDCD4), and ultimately inhibiting apoptosis. We have previously demonstrated an elevated abundance of MIR21 in metaphase II-arrested (MII) oocytes compared to immature oocytes with an intact germinal vesicle (GV), and that inhibiting MIR21 during oocyte maturation prevents PDCD4 degradation compared to control oocytes. The objective of this study was to characterize the abundance and regulation of MIR21 in relation to GVBD in the oocyte during in vitro maturation (IVM). GV stage oocytes were aspirated from 2 - 4 mm follicles and selected for IVM for 42 h. Relative abundance of mature MIR21 was quantified using quantitative reverse transcription PCR (qRT-PCR) at 0, 8, 16, 24, 32, and 42 hours of IVM with or without treatment with 3-isobutyl-1-methylxanthine (IBMX), a competitive nonselective phosphodiesterase inhibitor that prevents GVBD in oocytes. Culture with IBMX increased abundance of MIR21 at 24 hours approximately 6-fold compared to control oocytes ($P < 0.05$). To test whether IBMX induced MIR21 is able to regulate target mRNA, Western blotting was performed to detect relative changes in PDCD4 abundance at 0, 24, and 42 hours of IVM with or without IBMX treatment. IBMX induced increases in MIR21 abundance at 24 hours of IVM was concomitant with premature depletion of PDCD4 protein abundance in oocytes. Additionally, phosphorylated NF-κB, a potential regulator of MIR21, was increased in oocytes that underwent IVM in the presence of IBMX, compared to control oocytes after 24 hours in IVM media ($P < 0.05$). To characterize the effect of artificially increasing MIR21 on oocyte competence without inhibiting GVBD, a MIR21 mimic, scrambled microRNA negative control, or nuclease free water was micro-injected into denuded oocytes at 21 hours of IVM. The maturation rate of oocytes injected with either the microRNA negative control (40.3% ± 4.1%) or water (45.4% ± 2.2%) was not different ($P > 0.05$), while the maturation rate of oocytes injected with MIR21 (63.0 ± 7.5%) was higher than oocytes injected with either controls ($P < 0.05$). These data suggest that MIR21 is active and important during meiotic maturation of the oocyte.
**Introduction**

Maturation of the mammalian oocyte is a complex process involving internal checkpoints and bidirectional communication with the surrounding cumulus cells. The oocyte must maintain arrest at the diplotene stage until meiotic resumption occurs. During oocyte arrest at the diplotene stage there is continuous transfer of cyclic adenosine monophosphate (cAMP) to the oocyte through gap junctions (Dekel *et al.* 1981; Webb *et al.* 2002) and the level of cAMP is maintained within the oocyte as activation of G-protein coupled receptors (GPCR) on the plasma membrane activate adenylate cyclase (Masciarelli *et al.* 2004; DiLuigi *et al.* 2008). The regulatory role of cAMP in meiotic arrest is further illustrated by studies using selective phosphodiesterase 3A (PDE3A) inhibitors to block nuclear maturation (Tsafriri *et al.* 1996; Wiersma *et al.* 1998; Mayes and Sirard 2002; Lindbloom *et al.* 2008).

Following germinal vesicle break down (GVBD) the oocyte is transcriptionally quiescent until fertilization and activation of the embryonic genome, occurring at the four-cell stage of development in the pig (Oestrup *et al.* 2009). The inability to transcribe mRNA during this stage of development and the probable necessity for post-transcriptional gene regulation (PTGR) suggests an important role for non-coding RNA in the maturing oocyte. MicroRNA (miRNA) have been shown to be abundantly present in oocytes of multiple species during various stages of development (Watanabe *et al.* 2006; Watanabe *et al.* 2008; Tesfaye *et al.* 2009; Xu *et al.* 2011; Yang *et al.* 2012b; Abd El Naby *et al.* 2013).

Biogenesis of miRNA occurs in a step-wise manner starting with a double-stranded precursor (Lee *et al.* 2002; Denli *et al.* 2004; Lee *et al.* 2004). This double-stranded precursor, called the primary miRNA (pri-miRNA), is first cleaved by the DGCR8/Drosha
microprocessing complex, creating the second precursor form, the pre-miRNA (Gregory et al. 2004; Han et al. 2004). In somatic cells, the pre-miRNA is exported out of the nucleus by Exportin-5 (EXPO5) (Yi et al. 2003; Lund et al. 2004), where it is further processed by Dicer into the mature form (Hutvagner et al. 2001; Lau et al. 2001). The single-stranded mature miRNA can then associate with an Argonaute protein, which mediates its function of mRNA regulation through direct mRNA target cleavage or interactions with associated RNA-induced silencing complex (RISC) proteins (Filipowicz 2005).

Of particular interest is understanding the role of non-coding RNA in regulating meiotic checkpoints in the oocyte, particularly that of microRNA-21 (MIR21). The role for MIR21 in malignancy was first reported by Chan et al. (Chan et al. 2005), and is now known to be ubiquitously expressed in cancer cells (Li et al. 2012) due to the fact that MIR21 targets and inhibits pro-apoptotic proteins, programmed cell death 4 (PDCD4) and phosphatase and tensin homolog (PTEN) (Talotta et al. 2009; Zhou et al. 2010). Pri-MIR21 can be transcribed in coordination with the expression of vacuole membrane protein 1 (VMP1; formerly TMEM49), as MIR21 resides in the tenth intron of VMP1, although it appears MIR21 can be regulated independently as well due to a co-localized promoter within the VMP1 intron (Fujita et al. 2008).

MIR21 is a component of at least two feedback loops containing well characterized transcription factors, activator protein 1 (AP1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Talotta et al. 2009; Zhou et al. 2010). In one regulatory loop, MIR21 abundance is increased by AP1 activation, and subsequently interacts with the PDCD4 mRNA, causing its degradation (Talotta et al. 2009). Similarly, NF-κB has been shown to drive MIR21 expression directly (Zhou et al. 2010), culminating
in MIR21-targeted inhibition of PDCD4 or PTEN (Vinciguerra et al. 2009; Ruan et al. 2011).

