DISSERTATION

LUTEINIZING HORMONE INDUCED OOCYTE MATURATION INITIATES mRNA DECAY IN CATTLE

Submitted by

David Joshua Walker

Department of Biomedical Sciences

In partial fulfillment of the requirements

for the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2010

COLORADO STATE UNIVERSITY

December 21, 2009

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY DAVID JOSHUA WALKER ENTITLED LUTEINIZING HORMONE INDUCED OOCYTE MATURATOIN INITIATES mRNA DECAY IN CATTLE BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate work

Colin M Clay

Jason E Bruemmer

Russell V Anthony

Advisor: George E Seidel, Jr.

Acting Department Head: Thomas R Hansen

ABSTRACT OF DISSERTATION

LUTEINIZING HORMONE INDUCED OOCYTE MATURATION INITIATES mRNA DECAY IN CATTLE

Oocyte maturation is a complex process consisting of signal transduction, ultrastructural changes, and mRNA transcription, translation, storage, and degradation. In vitro-matured oocytes initiate maturation in response to removal from an inhibitory follicular environment while in vivo-matured oocytes mature in response to the LH surge. Oocytes matured in vivo lead to more successful embryo production than those matured in vitro. This research concerned study of expression levels and action of selected transcripts involved in RNA processing that occur in in vivo oocyte maturation.

The first experiment focused on the inability of GnRH to induce oocyte maturation in superstimulated cows during the luteal stage of the estrous cycle. Superstimulated cows were treated with PGF2α and GnRH to induce in vivo maturation or were treated with GnRH without PGF2α to induce an LH surge at 0, 3, 12, or 24 h before aspiration. While treatment with GnRH caused an increase in LH over no GnRH treatment, it was a smaller increase than that observed in cows treated with PGF2α before GnRH treatment ($P<0.001$) (No GnRH: 0.84 ng/mL, GnRH: 9.45 ng/mL, PGF2α: 93.86 ng/mL). Thus, increases in LH were sufficient to initiate epiregulin mRNA transcription in granulosa cells ($P<0.06$) with the greatest expression levels after 6h. However, germinal vesicle breakdown did not occur as reliably in cows with an intact corpus luteum 23 h after GnRH (treated with PGF2α 36 h prior to GnRH injection) (GV stage

oocytes; 0h: 79%, 6h: 58%, 12h: 83%, 24h: 60%, vs. controls 6%)($P<0.001$). In addition, cAMP levels remained stable in oocytes from cows treated with GnRH in the presence of progesterone regardless of post injection time, while control oocytes had a slightly elevated level of cAMP (0h: 4.95, 6h: 3.98, 24h: 4.12, control: 7.41 fmol) ($P>0.1$). Phosphodiesterase 3A mRNA levels were unaffected by any treatment ($P>0.10$). These data suggest that although the stimulatory signaling of LH and epiregulin occur, cAMP levels are unaffected by GnRH treatment in the presence of progesterone.

The second experiment evaluated mRNA concentrations in bovine oocytes of four transcripts involved in RNA regulation in mammalian cells; CUG-BP, PARN, eIF-4E, and PAP-1. In vivo- and in vitro-matured oocytes were collected 0, 3, or 6 hours after initiation of maturation via GnRH injection for in vivo-maturation and aspiration from follicles for in vitro-maturation. eIF-4E and PARN mRNA concentrations increased over time in both in vitro- and in vivo-matured bovine oocytes ($P<0.05$). In vivo-matured oocytes contained more eIF-4E mRNA molecules than in vitro-matured bovine oocytes ($P<0.10$). CUG-BP and PAP-1 concentrations remained stable over the first 6 h of maturation and were similar in the in vivo- and in vitro-matured oocytes.

The final experiment concerned deadenylation patterns for the cyclin B1 3' untranslated region (UTR) and GDF-9 3'UTR with a poly(A) tail of 60 adenosines. Bovine oocytes were injected with radiolabeled constructs after 0, 5, or 19 h of maturation and then cultured for 0, 1, or 3 h. Radiolabeled RNA was recovered from oocytes after culture and analyzed for changes in construct size, reflective of deadenylation or general degradation. Cyclin B1 underwent deadenylation before being degraded regardless of the stage of oocyte maturation. Analysis of gels showed an

intermediate with 0 adenosines (A0) in the Cyclin B1 injected oocytes, while those injected with GDF-9 displayed no such intermediate. These findings indicate that injected GDF-9 transcript remains stable with a poly(A) tail of 60 adenosines or is simply degraded randomly in bovine oocytes matured for 0, 5, or 19 h. Deadenylation of Cyclin B1 mRNA begins immediately in bovine oocytes resulting in degradation of the Cyclin B1.

David Joshua Walker
Department of Biomedical Sciences
Colorado State University
Fort Collins, CO 80523
Spring 2010

Acknowledgements

There are numerous people I would like to thank. I spent 5 years at Colorado State University and have made many friendships that will last a lifetime. First, I would like to thank my family for all of their support. My parents have always stood beside me during any decisions I have made, good or bad. I appreciate all of the sacrifices they have made for me over the years. I also need to thank my wife, Jennifer. She has been an integral part of my Ph.D. and without her I would have never finished up. She was always there for me during the good and bad experiences of our lives together. My children, Joshua and Julie make my life worth living and mean the world to me. I would also like to thank my advisor Dr. George Seidel, Jr. He has been instrumental in my professional development and has really changed the way I think about things. Without his advice and training, I would not be where I am today. I would also like to thank the graduate students and other faculty members. I spent most of my time in other labs and used equipment, materials, and intellectual property of almost every person at the Animal Reproduction Laboratory during my time here. I would also like to thank John Anderson and Dr. Carol Wilusz for helping me understand molecular techniques. Before I met them, my understanding of this side of research was extremely elementary. I greatly appreciate all of their input. All of these people mentioned above have been a part of my degree and I greatly appreciate their help.

Table of Contents

Chapter I

Review of Literature

Introduction

Mammalian oocytes are routinely recovered from donors and matured, fertilized, and cultured in vitro to produce embryos for transfer into the uteri of recipients. In cattle, some 240,000 commercial in vitro-produced embryos were transferred worldwide in 2005 (Thibier, 2006). In vitro-produced embryos are also used to circumvent certain infertility pathologies in human reproductive clinics, with an estimated 2,000,000 live births being attributed to IVF embryos though 2004 (Rinehart, 2005). In addition to commercial production, research groups produce millions of embryos in vitro annually for use in various experiments. These numbers could be much larger with improved methods of production. Current methods for in vitro-production of embryos remain inefficient and expensive, with less than half of the oocytes recovered developing into a transferable quality blastocyst, and less than half of those developing to term.

Primarily for research purposes, bovine oocytes from slaughterhouse-derived ovaries are aspirated from 3-8 mm diameter follicles. The cumulus-oocyte complexes recovered from these follicles result in about 30% blastocysts/oocyte when matured, fertilized, and cultured in vitro. Blastocyst development can be increased to 50% if oocytes are matured in vivo and recovered from >8mm follicles before being fertilized and cultured in vitro (Rizos et al., 2002). The different developmental rates of in vivo- and in vitro-matured oocytes have been documented in several studies (van de Leemput et al., 1999; Hendriksen et al., 2000); however, the reasons for these differences are not clearly understood. Current research has focused on understanding which molecules

stimulate oocyte maturation and what events occur after the initiation of maturation before the second meiotic arrest and subsequent fertilization. The following review will focus on mechanisms of oocyte maturation in cattle, with information from other species where appropriate.

Reproductive Hormones and Their Roles in Oocyte Maturation

The following section will address the roles of the main hormones involved in regulation of the female estrous cycle and their effects in vitro. Although their roles in controlling estrous cycles have been defined, their effects during in vitro-maturation are poorly understood. They are often included in maturation media simply because they are present in follicular fluid.

FSH

Follicle stimulating hormone (FSH) has an important role in folliculogenesis. Its secretion by the anterior pituitary controls growth of follicles from 3 mm to preovulatory size, about 15 mm in cattle (Sirois and Fortune, 1988). Exogenous FSH can induce superstimulatory effects on the ovary. Treatment of animals with FSH increases the number of follicles that can ovulate or be aspirated. This technique is commonly used in the field of bovine embryo transfer to increase the number of ova collected during a uterine flush or a transvaginal aspiration (Elsden et al., 1978).

In addition to its use for superstimulation of ovaries, recombinant FSH is also often included in oocyte maturation medium (Ali and Sirard, 2002). FSH is found in follicular fluid of heifers at a concentration of roughly 0.5 ng/mL (Fortune and Hansel, 1985). However, little is known about the influence of FSH on oocyte maturation. Ali and Sirard (2002) showed that addition of recombinant FSH with estradiol 17β to

chemically defined medium increased oocyte competence by increasing blastocyst yield from 19% to 40%. In 2005, they showed that culture of oocytes with recombinant FSH for the first 6 h of oocyte maturation increased blastocyst yield to 45%, whereas culture with recombinant FSH for 24 h (the traditional approach to oocyte maturation) yielded only 28% blastocysts. A study by Izadyar et al. (1998) also showed that simple addition of FSH to maturation medium could increase blastocyst development in vitro. Their results showed that FSH reduced the number of metaphase II (MII) oocytes present 16 h after induction of maturation, while blastocyst development was actually improved. These findings suggest that FSH aids cytoplasmic maturation by delaying nuclear maturation in vitro. Still, the mechanism of action of FSH in oocyte maturation remains controversial. Studies have linked FSH to activation of both the PKA and PKC signaling cascades.

Su et al. (1999) demonstrated that inhibiting protein kinase C and its related pathway blocked FSH-induced stimulation of oocyte maturation in pigs. Aberdam and Dekel (1985) showed that activation of PKC increased oocyte maturation in rat cumulus cell-enclosed oocytes. Although these results may suggest that PKC is the pathway used by FSH, similar results have been obtained when studying effects of FSH on cAMP and PKA. Eppig and Downs (1984) showed that oocytes treated with FSH in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methyl xanthine (IBMX) had more cAMP than oocytes not treated with FSH. They proposed that FSH aids in inhibiting oocyte maturation by maintaining cAMP, although they were unable to demonstrate significant changes in cAMP concentrations in the oocyte itself. In 1987, Eppig and Downs contradicted their earlier studies showing that FSH could induce oocyte maturation in

murine oocytes arrested with hypoxanthine during the final oocyte growth phase. Although Eppig's studies suggest a role for FSH, likely acting though a cAMP/PKA pathway, they failed to consistently prove that FSH plays a role in oocyte maturation. Again Eppig et al. (1998) showed that FSH increased cAMP production. They also showed that FSH treatment of growing cumulus oocyte-complexes in vitro increased LH receptor expression in granulosa cells. However; little change in embryonic development was observed when bovine oocytes were matured in the presence or absence of FSH (Walker et al., 2003). We also were unable to repeat results shown by Ali and Sirard (2002) in which oocytes were matured with FSH for only 2, 6, or 23 h (unpublished data). One explanation for the discordance among these studies is contamination of most FSH preparations with LH (Lynch et al., 1988).

Although actions of FSH on granulosa cells to increase cAMP production within those cells identifies FSH as a candidate for induction of oocyte maturation, FSH concentrations in follicular fluid and plasma remain fairly stable throughout the estrous cycle, with only a slight increase in follicular concentrations of FSH occurring during estrus (Fortune and Hansel, 1985). With a constant signal being applied in vivo, it is unlikely that FSH is responsible for inducing oocyte maturation. Also, results have been inconsistent regarding the true effect of FSH on oocyte maturation and subsequent embryonic development. However, FSH may be able to mimic in vivo signals in vitro when applied in a timely fashion, as was seen with mice in Eppig and Downs study from 1987 in which increasing cAMP in cumulus cells could have led to PKA activation and downstream phosphorylation events that will be discussed later. Ali and Sirard (2005) provide further evidence that timing of FSH treatment in vitro may be critical for its

action in stimulating oocyte competence as determined from the results that exposure of bovine oocytes to recombinant FSH for the initial 6 h of maturation. More work is needed to determine what effect if any FSH has in vitro.

LH

Unlike FSH, LH concentrations increase dramatically as the cow enters estrus (Fortune and Hansel, 1985). The preovulatory LH surge is responsible for inducing ovulation and luteinization (Channing et al., 1980) in vivo. The timely increase of LH corresponds with maturation of oocytes and suggests that LH is a key player in oocyte maturation. In fact, researchers have settled on the preovulatory LH surge as the trigger for oocyte maturation in vivo (Baker, 1982). Once the preovulatory surge occurs, oocytes arrested in the first prophase of meiosis I resume meiotic cell divisions and progress to metaphase II where they again arrest and await fertilization. The exact mechanism for initiating resumption of meiosis has not been clearly identified, although it appears that LH acts on thecal cells to induce intermediate signals that bind receptors on cumulus granulosa cells (Park et al., 2004).

Like FSH, LH is a common ingredient in maturation medium due to its presence in follicular fluid. And like FSH, LH is believed to play a role in inducing oocyte maturation. Interestingly, however, LH receptors are not present on cumulus cells. Instead, LH receptor mRNA is expressed at very low concentrations in immature rat granulosa cells. Concentrations were upregulated by treatment of rats with eCG (Peng et al., 1991). Eppig et al. (1998b) demonstrated increased expression of LH receptor in granulosa cells of mice cultured for 10 days in vitro in the presence of FSH. These studies demonstrate that FSH may "prime" preovulatory follicles for LH induction of

oocyte maturation by increasing the number of LH receptors present in granulosa cells for response to the LH surge. Molecular events of LH signaling that mediate the initiation of oocyte maturation will be discussed later.

Estradiol 17 β

Estradiol 17β is produced by the granulosa cells of growing follicles. Its classical role in the estrous cycle is stimulation of GnRH release to induce the LH surge once a follicle has reached the appropriate size for ovulation, about 15 mm in the cow. Estradiol has been used in several studies to regulate follicle wave synchrony during estrus synchronization (Lane et al., 2000; Gouveia Nogueira et al., 2002; Cavalieri et al., 2006).

Although not clearly understood, injection of estradiol (17β, benzoate, or valerate) in the luteal phase of cattle, often at the time of insertion of progesterone releasing device, induces follicle turnover, ensuring that a new dominant follicle will be ready for ovulation once the progesterone releasing device is removed and prostaglandin F2α is administered. Studies suggest that the dominant follicle simply undergoes atresia, allowing the next dominant follicle to take over later during the cycle due to inhibition of LH (Bo et al., 1993; Burke et al., 1999; Martinez et al., 2005). Bo et al. (1993) suggested that estradiol stops follicular growth of the dominant follicle and subsequently delays the spike in follicle stimulating hormone responsible for initiating the next follicle wave. Regardless of whether estradiol was given to heifers with growing dominant follicles or 15 mm dominant follicles (preovulatory size in cattle), follicular atresia eventually occurred and a new wave emerged. The difference between the two groups with different size dominant follicles was the timing from estradiol treatment to new wave emergence. Regardless, estradiol 17β acted as a potent regulator of follicle growth in their studies.

Estradiol 17β is present in follicular fluid during proestrus at 1000 ng/mL (Fortune and Hansel, 1985). Therefore, it is often included in maturation medium. Ali and Sirard (2002) showed that concentrations of estradiol 17β of 1000 ng/mL significantly improved blastocyst development over lower concentrations, suggesting that estradiol 17β plays a role in oocyte maturation. Although multiple authors have shown a beneficial effect of estradiol 17β in terms of blastocyst development (Younis et al., 1989; Ali and Sirard, 2002), little research has been done to investigate the mechanism responsible for this improvement.

Progesterone

Progesterone suppresses the endogenous LH surge. The mechanism for blocking LH release likely involves regulation of GnRH. Administration of progesterone decreases GnRH mRNA levels and receptor numbers in the anterior pituitary (Sakurai et al., 1997). In addition, progesterone downregulates production of GnRH mRNA in the brain of heifers in the luteal phase (Weesner et al., 1993). By preventing release of GnRH in response to increasing estradiol from follicles from the first or second follicular wave, progesterone regulates when the cow will ovulate a developmentally competent oocyte.

Although progesterone can block the endogenous LH surge via blocking the GnRH surge, injection of exogenous GnRH can bypass this regulation, inducing the release of LH. This is the basis of many estrus synchronization protocols. Use of an inserted progesterone releasing device blocks endogenous GnRH, preventing ovulation in cows that are in the follicular phase of the estrous cycle and having little effect on cows in the luteal phase. Atkins et al. (2008) recently showed that the LH response to GnRH is

dependent on the stage of the reproductive cycle in beef cattle. Their study showed that injection of GnRH between day 5 and 18 of the cycle resulted in increased LH production within 30 min of injection. This study shows that injection of GnRH in the presence of progesterone, either from the corpus luteum or a progesterone releasing device, results in a LH surge.

Although progesterone is present in follicular fluid at concentrations of 20 ng/mL, it is often not included in maturation medium. Results from studies examining the effect of progesterone in vitro have been mixed. While Sirotkin (1992) and Karlach (1986) showed beneficial effects of progesterone with increased incidence of maturation in bovine oocytes but little effect on blastocyst rates, Smith and Tenney (1980) and Silva and Knight (2000) showed that progesterone inhibited maturation in murine and bovine oocytes. These conflicting results have done little to convince researchers to include progesterone in medium for bovine in vitro-maturation.