We have previously demonstrated an elevated abundance of MIR21 in metaphase II-arrested (MII) oocytes compared to immature oocytes and that inhibiting MIR21 during oocyte maturation decreases the number of oocytes capable of reaching the MII arrest (Wright et al. 2016). Based on the apparent role of MIR21 in maintaining meiotic competence in the pig oocyte and that the oocyte undergoes a dramatic change in chromatin organization during meiotic maturation, we hypothesized that inhibition of GVBD would affect the abundance of mature MIR21 in the oocyte. MiRNA biogenesis is dependent on the transport of pre-miRNA out of the nucleus. We therefore predicted that the occurrence of GVBD in the oocyte potentially releases precursor miRNA out into the ooplasm and allows for further processing by Dicer to generate a mature miRNA. Our data suggest that this is likely not the case. The comparison of MIR21 abundance throughout *in vitro* maturation (IVM) with or without inhibition of GVBD suggests that miRNA biogenesis occurs before GVBD.

**Materials and Methods**

*In vitro maturation*

Sow ovaries were obtained from a local abattoir for isolation of cumulus-oocyte-complexes (COCs) for *in vitro* maturation (IVM) as previously described (Zhao et al. 2009; Yang et al. 2012a). Briefly, follicles (2-4 mm) were aspirated and COCs were collected and washed in TL-Hepes with 0.1% polyvinyl alcohol (PVA). COCs 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 at most 42 hours at 38.5 °C in 5% CO₂. Prior to IVM, an aliquot of germinal vesicle-intact (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 (5 to 7 minutes) in 1% hyaluronidase in TL-Hepes-PVA. COCs that were collected at 8, 16, 24, 32, or 42 hours of IVM were stripped of cumulus cells in the same manner.

**Utilization of IBMX to Inhibit Germinal Vesicle Breakdown**

Pools of approximately 70 COC were cultured under normal IVM conditions (as previously described) with media containing 1.0 mM IBMX or DMSO vehicle control. The concentration of 1.0 mM IBMX was selected based on a titration of 0.5 mM, 1.0 mM, or 2.0 mM of IBMX, for which the range was selected based on previous studies (Fan et al. 2002). Oocytes were stripped of cumulus cells as described above before initial culture (0 hour) or 8, 16, 24, 32, or 42 hours past initial culture. Denuded oocytes were collected based on the presence of an extruded polar body and morphologically healthy appearance, based on an appropriate zona pellucida integrity, the absence of ooplasm degradation, and proper perivitelline space. For each time point, oocytes randomly divided into two pools of 25; one pool was flash frozen for RNA extraction, and the other was fixed in 4% paraformaldehyde (PFA)/PBS at 4°C for 24 hours, as in previous studies (Yang et al. 2012c). Fixed oocytes were washed and mounted on slides with VectaShield mounting media containing DAPI stain (Vector Laboratories®, Burlingame, CA). Chromatin configurations were viewed for each of the 25 oocytes from vehicle control or IBMX containing culture media. Mounted oocytes were scored as either germinal vesicle-intact
(GV) or having undergone or undergoing GVBD. The percentage of either GV or GVBD was calculated for each time point.

**Injection of MIR21 mimic during IVM**

After approximately 21 hours of IVM, COCs were denuded in 0.5 mg/mL hyaluronidase with gentle vortexing. The oocytes were washed in manipulation media after cumulus cells were removed, and oocytes of good or excellent morphology were selected for injection. Injection occurred after 21 hours of IVM because injection of porcine GV-intact oocytes is more likely to be detrimental and cause oocyte demise (data not shown) and to mimic the artificial increase of MIR21 abundance due to IBMX between 16 and 24 hours of IVM. Denuded oocytes were injected using 0.7 µm diameter Femtotip®II tips (Eppendorf™, Hamburg, Germany) with 10 pL of nuclease-free water, microRNA mirVana™ negative control (Ambion™, Connecticut, USA), or mature 5´ MIR21 mimic (Integrated DNA Technologies, New Jersey, USA). Injected oocytes were cultured for an additional 21 hours. After a total of 42 hours of IVM, oocytes were assessed for their ability to achieve MII arrest based on the presence or absence of a polar body and a morphologically healthy appearance. Also at this time, 50 MII-arrested oocytes per treatment were pooled and flash frozen in liquid nitrogen. Oocytes were flash-frozen in liquid nitrogen and stored at -80°C for SDS-PAGE separation and Western blotting.

**Quantification of MIR21 abundance**

Oocytes were collected and denuded of cumulus cells as described above. Oocytes from each time point and treatment were collected in pools of exactly 25 oocytes with minimal amount of TL-Hepes/PVA. As previously established, a precise number of denuded oocytes were used per reaction to avoid the introduction of additional variation.
associated with reference genes (Yang et al. 2012a; Wright et al. 2016). TaqMan™ Gene Expression Cells-to-Ct™ Kit (Applied Biosystems, Carlsbad, CA) was used to lyse oocytes and prepare samples for quantitative real time polymerase chain reaction (qRT-PCR). Lysis solution and DNase from the Cells-to-Ct™ kit (Invitrogen™ Ambion™) were added to each pool at 4.95 and 0.05 µL, respectively, and incubated at room temperature for 5 minutes. Stop solution (0.5 µL) was added and the samples were incubated for an additional 2 minutes.

MIR21 was quantified using a TaqMan® MicroRNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA) for the reverse transcription (RT) reaction and the primers and probe used were TaqMan® MicroRNA Assay for hsa-MIR21 (Applied Biosystems, Carlsbad, CA) as per manufacturer’s recommendations. The RT reaction volume was 20 µL consisting of 13 µL master mix, 3 µL primers, and 4 µL sample lysate. Reverse transcription conditions were 16°C for 30 minutes, 42°C for 30 minutes and 85°C for 5 minutes. The final volume for all quantitative RT-PCR reactions was 20 µL, which included 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°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Fluorescent data acquisition was during the 60°C extension step.