Prostaglandin F2α

As discussed previously, progesterone inhibits the endogenous production of GnRH. Large luteal cells, which are responsible for the majority of progesterone production, contain Prostaglandin (PG) F2α receptors (Fitz et al. 1982). PGF2α binds large luteal cells to induce regression of the corpus luteum. As the corpus luteum regresses, circulating progesterone levels decline. The decline in progesterone permits GnRH release, LH release, and eventually ovulation. Injection of PGF2α is useful for controlling the estrous cycle in females. Animals injected with PGF2α after day 5 of the estrous cycle usually exhibit estrus within a few days and can then be bred in a synchronized fashion.

PGF2α levels increase markedly in the follicular fluid within 24 h of the LH surge to a concentration of about 0.3 ng/mL (Algire et al., 1992). In vitro, cumulus-oocyte complexes produce 0.7 ng/mL PGF2α during maturation and continue producing PGF2α following fertilization for up to 48 h (Gurevich et al. 1993). Addition of PGE2 to fertilization medium increased cleavage rates from 53% to 81% in bovine oocytes (Gurevich et al., 1993). Viggiano et al. (1995) showed that murine cumulus-oocyte complexes produce PGF2α during the same period identified in bovine oocytes by Gurevich et al. (1993). They suggest that prostaglandins from the cumulus-oocyte complex increase the sperm's ability to penetrate oocytes in vitro, possibly though stimulation of the acrosome reaction.

Effect of Follicle Size on Oocyte Competency

Follicle size can have profound effects on the success of embryo production. In cattle, oocytes obtained from follicles smaller than 2 mm in diameter yielded 0 blastocysts, whereas oocytes from 2-4 mm follicles resulted in 21% blastocysts, and oocytes from 4-8 mm follicles resulted in 29% blastocysts (Pavlok et al. 1992). As antral follicles increase in size, oocytes increase in diameter. Bovine oocytes reach their full diameter, 110 μm, once the follicle reaches 3 mm in diameter (Fair et al., 1995). Although oocytes from 3 mm follicles obtain developmental competence at 110 μm, they still develop at lower rates than oocytes from larger follicles. Oocytes recovered from follicles larger than 6 mm in diameter result in twice as many blastocysts as those from 3 mm follicles (Lonergan et al., 1994). Oocytes reach maximal developmental competence once the follicle reaches 8 mm in diameter (Hendriksen et al. 2000). Although oocytes with a diameter of 110 μm are developmentally competent, developmental differences

observed when culturing oocytes from varying sizes of follicles greater than 3 mm indicate that oocyte diameter is not the primary factor in oocyte competence, but instead events that occur during and after the plateau in growth.

Differences in development between oocytes from follicles less than 3 mm and those larger than 3 mm are likely due to differences in mRNA content. As the oocyte increases in diameter, a maternal pool of mRNA is transcribed and stored for use during early embryonic development. Transcription levels decline markedly once the oocyte becomes meiotically competent, at a diameter of 110 μm. Recovering oocytes before the decrease in transcription yields oocytes that do not have sufficient material to successfully complete meiosis I, while isolation of bovine oocytes from 3 mm follicles results in an oocyte that is capable of resuming meiosis and producing a transferable embryo. Yet, these oocytes with intact maternal pools of mRNA still develop less often than oocytes collected from larger follicles. This phenomenon likely occurs because once an oocyte reaches 110 μm and transcription levels fall to near 0; specific, timely events occur as the follicle grows to preovulatory size (13-15 mm in cattle). These changes have been referred to as oocyte capacitation (Hyttel et al., 1997).

In Vitro Maturation of Oocytes

The preovulatory surge of luteinizing hormone induces the oocyte to resume meiotic cell divisions within the follicle, permitting ovulation of an oocyte arrested at metaphase II of the second meiotic cell division until fertilization. In vivo-maturation is often referred to as induced maturation due to the need for a triggering event within the follicle, the LH surge. Although timing of maturation events of bovine oocytes have not been studied thoroughly; in vivo, ovulation occurs around 28 h after the LH surge. Using

superstimulation and timed administration of GnRH and prostaglandin F2α, an average of 80% MII oocytes can be recovered with ovum pick up (Callesen et al., 1986). These oocytes are in vivo-matured and when exposed to in vitro-fertilization and culture, will result in blastocyst production rates of 50% per oocyte or greater (Rizos et al., 2002). Although such embryos can be produced, superstimulation is expensive, and transvaginal guided ovum pick up is somewhat difficult and time consuming.

When in vitro-maturation is coupled with in vitro-fertilization and in vitro-culture, embryo production becomes much less efficient. Oocytes matured in vitro reach the blastocyst stage at a rate of only about 30%. To induce in vitro maturation, simply removing the cumulus-oocyte complex from the follicle is sufficient (Pincus and Enzmann, 1935). Therefore, in vitro-maturation is called spontaneous maturation. The major advantage of using in vitro-maturation stems from the ability to collect oocytes from slaughterhouse-derived ovaries and then aspirate follicles to recover thousands of oocytes each day without the cost of live cattle. Even when coupled with live cattle and superstimulation, oocytes not matured in vivo are easier to work with because their cumulus masses are less expanded and less sticky.

However, Van de Leemeutt et al. (1999) proved that in vivo-matured oocytes developed into an embryo at twice the rate of in vitro-matured oocytes. Furthermore, the study by Rizos et al. (2002) showed that maturation was of primary importance for subsequent blastocyst production rates by examining all phases of in vitro-embryo production. They matured oocytes in vivo and in vitro and then fertilized and cultured them in vitro. They also fertilized in vivo-matured oocytes in vitro and in vivo before using in vitro culture to produce embryos. Finally, they cultured in vitro-matured and

fertilized oocytes and in vivo matured and fertilized oocytes both in vitro and in vivo. Their results showed that the method of maturation had the largest impact on blastocyst production. This study has only solidified thinking in the field that the oocyte maturation is key in developmental competence. The fundamental difference in the two forms of maturation likely stems from events occurring early in maturation.

Oocyte Capacitation

Oocyte capacitation is a term applied to events that take place once the oocyte has reached its full size and prepares to respond to a signal for resumption of meiosis (Hyttel et al. 1997). Most of the changes observed during oocyte capacitation are ultrastructural, including decreases in Golgi complex size, increased size of the perivitelline space, vacuolization of the nucleolus, increased folding of the nuclear membrane, and clustering of the cortical granules (Hendriksen et al. 2000). In addition to structural changes seen in the oocyte, some transcriptional events occur during capacitation and maturation (resumption of meiosis) (Crozet et al., 1986). Limited transcription during this time likely completes the oocyte's stock of maternal mRNA and proteins for successful early development. As the follicle reaches preovulatory diameter, the oocyte progressively obtains competence though mRNA transcription, protein production, and ultrastructural modifications; however, production of a fully competent oocyte requires even more signals than those developed during folliculogenesis and capacitation.

Initiating Signaling Events of Oocyte Maturation

In a normal estrous cycle, the corpus luteum regresses, dropping progesterone levels and allowing the LH surge if estradiol 17β levels are high. LH then binds receptors on the mural granulosa cells and induces production of the EGF-like factors

amphiregulin and epiregulin (Johnson et al., 1993; Park et al., 2004; Lindbloom et al. 2008). Binding of amphiregulin and epiregulin to the EGF receptor on cumulus cells activates a tyrosine kinase cascade responsible for producing signals that lead to maturation. These signals are passed though gap junctions that exist between the cumulus cells and the oocyte (Vozzi et al., 2001). Once in the oocyte, the signals likely set into motion a series of events that lead to oocyte maturation. Oocyte maturation has been divided into thee phases: nuclear, cytoplasmic, and molecular maturation (Sirard et al., 2003).

Cytoplasmic Maturation

Nuclear maturation occurs in more than 80% of in vivo- and in vitro-matured oocytes (Sirard et al., 1989), yet the majority of those oocytes do not develop into blastocysts. Therefore, scientists have defined another phase of maturation, cytoplasmic maturation to explain some of the variation in embryo production. Cytoplasmic maturation consists mainly of ultrastructural changes in addition to those seen in capacitation, as the oocyte prepares itself for the first meiotic cell division and subsequent fertilization. These events include further decrease in Golgi size over that seen in oocyte capacitation, accumulation of lipids for energy, cortical granule migration for the polyspermy block, and the appearance of numerous ribosomes for protein synthesis (Hyttel et al., 1997). Incomplete cytoplasmic maturation could cause the deficiencies observed with in vitro-matured oocytes. Thus, many researchers have studied ways to delay in vitro nuclear maturation to allow sufficient time for cytoplasmic maturation to proceed (Franz et al., 2003).