**Western blot analysis**

Pools of 100 denuded oocytes within a replication were collected as described above for 0, 24 and 42 hours past initial culture with either DMSO loading control or 1.0 mM IBMX, and stored at -80°C until used for Western blot analysis. Oocyte pools were
lysed in 2.5 µL of 10% SDS (total sample volume 12.5 µL) at 95°C for 4 minutes followed by 1 minute on ice and centrifugation at 1000 rpm for 1 minute at room temperature. Samples were then loaded into a 4-20% Tris glycine gel (Lonza PAGEr® Gold Precast Gels). The BioRad Mini PROTEAN Tetra System was used to separate proteins at 60 volts for 30 minutes followed by 120 volts for 90 minutes. The proteins were transferred to a nitrocellulose membrane for 1 hour at 100 volts at 4°C. Membrane blocking was conducted using 5% milk in PBST (PBS with 0.5% Tween 20) for 1 hour at room temperature. A rabbit anti-PDCD4 monoclonal antibody (Abcam, ab79405), rabbit anti-JUN (pSer73; Calbiochem PhosphoDetect™ 420114), or rabbit anti-NF-κB RELA (pSer536 ThermoFisher Scientific™, MA5-15160) was added (1:1000 dilution) to the membrane in 0.5% milk in PBST overnight at 4°C. A negative control membrane lacking primary antibody was included to control for secondary antibody nonspecific binding. Following primary antibody incubation, the membranes were washed with PBST three times at room temperature for 10 minutes. Donkey anti-Rabbit IgG (Amersham™ ECL™ NA934) was incubated (1:2000) with the membrane for 1 hour at room temperature. The membrane was washed three times for 10 minutes each at room temperature. Horseradish peroxidase substrate (Millipore, Billerica, MA) was added to the membrane for 1 minutes in the dark. The membrane was exposed to x-ray film and developed for visualization. Average pixel intensity for the protein corresponding to 52 kDa for PDCD4 molecular weight, 45 kDa for corresponding molecular weight of JUN phosphorylated at Ser73, and 64 kDa corresponding molecular weight of NF-κB subunit RELA was conducted using Image Studio™ Lite (Li-Core®). Alpha-tubulin was used as a loading control to normalize oocyte Western blot data.
Statistical Analysis

Statistical analysis of oocyte maturation rate, qRT-PCR relative RNA abundance, and Western blot data was conducted using PROC MIXED in SAS® (Carry, NC), where a standard student t-test was used to compare statistical differences. Statistical significance was determined when $P$ values were less than or equal to 0.05. Analysis of oocyte maturation rate after injection utilized repetition number as a covariate to account for differences between repetitions.

Results

IBMX Inhibition of Oocyte Nuclear Maturation Increases MIR21 Abundance

To compare the increase of MIR21 abundance directly to the percentage of oocytes undergoing GVBD, oocytes were scored as GV-intact, GVBD, or post GVBD at 8 hour intervals during IVM (Fig 5.1A and B). There was a marked increase in the occurrence of GVBD between 0 and 16 hours of IVM, by which approximately 63% of the oocytes had undergone GVBD (Fig 5.1B).

To characterize the inhibition of GVBD under different concentrations of IBMX, vehicle control, 0.5 mM, 1.0 mM, or 2.0 mM IBMX was added to IVM media, and oocytes were collected at 0, 24, and 42 hours of IVM to be scored as either GV-intact or having undergone GVBD. As expected, all three concentrations of IBMX in IVM media inhibited GVBD (Fig 5.1C).

Compared to the percentage of GV-intact oocytes after IVM in the presence of 0.5 mM IBMX (68.0% ± 12.9%), there was a decreased percentage of oocytes with an intact GV after 42 hours of IVM with 1.0 mM IBMX (89.3% ± 5.9%; $P = 0.047$) and a tendency
for decreased GV-intact oocytes with 2.0 mM IBMX (88.0% ± 2.8%; \( P = 0.06 \)). The inclusion of 2.0 mM IBMX did not further decrease the number of GV-intact oocytes, compared to oocytes that underwent IVM in the presence of 1.0 mM IBMX (\( P = 0.894 \)). For all further experiments 1.0 mM IBMX was used.

To assess potential mechanisms contributing to the increase of MIR21 occurring in the maturing oocyte, IBMX was used to inhibit the nuclear maturation of oocytes in IVM conditions by preventing GVBD. The IBMX supplemented IVM media contained the hormonal signals and nutrients necessary for cytoplasmic maturation. The inclusion of 1.0 mM IBMX increased the abundance of MIR21 (29.6 ± 1.5-fold) by 24 h of IVM compared to DMSO loading control (4.5 ± 1.8-fold; \( P < 0.01 \); Fig 5.2A). There was a subsequent decrease in PDCD4 protein (0.75 ± 0.05 relative band intensity) in oocytes under IBMX inhibition at 24 h compared to control (1.2 ± 0.04 relative band intensity; \( P = 0.03 \); Fig 5.2B). This suggests that IBMX inhibition of GVBD increased the abundance of MIR21 in the oocyte, which increased the MIR21-driven decrease in PDCD4 abundance.

**NF-κB activation corresponds with increased MIR21 abundance**

Transcription factors contributing to the regulation of MIR21 have been well characterized in somatic cells, particularly via AP1 or NF-κB (Talotta *et al.* 2009; Vinciguerra *et al.* 2009). The induced MIR21 abundance above the level of control IVM oocytes, via IBMX, was hypothesized to be temporally associated with increased activated AP1 and NF-κB. Western blotting was used to detect activated AP1 (phosphorylated JUN) or activated NF-κB (phosphorylated RELA) at 0, 24, or 42 hours with or without IBMX. A minimal amount of phosphorylated JUN was detected in 0 hour GV-intact oocytes,
though not at later time points regardless of IBMX treatment (Fig 5.3A). The 24-hour time point for phosphorylated JUN is not shown, as only two replications were performed due to limited oocyte quantity, but of the two replications no phosphorylated JUN was detected.