Nuclear Maturation

Nuclear maturation occurs as the oocyte progresses though meiosis I. During this time, the nucleus of the oocyte, known as the germinal vesicle, breaks down. This event is commonly referred to as germinal vesicle breakdown (GVBD). GVBD has become a benchmark for evaluation of nuclear maturation completion along with subsequent extrusion of the first polar body.

After the LH surge, cAMP levels in the oocyte decrease, activating maturation promotion factor (MPF). Decreased PKA activity as a result of decreased cAMP allows dephosphorylation of Cdc25, which can in turn dephosphorylate CDK1, a component of MPF (Eppig et al., 2004). Dephosphorylated CDK1 contributes to activating MPF. Active MPF plays a role in condensing chromosomes, initiating microtubule formation, and inducing migration of the microtubules to the poles of the oocyte spindle for metaphase I (Kotani and Yamashita, 2002).

Decreased cAMP is also associated with the activation of MAPK (Fan and Sun, 2004). MAPK's role in activation of oocyte maturation is extremely important, but not yet fully understood (Su et al., 2002). MAPK is known to aid formation of spindles for cell division and in activation of cyclin B, a component of MPF (Fan and Sun, 2004). MAPK may also be involved in increasing transcription of specific genes during meiosis I.

Molecular Maturation

A third aspect of maturation, molecular maturation, has recently been described (Sirard et al., 2003). This form of maturation is also the least understood, although some of the components of molecular maturation have been defined. These transcriptional and

translational events which occur late in oocyte maturation are likely key factors in producing competent oocytes.

Although very limited, both transcription (Moore and Lintern-Moore, 1978; Kastrop et al., 1991a; Memili and First, 1999) and translation (Simon et al., 1989; Kastrop et al., 1991a; Kastrop et al., 1991b; Memili and First, 1999) occur after the oocyte reaches its full diameter and in the early embryo before the maternal-zygotic transition. The mRNA and proteins produced during this time are important components of successful oocyte maturation and subsequent embryo development. Corcoran et al. (2006) showed that oocytes removed from their follicular environment before completion of all aspects of maturation and placed into an in vitro system had altered expression of mRNA of genes involved in translation and transcription compared to embryos produced in vivo. Without proper expression of genes involved in regulating transcription and translation, proteins such as cyclin B1 that are translated from mRNA stores (Tay et al., 2000; Robert et al., 2002) will not be produced when they are needed, or at the levels necessary for efficient maturation.

Gene Expression in Mammalian Oocytes

With oocyte maturation identified as the critical step in determining overall success of embryonic development, recent research has focused on gene expression in oocytes. Specifically, which genes are upregulated after induction of maturation by LH (in vivo-maturation) or after spontaneous maturation (in vitro-maturation). The recent development of microarray technology combined with arrays designed to cover the bovine genome or the embryonic genome has allowed researchers to more closely examine overall gene expression. This has increased the rate of discovery of potential

candidates involved in developmental competence by allowing study of hundreds of genes at a time instead of focusing on one or two.

A major problem with microarray data stems from the amount of information produced. Most papers published simply provide lists of genes that are differentially expressed between treatments (Dalbies-Tran and Mermillod, 2003). These lists are long and often labeled with nomenclature specific to the microarray, making it difficult to pull out specific genes from these studies. However, development of computer programs to link genes to cell function has increased the usefulness of information from microarray studies. It is now possible to determine what key pathways are up or down regulated with different treatments. This information is very useful as differences in developmental competence likely are not due to a specific gene, but instead changes in expression of many genes and deregulation of specific pathways.

Current microarray analysis shows that the bovine oocyte expresses mRNA for about 23,000 genes (Fair et al. 2007). Of these 23,000 genes, Fair et al. (2007) showed that 821 genes were differentially expressed after maturation when compared to immature oocytes. Many of the genes identified by Fair et al. (2007) are involved in regulation of MAPK, transcription, and translation. Corcoran et al. (2006) also used microarray analysis to compare in vitro and in vivo-cultured bovine embryos. Similarly to Fair et al. (2007), Corcoran et al. (2006) showed that many of the genes differentially expressed were involved with regulation of transcription and translation. These experiments suggest that many differences in gene expression observed by other microarray studies may be due to deregulation or over regulation of mRNA production (transcription) or mRNA processing including translation. Deregulation of the maternal

pool of mRNA could alter gene expression levels in oocytes. This is especially important during early embryonic development when the oocyte uses the maternal pool of mRNA for determining when to produce specific products.

Maternal Pool of mRNA

As bovine oocytes grow to a final diameter of about 110 μm, they produce mRNA that is stored for use during oocyte maturation and early embryonic development until the 8-16 cell stage when the embryonic genome is activated. Most of the RNA and proteins required by early embryos are produced during the stages of follicular and oocyte growth. Once oocytes reach their full size, transcription decreases to basal levels. Memili et al. (1998) showed that immature oocytes display ^{35}S-UTP uptake above background levels, which was used to measure new RNA production. Isolation of the mRNA and measurement of radioactivity show to what extent new RNA has been produced. Memili et al. (1998) showed that low levels of transcription occurred in immature oocytes compared to those of mature oocytes or 4 cell embryos. Furthermore, western blot analysis showed that immature oocytes contain levels of RNA polymerase IIA comparable with germinal vesicle and 2-cell embryos (Memili and First, 1998). RNA polymerase isoform IIA is an unphosphorylated isoform that is involved in initiation of transcription (Dahmus, 1983; Corden et al., 1985). These studies support the hypothesis that RNA is transcribed throughout oocyte maturation and likely during the time needed for the follicle to grow from 3 mm (the size when the oocyte reaches its full diameter) to a diameter of 15 mm (preovulatory size) in cattle. These final transcripts likely are involved in early regulation of cell cycle kinetics and possibly RNA regulation.

Once an oocyte has transcribed the maternal pool of mRNA, it must regulate this pool of mRNA and proteins amassed during growth. Lequarre et al. (2004) measured all mRNA containing poly(A) tails in oocytes before and after maturation. They found that the total amount of poly(A) RNA decreased over the maturation period. Research has proven that the overall levels of mRNA and poly(A) tail length decrease until the maternal-zygotic transition (Lequarre et al., 2004), yet studies focusing on individual genes have revealed a more complex regulation. Some genes undergo polyadenylation during maturation while others undergo deadenylation (Brevini-Gandolfi et al., 1999; Brevini et al., 2002). Regulation of the poly(A) tail is critical for determining RNA fate.

mRNA Fate

Once transcribed and exported to the cytoplasm, RNA has to be processed. This processing comes in the form of translation, storage, or degradation. Which pathway RNA is processed though depends heavily on poly(A) tail length (Garneau et al. 2007). Most mRNA molecules consist of a 5' untranslated region (UTR) with a cap, an open reading frame (ORF), and a 3' UTR containing a poly(A) tail. Extension of the poly(A) tail often results in increased translatability of RNA and increased protein production. Intermediate poly(A) tail length results in storage and stability of the RNA, while shortening of the poly(A) tail results in degradation. Poly(A) tail length is regulated by interaction between proteins and the 3' UTR. mRNA stability in mammalian oocytes likely is regulated by mechanisms similar to those in somatic cells.

Translation of maternal mRNA

mRNA provides an amino acid sequence code for protein production; there are also regulatory and structural RNAs. Ribosomal translation mechanisms are well

understood, as most of the proteins involved in recruiting the 60S and the 40S ribosomal subunit to the 5' methyl-cap have been identified. This section of the review will focus on interactions between the 3' UTR, the poly(A) tail, and proteins responsible for recruiting the ribosomal subunits for translation.

The mechanisms for RNA translation in oocytes were reviewed by de Moor et al. (2005). The 3' UTR usually contains a sequence termed the cytoplasmic polyadenylation element (CPE), UUUUUAUAAUAA. Cytoplasmic polyadenylation element binding protein (CPEB) binds the CPE and recruits cleavage and polyadenylation specific factor (CPSF) to the 3' UTR. These factors work to recruit poly(A) polymerase (Gld-2). Gld-2 elongates the poly(A) tail, which allows multiple poly(A) binding proteins (PABP) to bind the elongating poly(A) tail. An oocyte specific form of PABP known as ePABP has been identified in Xenopus (Copeland and Wormington, 2001) and murine (Seli et al., 2005) oocytes. ePABP seems to play a similar role in translation as PABP, but its expression is limited to a window of time between growth of the oocyte and embryonic genome activation. With either type of PABP, when the mRNA poly A tail reaches 200-300 nucleotides in length, PABP binds eukaryotic initiation factor 4G (eIF-4G) and these proteins, in combination with phosphorylation of CPEB, allow PABP to displace maskin, a protein that binds eIF-4E to mask mRNA from translation machinery. The mRNA is now in a circular form ready for translation by the ribosome (Wells et al., 1998). Events similar to these described above likely occur in mammalian oocytes.