An increase in phosphorylated RELA was observed at 24 hours of oocytes in IBMX IVM media (1.0 ± 0.21 relative band intensity) compared to oocytes in IVM media with DMSO vehicle control (0.39 ± 0.17 relative band intensity; $P = 0.02$; Fig 5.3B). Due to this increase in NF-κB activation is at a time point where artificially increased mature MIR21 abundance was also seen, NF-κB is potentially driving MIR21 expression in the oocytes.

**Injection of MIR21 at 21 hours of IVM increases oocyte maturation**

After 21 hours of IVM, oocytes were denuded and injected with either a MIR21 mimic, microRNA scrambled negative control, or nuclease-free water and allowed to continue IVM until a total of 42 hours. The IVM oocytes were injected at 21 hours to mimic the increase in MIR21 abundance observed in control oocytes by 24 hours of IVM (Fig 5.2A). The time point of 21 hours was chosen because it was prior to the observed increased in MIR21, so that it can be utilized for target mRNA inhibition at or earlier than 24 hours of IVM. Injections were not done earlier than 21 hours of IVM as it was a concern that exogenous single-stranded RNA would be degraded if unutilized (Montreau et al. 2004); though degradation of exogenous RNA was not specifically quantified in this study.

Maturation rate for each treatment was scored via the presence of a morphologically healthy metaphase II arrested oocyte containing an extruded polar body. The injection of MIR21 increased the maturation rate of oocytes (61.6% ± 0.75%) compared to the injection of either scrambled microRNA (40.3% ± 0.02%; $P = 0.004$) or water (45.4% ± 0.4%; $P =$
The oocyte maturation rate was not different between the two negative controls ($P = 0.15$; Fig 5.4A). Artificial increase of MIR21 abundance at 21 h of IVM caused a subsequent decrease in PDCD4 abundance at 42 h of IVM ($0.02 \pm 0.01$ relative band intensity), compared to injection of either scrambled microRNA ($0.14 \pm 0.6$ relative band intensity; $P < 0.01$) or water ($0.10 \pm 0.03$ relative band intensity; $P = 0.04$; Fig 5.4B). These results indicate that MIR21 is active and affects meiotic maturation in the porcine oocyte.

**Discussion**

MIR21 is an anti-apoptotic factor that is ubiquitously found in cancer cells (Li *et al.* 2012). This is in part due to its ability to target and suppresses pro-apoptotic proteins, including PDCD4 and PTEN (Talotta *et al.* 2009). Predictions using TargetScan v6.2 suggest there are currently 382 predicted MIR21 targets identified in the human genome (Lewis *et al.* 2005). Besides targets regulating apoptosis, MIR21 also has the potential to regulate pathways associated with cell cycle arrest, suppression of tumor growth, and chromosome assembly (Yang *et al.* 2009; Li *et al.* 2012). We have previously discovered increased MIR21 abundance in MII porcine oocyte compared to immature oocytes (Yang *et al.* 2012a), and that inhibition of MIR21 function in the maturing oocyte increased PDCD4 abundance and decreased developmental competence (Wright *et al.* 2016). Furthermore, increased MIR21 in the maturing potentially originates from the oocyte and not solely from the surrounding cumulus cells (Wright *et al.* 2016). Due to the important role of MIR21 in regulating cell cycle decisions in somatic cells, and its abundance in the
maturing oocyte, it is likely that MIR21 is involved in meiotic maturation in the female germ cell.

In this project, IBMX, a phosphodiesterase inhibitor, was used to prevent GVBD during IVM of oocytes to test the hypothesis that GVBD affects MIR21 abundance. In somatic cells, miRNA are first transcribed as primary miRNA (pri-miRNA), which form secondary RNA structures producing 60-75 nucleotide (nt) hairpins (Lee et al. 2002; Lee et al. 2004). The canonical miRNA hairpins are recognized by DGCR8 (DiGeorge syndrome critical region 8), which directs the RNase III enzyme Drosha to cleave the base of the hairpin resulting in a pre-miRNA (Denli et al. 2004; Gregory et al. 2004; Han et al. 2004). This pre-miRNA structure, composed of a hairpin with a 3′ overhang is exported out of the nucleus via EXPO5 (Yi et al. 2003; Lund et al. 2004) and then further processed by Dicer in complex with transactivating response element RNA-binding protein (TRBP), resulting in a functionally mature miRNA (Lau et al. 2001; Lee et al. 2002; Castellano and Stebbing 2013; Kim et al. 2014; Wilson et al. 2015).

During maturation, the oocyte experiences a period following GVBD where the nuclear material is no longer encapsulated by the nucleolus, potentially affecting microRNA biogenesis due to the reduced requirement of EXPO5 to facilitate pre-miRNA out of the nucleus. Further supporting this concept is that EXPO5 mRNA is relatively lowly expressed in the maturing pig oocyte relative to the other molecules known to affect canonical miRNA biogenesis (Whitworth et al. 2005). Based upon this knowledge, we developed our hypothesis that inhibition of GVBD could do one of two things: 1) decrease the abundance of mature MIR21, since immature MIR21 would be sequestered within the GV, or 2) increase the abundance of mature MIR21, since the oocyte would have a
prolonged ability to transcribe immature MIR21. To decipher this, nuclear maturation of the oocytes was inhibited through IBMX and the abundance of MIR21 was determined.

IBMX is a nonspecific phosphodiesterase inhibitor which inhibits adenylate cyclase within the oocyte (Soderling and Beavo 2000). This prolongs the elevated levels of cAMP in the oocyte and obstructs oocyte nuclear maturation (Schultz et al. 1983; Luciano et al. 1999). Though the process of GVBD and the inhibition of GVBD via IBMX has been well characterized in the pig oocyte during IVM (Fan et al. 2002; Sun et al. 2004; Laforest et al. 2005), the approach used herein enabled the temporal characterization of MIR21 changes in abundance and GVBD at 8-hour intervals.

In control oocytes, mature MIR21 was elevated by 24 hours of IVM and remained elevated until 42 hours. Interestingly, oocytes matured in the presence of IBMX had much greater MIR21 than control oocytes at 24 hours of IVM. This 24 hour time point corresponds to when approximately 75% of control oocytes undergo GVBD which is synonymous with transcriptional quiescence (Sirard 2012). The greater abundance of MIR21 in IBMX oocytes compared to control oocytes at 24 hours of IVM could be explained by the fact that 75% more oocytes in the IBMX treatment are maintaining the ability to produce nascent RNA. This increase of MIR21 abundance between 0 and 24 hours of IVM could be important for maintaining oocyte viability during maturation.

The IBMX-induced increase of MIR21 at 24 hours of IVM allowed for a model where the upstream regulation of MIR21 transcription could be detected. The transcription factors that drive MIR21 in somatic cells are well characterized (Fujita et al. 2008; Talotta et al. 2009; Vinciguerra et al. 2009; Hu et al. 2013). The MIR21 promoter contains enhancer elements for AP1, Ets family transcription factor PU.1 (Ets/PU.1), CCAAT-
enhancer-binding protein α (C/EBPα), and signal transducer and activator of transcription 3 (STAT3) (Talotta et al. 2009).

We therefore characterized the abundance of phosphorylated JUN, indicating activated AP1 complex, and phosphorylated RELA, indicating activated NF-κB complex (Sakurai et al. 1999), in oocytes during IVM with or without IBMX. Despite observing detectable levels of phosphorylated JUN in oocytes collected before introduction into IVM media, it was substantially lower during IVM.

The abundance of activated NF-κB, as detected by phosphorylated RELA, was increased in control oocytes at 24 hours of IVM although not statistically significant. However, there was a significant increase in activated NF-κB at 24 hours of IVM in oocytes in the presence of IBMX compared to control oocytes. This observation of IBMX induced activation of NF-κB corresponds to the substantial increase of MIR21 in oocytes undergoing IVM in the presence of IBMX and implicates the potential involvement of NF-κB in mediating MIR21 transcription in the oocyte.

Generally, genes whose transcription is controlled by NF-κB participate in the regulation of apoptosis, cell adhesion, proliferation, innate- and adaptive-immune responses, and cellular stress response (Perkins 2007). In the oocyte, the transcription of the mRNA for the inhibitor of NF-κB alpha (NFKBIA) is decreased in MII oocytes compared to GV stage oocytes in the mouse (Paciolla et al. 2011), suggesting that NF-κB activity increases during maturation. This increase in NF-κB activity is consistent with the numerical increase of phosphorylated RELA in control oocytes observed in this study. The results presented here suggest that increased MIR21 transcription may be in part the result
of NF-κB activation in the maturing oocyte functioning to decrease pro-apoptotic signals prior to GVBD.

We further aimed to characterize the importance of the MIR21 increase from 0 to 24 hours of IVM. If this increase is paramount to maintaining viability, then artificially increasing MIR21 before 24 hours of IVM without inhibiting GVBD could increase the maturation rate of IVM oocytes. To characterize the effect of artificially increasing MIR21 on oocyte competence without inhibiting GVBD, a synthesized MIR21 mimic or controls, were micro-injected into the oocyte at 21 hours of IVM. In comparison to the control oocytes, the maturation rate of oocytes injected with MIR21 mimic was increased. This further indicates the biological machinery available for MIR21-mediated PTGR in the oocyte is active. Injection of the MIR21 mimic decreased the protein abundance of the MIR21 target, PDCD4, as we predicted based on previous studies (Wright et al., 2016). These results suggest that MIR21 is active within the porcine oocyte and that it has a role in regulating oocyte competence during maturation.

By taking a targeted approach, we have previously shown that inhibition of a specific miRNA, MIR21, in the oocyte decreases oocyte maturation (Wright et al. 2016). Here we characterize the increase of MIR21 in relation to GVBD, and how the effect of artificially increasing MIR21 increases oocyte maturation rate. These cumulative results suggest a functional role of miRNA in porcine oocyte maturation. Using conditional knock-out models, it has been suggested that miRNA function is repressed during oocyte maturation in rodents (Ma et al. 2010) and our findings suggest some potential species differences with respect to miRNA function.
The cumulative results of this study fortify the argument that miRNAs are active and important for meiotic maturation of the female germ cell. After GVBD the oocyte is transcriptionally quiescent until after fertilization and activation of the embryonic genome (Oestrup et al. 2009). The level of nascent mRNA produced after GVBD is almost nonexistent, and the oocyte must rely on PTGR and other mechanisms to regulate the dynamic processes involved in chromosomal migration as well as react to cellular or environmental stress. MiRNA is potentially a mechanism the oocyte utilizes to enact PTGR.

Morphological markers for the integrity of immature oocytes are imperfect at predicting whether the oocyte contains the necessary material to mature and undergo fertilization (Coticchio et al. 2015). MiRNA potentially represent more informative markers of oocyte viability, and increasing the understanding of the components necessary for oocyte viability is necessary for improving the efficiency of assisted reproductive techniques (Jurema and Nogueira 2006; Whyte and Prather 2011).
Literature cited