Storage of maternal mRNA

The oocyte is a unique cell, remaining relatively quiescent during the late stages of folliculogenesis and early embryogenesis. During these stages of development,

mRNA is stored until it is needed. This storage period can last six days or more. Like translation, storage is driven by interactions between specific proteins and the 3' UTR of mRNA. Currently, researchers are trying to determine how proteins identify their specific targets and how binding is regulated to permit translation of each individual mRNA at the appropriate time. There are two basic types of protein interaction phenomena that drive mRNA translational repression, or storage.

The first form is a nonspecific mRNA silencing where mRNA is packaged into maternal ribonucleoprotein (mRNP) complexes similar to condensed chromosomes. These mRNPs are masked from translational machinery by several proteins that block steric interactions between mRNA molecules and RNA processing machinery. A major component of mRNPs is a class of y-box domain binding proteins (de Moor and Richter, 2001). In Xenopus oocytes, FRGY-2 is a germ-cell specific y-box binding protein that is responsible for binding mRNA molecules (Marello et al., 1992). In mice, a homologue to FRGY-2 has been identified as MSY-2. MSY-2 is transcribed during oocyte growth and degraded along with other maternal mRNA molecules by the two cell stage in mice (Paynton, 1998). FRGY-2 is phosphorylated by protein kinases that are associated with mRNPs, and this phosphorylation appears to increase the stability of the protein-RNA interaction (Cummings and Sommerville, 1988). Microinjection of protein kinase inhibitors into growing xenopus oocytes stimulated the rate of translation two- to three-fold, indicating that disruption of FRGY-2 binding to mRNA unmasks the RNA (Summerville, 1990). These studies suggest that y-box domain proteins play a key role in regulating mRNA silencing. Degradation of MSY-2 in murine oocytes coincides with the bulk degradation of other mRNA's (Gu et al., 1998), and exposure of recombinant

MSY-2 bacterially expressed mRNA molecules results in suppressed translation (Yu et al., 2002).

The other method for mRNA storage involves interactions between the cytoplasmic polyadenylation element (CPE) and proteins needed for blocking translation though blocking binding sites for proteins involved in RNA translation. As stated previously, the CPE is a sequence of UUUUUAUAAUAA responsible for recruiting factors for poly(A) tail elongation. The most studied protein involved in interaction with cytoplasmic polyadenylation element binding protein (CPEB) is maskin. Maskin binds to CPEB and eIF-4E (Stebbins-Boaz et al., 1999). By binding eIF-4E, maskin blocks interactions between eIF-4E and eIF-4G which are necessary for initiating translation, thus suppressing translation to induce mRNA storage. Upon oocyte maturation, phosphorylation of CPEB (Tay et al., 2003) and maskin (Stebbins-Boaz et al., 1999) releases eIF-4E to initiate translation in mRNAs containing the CPE sequence. Although maskin was initially discovered as the primary protein involved in storing mRNA molecules containing a CPE sequence, recent studies have identified another set of proteins that drive storage of these RNA molecules via a different mechanism.

In 2006, Kim and Richter proposed an opposing action of cytoplasmic poly(A) polymerase (Gld-2) and poly(A) ribonuclease (PARN) to maintain the poly(A) tail of CPE containing RNAs at around 50 nucleotides in Xenopus oocytes. Deadenylation studies showed that the CPE was necessary for deadenylation of cyclin B1 mRNA. Previous work by Barnard et al. (2004) showed that symplekin, a scaffolding protein discovered in Xenopus oocytes, binds CPEB and cleavage specificity factor (CPSF) along with Gld-2. Their study showed that this anchoring was present even before

poly(A) elongation and eventual translation, suggesting a new mechanism for Gld-2 in posttranscriptional regulation. Kim and Richter (2006) followed up this work by showing that symplekin indeed co-immunoprecipitated with Gld-2. They also found an interaction with PARN. CPE containing mRNA recruits factors involved in both polyadenylation and deadenylation together to their 3' UTRs. Kim and Richter (2006) suggest that RNA storage of mRNA molecules containing the CPE sequence is driven by a constant lengthening and shortening of the poly(A) tail that works to cancel out any net shortening or lengthening during early maturation. Upon phosphorylation of CPEB by Aurora A kinase during maturation, PARN is released from the complex, allowing Gld-2's action of elongating the poly(A) tail to take over. With poly(A) elongation becoming the predominant action, the mRNA is activated for translation though previously discussed mechanisms. Currently, little research has focused on these interactions in mammalian oocytes and embryos nor has work completely linked maskin with the symplekin driven RNA storage. The subject of mRNA storage remains somewhat unexplained and poorly understood, although it is one of the most interesting aspects of oocyte biology.

Degradation of maternal mRNA

The final pathway for RNA processing is RNA degradation. Two basic pathways exist for RNA degradation (for review see Garneau et al., 2007). Both pathways involve removing protective structures from the RNA such as the 3' poly(A) tail and the 5' cap. Removal of these structures exposes the RNA to exoribonucleases or the exosome, a 10-12 subunit protein complex with helicase and ribonuclease activity. Like storage or

translation, degradation machinery is targeted to mRNA sequences in the 3' and 5' untranslated regions.

5' to 3' degradation occurs after the 5' 7-methylguanosine cap structure is removed. This form of RNA degradation is independent of poly(A) tail length. The mRNA is decapped by a combination of proteins known as decapping proteins 1 and 2 (DCP-1 and DCP-2) (Steiger, 2003). Once the cap structure is removed, exoribonuclease 1 is able to degrade the mRNA in a 5'-3' direction (Larimer, 1992). This form of degradation occurs with less frequency than deadenylation-dependent decay in yeast, yet in mammalian cells less is known about the number of RNA molecules that actually undergo deadenylation-dependent decay. In mammalian cells, deadenylation-dependent decay does appear to occur more readily in focused areas known as P bodies. P bodies are granular cytoplasmic focal points that contain machinery involved in posttranscriptional modification of mRNA. P bodies exist in a variety of cells, although it is unclear whether most RNA degradation actually occurs in these focal points or in the cytoplasm of cells. In addition, P bodies do not exist in all cells, and in some they exist only during times of stress (Wilusz and Wilusz, 2004). Another interesting observation involving P bodies is that they seem to disappear when there is no longer an abundance of mRNA for degradation. This observation suggests that they form in response to overstimulation of RNA degradation pathways. Deadenylation-independent decay may become more important during these times of overstimulation or overabundance of mRNA to allow the cell to degrade mRNA products that need to be removed from the system more efficiently.

However, deadenylation-dependent decay is responsible for most of mRNA turnover in cells. This form of decay can be a targeted type of degradation or it can be a nonspecific decay; it occurs when the poly(A) tail is shortened though deadenylation, and then decapping follows. Removal of the protective features of mRNA, the cap and poly(A) tail, allows 3'-5' exonucleases access to the 3' end of mRNA, and 5'-3' exonucleases gain access to the open reading frame. One key enzyme in deadenylation is poly(A) ribonuclease (PARN) (also known as deadenylating nuclease). PARN binds to the mRNA cap (Dehlin et al., 2000), competing with eIF-4E for cap binding. Once PARN is bound to the cap, the rate of deadenylation is increased. As the poly(A) tail is shortened, PABP dissociates from the mRNA, exposing the 3' end to exonucleases for degradation. Another player in degradation, first recognized in xenopus embryos, appears to have a role in targeted degradation. Dephosphorylation of embryonic deadenylation element binding protein (EDEN-BP) in response to calcium increases EDEN-BP's affinity for the embryonic deadenylation element (EDEN) sequence (Detivaud et al., 2003). Once bound, EDEN-BP increases the deadenylation of mRNA containing the EDEN sequence. Possibly, EDEN-BP acts in conjunction with PARN to stimulate deadenylation of mRNA. EDEN-BP appears to be a key element to target specific sequences for degradation in oocytes.

Still, it is unclear what pathway is predominantly used in mammalian oocytes. Disrupting binding of proteins involved in polyadenylation to the 3' UTR in mice delayed maturation of oocytes (Tay et al., 2002). Further evidence for involvement of the 3' UTR in posttranscriptional regulation has been reported in Xenopus embryos. Richter (1999) proved that EDEN-BP targets A-rich elements (AREs) and CUG elements in the

3' UTR to direct deadenylation of specific genes after fertilization. Although EDEN-BP-bound targets were increasingly deadenylated in Xenopus embryos, it is unclear how EDEN-BP directs deadenylation. Recent research by Moraes et al. (2006) has demonstrated that CUG-BP, the human homologue of EDEN-BP, actually interacts with poly A ribonuclease (PARN), suggesting that EDEN-BP targets PARN to deadenylate specific messages during development.