Figure 5.1. Quantification of Germinal Vesicle Breakdown during *In Vitro* Maturation.
Figure 5.1. Quantification of Germinal Vesicle Breakdown during *In Vitro* Maturation. Cumulus-oocyte-complexes (COCs) were aspirated from 2 to 4 mm follicles and allowed to undergo *in vitro* maturation (IVM). COCs were collected at 8 hour intervals, denuded, fixed, and the chromatin was stained with DAPI. Oocytes were scored as containing an intact germinal vesicle (GV) or undergoing or have undergone germinal vesicle breakdown (GVBD; A). GVBD increased rapidly from 8 to 24 hours of IVM, where by 24 hours of IVM approximately 75% of the oocytes collected were scored as GVBD (B). Vehicle control, 0.5 mM, 1.0 mM, or 2.0 mM IBMX was added to IVM media, and oocytes were collected at 0, 24, and 42 hours of IVM to be scored at either GV-intact or having undergone GVBD. All three concentrations of IBMX inhibited GVBD. White scale bars represent 25 microns.
Figure 5.2. Inhibition of GVBD Increased MIR21 Abundance.
Figure 5.2. Inhibition of GVBD Increased MIR21 Abundance. Collected COCs underwent IVM in the presence of a vehicle control or 1.0 mM IBMX, and then collected and denuded at 8 hour intervals. The oocytes that underwent IVM in the presence of IBMX had approximately 30-fold greater MIR21 abundance at 24 hours of IVM compared to control oocytes (A). This increase of MIR21 at 21 hours of IVM was temporally associated with decreased PDCD4 abundance (B) suggesting the increased MIR21 due to IBMX prevented GVBD is biologically active within the oocyte. Superscripts denote significant difference ($P < 0.05$).
Figure 5.3. Increased Abundance of MIR21 Corresponds with Activation of NF-κB.
Figure 5.3. Increased Abundance of MIR21 Corresponds with Activation of NF-κB.

To characterize the potential transcription factors that could be driving transcription of MIR21, oocytes underwent IVM in the presence of a vehicle control or 1.0 mM IBMX. While little to no phosphorylated JUN, part of the AP1 complex, was seen after the 0 hour time point (A), there was an increase in phosphorylated RELA, a subunit of NF-κB, after 24 hours of IVM in the presence of IBMX compared to control (B). This suggests that NF-κB could be driving MIR21 transcription in the oocyte. Superscripts denote significant difference ($P < 0.05$).
Figure 5.4. Injection of MIR21 Increases Maturation Rate.

A

![Bar Chart](image)

Maturation Rate

Water | NC | MIR21

Injection Treatment

B

![Image](image)

Relative Signal

NC | H2O | MIR21

Injection Treatment
Figure 5.4. Injection of MIR21 Increases Maturation Rate. Collected COCs underwent IVM for 21 hours, when they were then denuded and injected with either scrambled miRNA negative control (NC), water loading control (water), or synthetic mature MIR21. The oocytes were then placed back into IVM media until a total of 42 hours. Injection of MIR21 increased maturation rate compared to oocytes injected with either control (A). Oocytes injected with MIR21 had decreased PDCD4 compared to controls, though all three treatments had decreased PDCD4 compared to oocytes collected before IVM (B).
CHAPTER 6: CONCLUSIONS

The purpose of the experiments described in this dissertation were to characterize two molecular processes that are potential components involved in maintaining developmental competence: autophagy induction in response to heat stress (HS) in the ovary (Chapter 3) and the oocyte (Chapter 4), as well as microRNA-21 (MIR21) in the maturing oocyte (Chapter 5).

Heat stress induced autophagy induction in the ovary

The detrimental effects of heat stress (HS) create an annual financial burden on animal agriculture [1, 2], and HS is well-known to compromise follicular and early embryonic development in a variety of animal models and agriculturally important species [3-7]. Heat stress has been associated with reduced oocyte developmental competence, and induces apoptosis in in vitro fertilized (IVF) and parthenogenetically-activated porcine embryos [8-10].

Since HS is known to be toxic to cells [11], we hypothesized that the ovary and oocyte utilize autophagy to mitigate HS-induced cellular damage. Autophagy is a mechanism by which dysfunctional intracellular machinery is disposed of, and is known to be activated by a variety of stressors [12]. Little is known concerning autophagy in the ovary and oocyte and we therefore studied the effect of HS on autophagy-related proteins and autophagy induction in the ovary and oocyte.

To characterize the effects of HS on autophagy in the ovary, an in vivo model was utilized where gilts were synchronized to the follicular stage of the estrous cycle and then
exposed to 5 days of cyclical HS. After the 5 days of HS, the ovaries were collected and used to visualize morphology of the ovary and abundance of autophagy-related proteins.

A drastic difference in cell morphology of follicular cells and oocytes between ovaries from gilts that experienced 5 days of HS compared to ovaries of gilts that experienced 5 days of TN conditions was observed. As observed through transmission electron microscopy, HS qualitatively increased the incidence of vacuolization of the oocytes and granulosa cells, observed as spherical white spaces, compared to TN oocytes and granulosa cells. The granulosa cells (GC) surrounding oocytes from HS ovaries had increased incidence of sequestered cytoplasm of smaller diameter while the oocytes from HS ovaries contained large vacuole-like structures. Strikingly, there appeared to be a distinct loss of interaction between the oocyte and granulosa cells of follicles from HS gilts. This is likely detrimental to the oocyte, as the oocyte is dependent on the GC to maintain proper regulation and competence of the oocyte. Interestingly, there was no effect of HS on tertiary follicle number or tertiary follicle diameter. This suggests that HS is potentially more detrimental on early stage follicles than later gonadotropin dependent stage follicles. Future studies characterizing follicle loss in the pig due to HS are warranted, either through in vivo experiments or ovarian culture assays.

The abundance of autophagy-related proteins was compared between total ovarian lysate from either TN or HS gilts. There was an effect of HS on the abundance of autophagy-related proteins, with increased beclin 1 (BECN1), increased microtubule associated protein 1 light chain 3 (LC3B)-II, and decreased autophagy related protein (ATG)-12 in complex with ATG5. The increased abundance BECN1 and LC3B-II due to HS, coupled with the increased vacuole-like structures, suggest that HS induces autophagy
in the ovary. Though, it was unexpected that the abundance of ATG12 in complex with ATG5 was decreased. This discrepancy could be due to differences in dynamics between ubiquitin-like conjugation pathways in autophagosomal extension [13].