Polyadenylation status is a key component of maternal mRNA regulation. Molecular maturation most likely includes transcription and translation of genes involved in the processes of posttranscriptional modification and translation. One possible explanation for the inefficiencies observed with in vitro-maturation compared to in vivo-maturation could arise from altered expression of these genes. Recently, Corcoran et al. (2006) used microarray analysis to identify differentially expressed genes in in vitro- and in vivo-cultured bovine embryos. They showed that 10 of 24 genes expressed at lower levels in in vitro-produced embryos were involved in transcription and translation. Smith et al. (2006 personal communication) also identified 6 differentially expressed genes involved in RNA processing though the use of microarray analysis. Both studies provide evidence that in vitro- and in vivo-matured oocytes exhibit different patterns of gene expression for genes involved in posttranscriptional modification of RNA. Recovery of oocytes before the LH surge and maturing them in vitro may lead to incomplete molecular maturation, specifically altering the expression of genes involved in RNA regulation. This may in turn lead to altered regulation of the maternal pool of mRNA and subsequent decreases in developmental competence.

Strategies for improving oocyte maturation

To date, research on improving oocyte maturation has focused on timing, as in vitro-maturation concentrates events into 1 day that occur over 3-5 days in vivo. The inefficiencies observed with in vitro oocyte maturation are likely due to deficiencies in cytoplasmic and molecular maturation. Research has focused on two strategies for dealing with these issues: 1. prevent spontaneous maturation, providing more time for cytoplasmic maturation; 2. manipulate the follicular environment before recovering the oocyte to initiate in vivo events for in vitro-maturation.

To prevent spontaneous maturation, pharmacological agents have been added to slow nuclear maturation in vitro. In theory, this strategy allows more time for events such as cytoplasmic and molecular maturation. Addition of phosphodiesterase inhibitors has proven effective at maintaining cAMP levels and preventing GVBD (Bilodeau et al., 1993; Mayes and Sirard, 2002; Thomas et al., 2002; Thomas et al., 2004). Another strategy has involved the use of forskolin or other activators of adenylyl cyclase to maintain production of cAMP (Homa, 1988; Sirard, 1990; Aktas et al. 1995a, b). In addition to cAMP manipulation, agents such as roscovitine (Franz et al. 2003), 6-DMAP (Fulka et al., 1991), and butyrolactone-1 (Lonergan et al., 2000) have been added to manipulate phosphorylation capacity within the oocyte. By preventing phosphorylation, MPF activity can be blocked and nuclear maturation prevented. Although these methods have proven to be both reversible and effective at preventing GVBD, they are inhibitory molecules that prevent events rather than set them up. They are also highly specific and artificial in nature. Little is known about the broad effects these artificial inhibitors may have on embryonic development. The other strategy for manipulation of maturation

focuses on manipulating the follicular environment to initiate events leading to maturation without inducing in vivo-maturation, producing more competent oocytes for subsequent in vitro procedures.

In commercial settings, donors are routinely superstimulated with exogenous FSH to increase the number of large follicles that can be recovered upon transvaginal aspiration. In cattle, 6 doses of 50-mg FSH at 12 h intervals results in an average recovery of 6 cumulus oocyte complexes per ovum pickup session (unpublished data from our laboratory). In vivo maturation can increase embryonic development (van de Leemput et al., 1999), but strategic recovery of oocytes without the use of prostaglandin F2α to reduce progesterone levels can also yield improved embryonic development after in vitro maturation, while allowing more aspirations per unit of time. Blondin et al. (2002) showed that recovering oocytes 48 h after the final FSH injection resulted in 52% blastocysts per oocyte recovered. Injection of LH 6 h prior to aspiration increased blastocyst development to 63%, although this increase was statistically insignificant. Blondin et al. (2002) were able to manipulate the follicular environment to produce oocytes that were comparable in terms of developmental competence to oocytes matured in vivo by Rizos et al. (2002) (63% vs. 80% blastocyst rates respectively). Their results suggest that the most effective protocol for maturing oocytes in vitro will consist of a combination of proper stimulatory and inhibitory events. Although manipulating follicular environments led to improved embryo production in a commercial, donor-based setting, this approach is cost prohibitive for use in research settings and certain commercial settings. Therefore, more research is needed to identify those events required to produce a competent oocyte from multiple follicular stages.

Chapter II

Inability of GnRH to induce bovine oocyte maturation during the luteal phase of the estrous cycle

Abstract

Oocyte maturation in vivo consists of a sequence of events occurring in response to the LH surge. These events prepare oocytes arrested in the germinal vesicle stage for fertilization. Oocyte maturation is not initiated via such an LH signal with in vitro-maturation protocols. Instead, the oocyte is removed from its inhibitory follicular environment. Oocytes can be aspirated transvaginally from follicles of cows that have been treated strategically to induce oocyte maturation, increasing the success of in vitro embryo production. The purpose of this study was to determine if inducing an LH surge in the presence of luteal phase levels of progesterone would induce LH signaling, germinal vesicle breakdown (GVBD), and cAMP reduction in bovine oocytes. Injection of GnRH into superstimulated cows resulted in an increase in LH, followed by upregulation of epiregulin mRNA concentrations within 6 h. Despite LH signaling, GVBD did not occur as reliably in oocytes recovered from cows with an intact corpus luteum treated with GnRH 0, 6, 12, or 24 h before aspiration as controls treated with PGF2α prior to injection of GnRH (% GII oocytes; 0 h: 79%, 6 h: 58%, 12 h: 83% and 24 h: 60% vs. PGF2α: 6%) ($P<0.001$). In addition, cAMP levels remained stable in oocytes from cows treated with GnRH in the presence of progesterone regardless of culture time post injection. Oocytes were capable of degrading cAMP as cAMP levels declined after 30 min of culture post aspiration (6.18 vs. 4.04 fmol/oocyte) ($P<0.10$). Phosphodiesterase-3A mRNA concentrations in oocytes were unaffected by treatment.

Oocyte maturation is blocked by sustained cAMP levels despite LH surge signaling in superstimulated cows not treated with PGF2α to regress the corpus luteum.

Introduction

Mammalian oocytes are arrested at the dictyate stage of prophase of meiosis I before being signaled to resume meiotic cell divisions. The source of the signal differs for in vivo-and in vitro-maturation. In vitro-maturation occurs spontaneously while in vivo-maturation is induced. Oocytes matured in vitro produce blastocyst rates of around 30% while oocytes matured in vivo and recovered before fertilization can result in blastocyst rates exceeding 60% (Rizos et al., 2002; Blondin et al., 2002). One of the fundamental differences between in vivo- and in vitro-maturation is how the maturation process begins. Recent research has focused on understanding fundamental differences between spontaneous and induced maturation.

In spontaneous oocyte maturation, simple removal of the cumulus oocyte complex from the follicular environment initiates maturation. Spontaneous resumption of maturation indicates presence of an inhibitory molecule or molecules in follicular fluid. Richard and Sirard (1996) showed that culture of bovine cumulus-oocyte complexes with theca cells prevented oocyte maturation in vitro, suggesting that theca cells produce some molecule that either binds cumulus cells or possibly even the oocyte itself to maintain cAMP at high levels. Exposing oocytes to phosphodiesterase 3A (PDE-3A) or 4 (PDE-4) specific inhibitors results in blocking resumption of meiosis only when PDE-3A inhibitors are used (Thomas et al., 2002; Thomas et al., 2004). PDE-3A is an oocyte-specific form of phosphodiesterase, further suggesting that control of cAMP levels is maintained within the oocyte itself. Either a receptor in the oocyte itself is

activated by the unknown inhibitor of cAMP production and/or the cumulus cells are activated, and cAMP is shuttled though gap junctions. Although this inhibitory substance has yet to be identified, recent studies have identified a G-protein-coupled receptor on the surface of murine oocytes (Mehlmann et al., 2004; Hinckley et al., 2005). Mehlmann et al. (2004) showed that knocking out this receptor, GPR3, in mice resulted in follicles that contained mature oocytes. GPR3 is a $G\alpha_s$ linked receptor that stimulates adenylate cyclase to produce cAMP within the oocyte. The recent discovery of GPR3 may have identified an additional part of the pathway responsible for maintaining meiotic arrest in oocytes though cAMP production. However, little is currently understood about what signal drives the decreases in cAMP levels within the oocyte. In in vitro systems, we simply remove the inhibitory environment from our cumulus-oocyte complexes, possibly starving the oocyte for the GPR3 ligand. In vivo, the trigger for maturation is the LH surge.