The *in vivo* model used in Chapter 3 accurately depicts the effects of HS on the ovary in terms of whole animal physiology, but a limitation of this model is that in order to compare multiple time points, an increased number of animals would be required, along with increased costs and demands of the experiment. We did not specifically characterize autophagic flux, or the turn-over of autophagosomes and their contents, in Chapters 3 or Chapters 4. However, by examining the differences in abundance of autophagy-related proteins in the ovary, the data presented in Chapter 3 suggests that HS induces autophagy in the ovary.

*Heat stress induced autophagy induction in the oocyte*

We then wanted to investigate if HS could induce autophagy specifically in the maturing oocyte before fertilization, as HS has been associated with reduced oocyte developmental competence [9]. To determine the effects of HS on induction of autophagy in the oocyte, *in vitro* maturation (IVM) was utilized to characterize autophagy-related proteins under different temperature treatments and different time points (Chapter 4). The use of IVM allowed us to detect changes in autophagy-related proteins under different temperature treatments and time points throughout oocyte maturation.

The HS model used in Chapter 4 is based off previous work from our group [14]. Wright et al. used similar temperature treatments during IVM (HS/TN and TN/HS), and maturation rate was decreased by the HS/TN temperature treatment but not the TN/HS
treatment during IVM [14]. This differs from the data presented in Chapter 4, as both temperature treatments decreased maturation rate, but the same trend was observed: the HS/TN temperature treatment was more detrimental to maturation rate than the TN/HS temperature treatment. However, Wright et al. have also shown that of the oocytes that were able to reach the metaphase II (MII) arrest under HS temperature treatments, and were fertilized via IVF, the oocytes that matured under the TN/HS temperature treatment had decreased blastocyst rates compared to oocytes that matured under the HS/TN temperature treatment. This suggests that even though oocytes appear to be morphologically healthy and have reached the MII arrest necessary for fertilization, there is still HS derived damage that decreases their developmental competence, as seen by decreased blastocyst development rate.

This model allowed us to characterize the effects of HS on autophagy induction directly in the oocyte. The ability of the oocyte to reach meiotic maturation and undergo fertilization to produce an embryo is dependent on processing the required molecular machinery within the ooplasm. It is known that HS is toxic to cells and increases protein misfolding as well as apoptosis in cells [11]. Therefore, the decreased maturation rate and developmental competence in oocytes caused by HS is potentially brought about by damage at the level of protein or organelle stability. Therefore, autophagy a potential mechanism for the oocyte to mitigate these detrimental effects of HS by degrading misfolded proteins and organelles.

The abundance of autophagy-related proteins was compared between oocytes that had undergone IVM during TN/TN, TN/HS, or HS/TN temperature treatments. There was increased abundance of ATG12 in complex with ATG5 in all three temperature treatments
compared to oocytes collected at 0 hour before IVM, and a further increase in oocytes that had experienced the HS/TN temperature treatments. There was no difference between the three temperature treatments in the abundance of LC3B-II, but treatments had decreased abundance of LC3B-II compared to oocytes collected at 0 hour.

This sharp decline in LC3B-II in oocytes after IVM was further characterized by comparing the abundance of LC3B-II in oocytes that had either experienced IVM under the TN/TN or HS/TN temperature treatments at 0, 21, or 42 hours of IVM. The decrease in LC3B-II abundance was exasperated by HS. The comparison of increased ATG12-ATG5 complex formation with decreased LC3B-II abundance is interesting, as the ATG12-ATG5 complex dissociates from the autophagosomal membrane before interaction with the lysosome, while LC3B-II incorporated directly in the autophagosomal membrane is degraded [15]. Also, the cleavage of LC3B-I to form LC3B-II is dependent on the formation of the ATG12-ATG5 complex [16]. Therefore, the decreased abundance of LC3B-II at 21 hours of IVM under HS is potentially due to increased autophagic flux.

Autophagy and apoptosis are regulated in tight coordination [17]. One mechanism by which both pathways are regulated is the BCL2 family member proteins. This regulation occurs through protein-protein interactions, with BCL2 or BCL2L1, interacting with BECN1 to inhibit autophagy or interacting with BAX to inhibit apoptosis [18-20]. In the in vivo model utilized in Chapter 3, HS increased the abundance of BCL2L1 in oocytes and somatic cells of primary follicles. This led us to characterize the effects of HS on the interactions of BCL2L1 with either BAX or BECN1 in oocytes during IVM through colocalization. While there appeared to be a high degree of colocalization between BCL2L1 and BAX with a low degree of colocalization of BCL2L1 and BECN1 in the
oocytes, this was independent of temperature treatment or time point of IVM. This suggests that BECN1 is not inhibited by BCL2L1 in the oocyte during IVM. Whether apoptosis is being inhibited by BCL2L1 is less conclusive, as we did not specifically look at the ratio of BAX to BCL2L1. BCL2L1 is likely interacting with BAX based on colocalization, and if the abundance of BAX reaches a certain point, apoptosis will still occur, as mitochondrial pores will be able to form and release cytochrome C [21]. The data presented in Chapter 4 strongly suggests that future studies concerning how BCL2L1 regulates the interplay between autophagy and apoptosis in the oocyte are warranted. It is likely that this mechanism is part of the process in determining whether the oocyte will be able to mature and maintain developmental competence.

If autophagy has protective effects on the oocyte, then chemically activating autophagy in oocytes undergoing HS should have a positive effect on meiotic maturation rate. Rapamycin has been shown to induce autophagy in yeast [22] and mammalian cells [23], via the inhibition of mammalian target of rapamycin (mTOR) complex 1 (mTOC1). It is well characterized that mTOR is a central regulator of cellular metabolism and cell fate [24] and acts as an inhibitor of autophagy [25, 26].