Rizos et al. (2002) exposed preovulatory follicles to an LH surge, increasing developmental competence two fold. In a normal estrous cycle, the corpus luteum regresses, and progesterone concentrations decline before the LH surge. LH then binds receptors on the mural granulosa cells and induces production of the EGF-like factors amphiregulin and epiregulin (Johnson et al., 1993; Park et al., 2004; Lindbloom et al. 2008). Binding of amphiregulin and epiregulin to the EGF receptor on cumulus cells activates a tyrosine kinase cascade responsible for producing signals that lead to maturation. These signals are passed though gap junctions between the cumulus cells and the oocyte (Vozzi et al., 2001), setting into motion a series of events that lead to oocyte

maturation. Future techniques for in vitro-maturation will need to mimic these in vivo steps more precisely to improve oocyte maturation and subsequent embryo production.

Treating cattle with FSH to superstimulate the ovaries for production of multiple preovulatory follicles is a routine practice in embryo transfer programs. Treating superstimulated cattle with prostaglandin F2α and GnRH at specific time points of the estrous cycle and then aspirating the cumulus-oocyte complexes from follicles within 24 h of GnRH treatment yields a high percentage of metaphase II oocytes (Callesen et al., 1986). These oocytes can then be fertilized in vitro and cultured to produce blastocysts for transfer. Injection of GnRH into cattle stimulates the surge of luteinizing hormone responsible for initiating oocyte maturation. However, little is known about how the LH surge induces the oocyte or the follicular environment to switch from an inhibitory system into a stimulatory one. Another key component of normal estrous cycles is the drop in progesterone that precedes the LH surge. The role of the progesterone decline in mammalian oocyte maturation has been less studied.

The purpose of this experiment was to determine if the LH surge was capable of stimulating oocyte maturation in the presence of progesterone. Cows were superstimulated and treated with GnRH to induce maturation of preovulatory follicles with or without luteal phase levels of progesterone. Recovered oocytes were then analyzed at several points to determine if known steps of oocyte maturation were taking place. We analyzed production of epiregulin, levels of cAMP, and germinal vesicle status. Recent unpublished studies in our laboratory led us to believe that progesterone influences cAMP levels in oocytes via some unknown mechanism. Perhaps, the decrease in progesterone is critical for allowing the oocyte to overcome its inhibitory environment.

Materials and Methods

Cloning of Bovine Homologues of Epiregulin and Phosphodiesterase 3A

Human and mouse DNA sequences of each gene of interest were blasted against the bovine genome to identify potential conserved regions for primer design. Intron spanning primers were designed using the Invitrogen OligoPerfect^{TM} primer design tool. Epiregulin sequence number NM001432 and phosphodiesterase 3A sequence number NM000921 were used for primer design. Sequences as well as primer sequence and primer location are shown in Figure 2.1. mRNA was isolated from bovine oocytes using the Qiagen viral RNA isolation kit following manufacturer's instructions and used for reverse transcription PCR using temperature gradients of 50˚C-64˚C for annealing of primers as described in more detail later. Reverse transcription was performed using the Qiagen Sensiscript Reverse Transcriptase according to manufacturer's instructions. Resulting PCR products were cloned into pGEM-T easy vectors following the manufacturer's instructions. DH5α cells were transformed with the ligated pGEM-T easy vectors and screened for mRNA incorporation via PCR. Colonies that tested positive via PCR using conditions described above were grown overnight and the plasmids recovered using the Qiagen maxi prep kit. The plasmids were sequenced by Macromolecular Resources at Colorado State University to ensure proper product amplification. This sequence information was used to design new intron spanning primers as previously described. These new primers were designed to melt at similar temperatures for each gene and to amplify around 150 base pairs for real time PCR.

Figure 2.1: Sequence for epiregulin and phosphodiesterase 3A mRNA along with primer location and sequence used for real time PCR.

EREG mRNA NM 001432: 150 base pairs amplified
TTTCTATCTTCTTCAAGCAGTCTTCAGTACAACTGTGATTCCTTCATGTATCCC
AGGAGAGTCGGGAGATAACTGCACAGCATTAGAGATAATCCACGTGTGGCTC
AAGTGTCAATAACAAAGTGTAGTGCTGACATGAATGGCTACTGTTTGCATGG
ACAGTGCATCTACCTGGTAGACATGAGTGAAAATTACTGCAGAGTAGGTTAC
ACTGGTGTCCGGTGTGAGCATTTCTTTTTAACCGTCCAAAAACCTCTGAGCAA
AGAATATGTGGCTTTGACTGTGATTCTTGTTATCTTGTTCCTTGTCATAGTTGC
TGGTTCTATATACTACTTCTGCAGAGAAATCGAAAAAGTAAAGAACCCAAGA
AAGAATATGAAAGAGTGACCTCAGGGGATCCGGCATTGCCGCAAGTCTGAAT
GGTGCCATTAAGCTTATGGGCAGGAATGCACTGCATGCCTGGTT

PDE3A mRNA NM000921: 166 base pairs amplified
GGAAAAACCAATTCTTGCTCCTGAACCTCTTGTCATGGACAACTTGGACTCAA
TTATGGAGCAACTGAATACCTGGAATTTTCCAATTTTTGATTTGGTGGAAAAA
ATAGGAAGAAAATGTGGCCGTATTCTTAGTCAGAGACTTTTTGAAGACATGG
GCCTCTTTGAAGCTTTTAAAATTCCAGTTAGGGAATTCATGAATTATTTTCAT
GCTTTGGAGATTGGCTATAGGGAAATTCCTTAACAGAATCCATGCCACGGAT
GTCTTACATGCTGTTTGGTATCTCACGACACAACCTATTCCAGGCCTCTCAAC
TGTGATTAATGATCACGGATCAGCAAGCGATTCAGAGTGGATTTACACATGG
CCATATGGGATATGTATTCTCAAAAATGTATAATGTGCCTGATGATAAATATG
GATGTTTGTCTGGAAATATCCCTGCCTTGGAGTTGATGGC

Actin mRNA NW001494327.2: 150 base pairs amplified
TTGCCAGAGGCATACAGGGACAGCACAGCCTGGATGGCCACGTACATGGCA
GGGGTGTTGAAGGTCTCGAACATGATCTGGGTCATCTTCTCACGGTTGGCCTT
GGGGTTCAGGGGGGCCTCGGTCAGCAGCACGGGGTGCTCCTCAGGGGCCACA
CGGAGCTCGTTGTAGAAGGTGTGGTGCCAGATCTTCTCCATGTCGTCAATCAC
TAGTGAATTCG

H2A-1 mRNA XM602557: 113 base pairs amplified
GTCGTGGCAAGCAAGGAGGTAAGGCGCGAGCAAAGGCCAAGTCTCGTTCTTC
GCGGGCGGGGCTCCAGTTCCCGGTGGGTAGAGTCCATCGTCTACTACGTAAG
GGCAATTACGCCGAGCGTGTAGGCGCTGGGGCCCCGGTGTATCTGGCGGCGG
TGTTGGAGTACCTAACGGCCGAGAT

Follicular Aspiration

Cows were injected with six 50-mg FSH injections of FSH-P (Folltropin V Bioniche Life Sciences, Athens, GA) i.m. at half-day intervals between days 8 and 13 of the estrous cycle (Blondin et al. 2002), resulting in an average of 12 preovulatory follicles per cow rather than the normal 1. Oocytes for in vitro maturation were collected using

ultrasound-guided transvaginal aspiration (TVA) 48 h after the last FSH injection. Oocytes were also collected 6, 12, and 24 h after administration of 100 μg GnRH i.m. to induce an LH surge. GnRH was given so that maturations time post GnRH combined with time after last FSH injection totaled 48 h. Blood was collected from the tail vein 30 min, 1 h, and 2 h post injection time from cows that received no GnRH, GnRH in the presence of progesterone, and GnRH in the absence of progesterone to document the occurrence of an LH surge. Positive control oocytes (in vivo matured) also were collected after treatment of cows with 25 mg/L prostaglandin F2α i.m. at the 6th FSH injection; 37 h post prostaglandin F2α, cows were injected with 100 μg GnRH i.m., and cumulus-oocyte complexes were recovered 23 h later.

Oocyte aspirations were performed with the aid of an Aloka 500 ultrasound machine with a 5-mHz probe and needle guide. A 2 inch 18 gauge hub lock needle attached to a needle guide was inserted into the transvaginal probe handle and follicles were punctured. Follicular fluid and cumulus-oocyte complexes were aspirated using a foot activated vacuum pump set at 80 mg/mL Hg of pressure. Oocytes were collected in a 50 mL conical tube containing prewarmed Dulbecco's modified PBS (Tervit et al., 1972) + 0.25% FrV BSA and 10 units/mL heparin. This procedure resulted in average recovery of 6-7 cumulus-oocyte complexes (COCs)/cow in our laboratory, but with great variation among cows.