The maturation rate of the oocytes in 10 or 100 nM rapamycin was not different from oocytes that matured in the presence of vehicle control, while the maturation rate of oocytes in 1 nM rapamycin was increased compared to control. These results are similar to other experiments that characterize pig oocytes undergoing IVM in normal conditions in the presence of rapamycin [27]. The higher concentrations of rapamycin may have had no overt effect on maturation rate because too much inhibition of mTOC1 could be toxic to the oocyte. Since mTOR is a major regulator of nutrient sensing and cell fate decision [24],
there could be a threshold that exists where the negative effects of inhibition of mTORC1 outweigh the beneficial effects of autophagy induction.

**MicroRNA-21 abundance and function during germinal vesicle breakdown**

The second mechanism characterized in this dissertation is the function of MIR21 in relation to the release of condensed chromatin from the nuclear envelope, or germinal vesicle. Following germinal vesicle break down (GVBD) the oocyte is transcriptionally quiescent until fertilization and activation of the embryonic genome, occurring at the four-cell stage of development in the pig [28]. The inability to transcribe mRNA during this stage of development and the potential necessity for post-transcriptional gene regulation (PTGR) suggests an important role for non-coding RNA in the maturing oocyte. MicroRNA (miRNA) have been shown to be abundantly present in oocytes of multiple species during various stages of development [29-34].

Though miRNA are expressed abundantly in the oocyte, it has been shown that Dgcr8 is not required for the maturing mouse oocyte, and loss of DGCR8 has no noticeable effect on mRNA regulation [35]. Loss of both Dicer and argonaute 2 (AGO2) does have an observable effect on oocytes and both are required for healthy oogenesis in mice [35-38]. This has led to the hypothesis that endogenous short interfering RNA (endo-siRNA) have a more critical role in oocyte development and maturation rather than miRNA. It is surprising, that everything required for miRNA regulation is present in the oocyte, but miRNA would have no function [39].

We therefore predicted that the occurrence of GVBD in the oocyte potentially releases precursor miRNA into the ooplasm and allows for further processing by Dicer to
generate a mature miRNA. During maturation, the oocyte experiences a period following GVBD where the nuclear material is no longer encapsulated by the nucleolus, potentially affecting microRNA biogenesis due to the reduced requirement of exportin-5 (EXPO5) to facilitate pre-miRNA out of the nucleus. Further supporting this concept is that EXPO5 mRNA is lowly expressed in the maturing pig oocyte relative to the other molecules known to affect canonical miRNA biogenesis [40]. Knowing that led to the hypothesis that inhibition of GVBD could do one of two things: 1) decrease the abundance of mature MIR21, since immature MIR21 would be sequestered within the GV, or 2) increase the abundance of mature MIR21, since the oocyte would have a prolonged ability to transcribe immature MIR21. To decipher this, nuclear maturation of the oocytes was inhibited through 3-isobutyl-1-methylxanthine (IBMX) and the abundance of MIR21 was determined.

In control oocytes, mature MIR21 was elevated at 24 hours of IVM and remained elevated until 42 hours. Interestingly, oocytes matured in the presence of IBMX had much greater MIR21 abundance than control oocytes at 24 hours of IVM. This 24 hour time point corresponds to when approximately 75% of control oocytes undergo GVBD which is synonymous with transcriptional quiescence [41]. There was a subsequent decrease in programmed cell death protein 4 (PDCD4) protein in oocytes under IBMX inhibition at 24 hour compared to control. This suggests that IBMX inhibition of GVBD increased the abundance of MIR21 in the oocyte as well as that MIR21 is active and interacting with target mRNA.

The elevated abundance of MIR21 in IBMX oocytes compared to control oocytes at 24 hours of IVM could be explained by the fact that 75% more oocytes in the IBMX
treatment are maintaining the ability to produce nascent RNA. This increase of MIR21 abundance between 0 and 24 hours of IVM could be important for maintaining oocyte viability during maturation.

The transcription factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), has been shown to drive MIR21 expression directly [42], culminating in MIR21-targeted inhibition of PDCD4 or phosphatase and tensin homolog (PTEN) [43, 44]. The abundance of activated NF-κB, as detected by phosphorylated RELA, was increased in control oocytes at 24 hours of IVM although not statistically significant. However, there was a significant increase in activated NF-κB at 24 hours of IVM in oocytes in the presence of IBMX compared to control oocytes. This observation of IBMX induced activation of NF-κB corresponds to the substantial increase of MIR21 in oocytes undergoing IVM in the presence of IBMX and implicates the potential involvement of NF-κB in mediating MIR21 transcription in the oocyte.

We further wanted to characterize the importance of the MIR21 increase from 0 to 24 hours of IVM. If this increase is paramount to maintaining oocyte viability, then artificially increasing MIR21 before 24 hours of IVM without inhibiting GVBD could increase the maturation rate of IVM oocytes. To characterize the effect of artificially increasing MIR21 on oocyte competence without inhibiting GVBD, a synthesized MIR21 mimic or control, were micro-injected into the oocyte at 21 hours of IVM. In comparison to the control oocytes, the maturation rate of oocytes injected with MIR21 mimic was increased. This further indicates the biological machinery available for MIR21-mediated PTGR in the oocyte is active. Injection of the MIR21 mimic decreased the protein abundance of the MIR21 target, PDCD4 as we would have predicted based on previous
studies (Wright et al., 2016). These results suggest that MIR21 is active within the porcine oocyte and that it has a role in regulating oocyte competence during maturation.

Conclusions

In conclusion, this body of work has provided novel insights to how the ovary and oocyte maintain developmental competence. HS affects the abundance of autophagy-related proteins in the ovary and oocyte, as well as the cellular morphology. The data presented herein suggests that HS results in autophagy induction in the ovary and oocyte. Additionally, characterizing a specific miRNA, MIR21, in the oocyte in relation to GVBD has filled a knowledge gap concerning the function of miRNA in the oocyte. Since MIR21 is functional and important for regulating events that relate to oocyte competence, future studies characterizing miRNA in the oocyte are warranted.
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