Oocyte Selection and Preparation

Tubes containing aspirate were emptied and rinsed into 25 μm Emcon mesh filters. Filters were rinsed with Dulbecco's modified PBS supplemented with 0.25% Fraction V BSA and 10 units/mL heparin. Cumulus-oocyte complexes with intact

cumulus cell layers and evenly granulated cytoplasm were recovered from the filters and isolated using a stereomicroscope. Once recovered, oocytes for reverse transcription real time PCR were vortexed in Dulbecco's modified PBS supplemented with 0.25% Fraction V BSA for 5 min to remove cumulus cells. Three oocytes from each of three different cows (9 total) were isolated from each treatment and individually loaded into 1.5 mL Falcon tubes in 240 uL of protein-free phosphate-buffered saline (PBS) supplemented with 0.2% 50,000 MW polyvinyl alcohol (PVA). Granulosa cell pools from aspirates of thee different cows were isolated and prepared in a similar manner as described for oocytes. Once loaded into tubes, oocytes were snap frozen in liquid nitrogen and stored at -80˚C until RNA isolation.

Germinal Vesicle Staining

Cumulus oocyte complexes were denuded by vortexing for 3 min, mounted on glass slides under cover slips, and fixed in a 3:1 absolute ethanol:acetic acid solution for 2 days to extract lipids. After 2 days, 1% aceto orcein (100 mg orcein (Sigma O-7380), 4.5 mL acetic acid, 5.5 mL deionized water up to 10 mL) stain was applied to slides to visualize germinal vesicles. At least 6 oocytes were evaluated per cow X 8 cows (the experimental units) for each of 5 treatments (Table 2.1). After counting nuclei, the percentage of oocytes maintaining their germinal vesicle was calculated for each cow, and compared among treatments.

cAMP Measurement

Cumulus oocyte complexes collected from follicles were placed into modified Dulbecco's phosphate-buffered saline (m-PBS) with 0.25% FAF-BSA. 0.5 mM 3-isobutyl-1 methylxanthine (IBMX) was added either immediately upon recovery or after

30 min in maturation medium (TCM-199-0.5% BSA) minus hormones. IBMX blocks phosphodiesterase activity, ensuring cAMP levels remain constant during measurement. At the 0 or 30 min time point, COCs were rinsed in m-PBS without IBMX and transferred in 3-5 μL into an Eppendorf tube to be snap frozen and stored at -20˚C until assay. Intracellular cAMP levels were determined using an enzyme immunoassay system, EIA Biotrak, from Amersham Life Sciences. At least 5 COCs were pooled per cow for each of the 8 groups X 4 cows/group (Table 2.3). Luciano et al. (2005) and Thomas et al. (2004) were able to measure cAMP in as few as 5 COCs using similar techniques as described above.

Isolation of RNA from Individual Bovine Oocytes

Total RNA isolation from individual oocytes and granulosa and cumulus cells was done using the PicoPure™ RNA Isolation Kit (Arcturus, Sunnyvale, CA). Samples for RNA isolation were treated with DNAse (Qiagen Inc, Valencia CA) and stored at -80°C in 10 μL RNase free water. Complementary DNA (cDNA) was generated using the Sensiscript® Reverse Transcription Kit (Qiagen Inc, Valencia CA) with random primers.

Real Time Reverse Transcription Polymerase Chain Reaction

Eight point standard curves (10^{-16} to 10^{-23} moles) were generated for the housekeeping gene (histone H2A.1 (H2A.1) for oocytes and actin for granulosa cells) and the genes of interest (EREG and PDE-3A) by serial dilutions of the calculated molarity of the plasmids. Real time PCR was performed with the LightCycler® 480 Real Time PCR System and the LightCycler® 480 SYBR Green I Master detection reagents (Roche Applied Science, Indianapolis IN). Standard curve dilutions for each gene, including the control were analyzed in duplicate while oocyte (PDE-3A) and granulosa cell (EREG)

mRNA levels for each gene were analyzed in triplicate in 10 μL reactions. The PCR amplification included dissociation for 10 min at 95°C, 45 PCR cycles [5 sec at 95°C, annealing for 5 sec at 57°C, and 20 sec at 72°C]. PCR products were analyzed on a 2% agarose gel with ethidium bromide to confirm specificity. The data were analyzed by the LightCycler® 480 Relative Quantification Software (Roche Applied Science, Indianapolis IN) and normalized to the endogenous housekeeping control (H2a.1 for oocytes or actin for granulosa cells). Use of housekeeping genes in early embryos has proven difficult due to the ever changing nature of early embryos. Vigneault et al. (2007) showed that H2a.1 levels remained stable throughout the early stages of oocyte development, whereas actin levels are constantly changing as the number of cells in early embryos changes during development. Therefore we chose to use H2a.1 for oocyte analysis and actin for granulosa cell analysis. Melting curves were determined for each sample to validate specificity of amplification. At least 3 oocytes/cow were evaluated individually for all 3 genes X 3 cows for each of the 3 treatments (Table 2.1).

Experimental Design

Oocytes were collected from FSH-superstimulated cows treated in 5 different ways to produce various stages of oocytes matured in the presence or absence of progesterone. After superstimulation, cows were given a 48 h coasting period before aspiration (Treatment 1); coasted 42 h, injected with GnRH, and aspirated 6 h later (Treatment 2); coasted 36 h, injected with GnRH, and aspirated 12 h later (Treatment 3); coasted 24 h, injected with GnRH and aspirated 24 h later (Treatment 4); or injected with PGF2α at the time of the final FSH injection, injected with GnRH 36 h afterwards, and then aspirated after 23 h (in vivo-maturation) (Treatment 5). Table 2.1 illustrates

experimental designs, identifying which treatments were used for each experiment. In experiment 1, germinal vesicle status was analyzed on oocytes collected from each of the five treatments. In experiment 2, cAMP measurements were performed on oocytes from only four of the five treatments. We chose to leave out the 12 h post GnRH treatment as we planned to measure cAMP 6 h and 24 h post GnRH, and believed the 12 h time point would be of less interest because cAMP would remain high when progesterone was present. Experiment 3 used real time PCR on oocytes and granulosa cells from three of the five time points of interest. We chose to evaluate one of the groups of interest in addition to the positive and negative control to determine if mRNA levels behave more like in vivo-matured or immature oocytes in the groups where progesterone was present.

Table 2.1. Experimental design

Trt	Progesterone	Treatment after 6th FSH Injection	Exp 1	Exp 2	Exp 3
1*	+	48 h coast-no GnRH	+ (48)	+ (40)	+ (9)
2	+	42 h coast + 6 h post GnRH	+ (48)	+ (40)	+ (9)
3	+	36 h coast + 12 h post GnRH	+ (48)	-	-
4	+	24 h coast + 24 h post GnRH	+ (48)	+ (40)	-
5**	-	PG, 36 h coast + 23 h post GnRH	+ (48)	+ (40)	+ (9)

*Negative control; **In vivo matured positive control
(number of oocytes/trt)

Statistical Analyses

After analysis of residual plots, differences in cAMP, mRNA, and percent MII oocytes were transformed (log10) to achieve homogeneity of variance when necessary and analyzed by ANOVA. One way ANOVA was used to analyze mRNA concentration and percent MII oocytes. cAMP concentrations were analyzed using a 2 way ANOVA with treatment (negative control, 6 h post GnRH, 12 h post GnRH, 24 h post GnRH, positive control) and culture time (0 or 30 min) as factors. Differences were compared using the lsd method for pre-planned comparisons. Oocytes were aspirated from 8 FSH-

treated cows for each treatment of each experiment. At least 6 oocytes/cow were stained for germinal vesicle status. 5 COCs/time point from each of 4 cows were used for cAMP measurement. RT-RT-PCR was performed on at least 3 individual oocytes and granulosa cell groups/cow/treatment. Real time data were normalized to an endogenous housekeeping gene before analysis to correct for differences in efficiencies of RNA isolation, reverse transcription, and PCR amplification. This also corrected for differences in granulosa cell numbers.

Results

Experiment 1: Germinal Vesicle Status

Means for germinal vesicle status are presented in table 2.2. Oocytes induced with GnRH in the absence of progesterone had fewer GV stage oocytes at the time of staining ($P<0.001$). Regardless of time after GnRH injection, the majority of oocytes maintained their germinal vesicle in the presence of progesterone.

Table 2.2. Means for percentage of oocytes maintaining germinal vesicle status.

Treatment	% Germinal Vesicle Stage Oocytes	cows/trt
Negative Control	79.2^{ab}	8
6 h post GnRH	58.3^{b}	8
12 h post GnRH	83.3^{a}	8
24 h post GnRH	60.4^{b}	8
Positive Control	6.3^{c}	8

^{ab} Values without common superscripts differ (<0.05)

Experiment 2: cAMP Concentrations

Main effect least squares means for cAMP levels in COCs exposed to GnRH in the presence or absence of GnRH are presented in Table 2.3. The positive control COCs had higher levels of cAMP compared to COCs exposed to progesterone during GnRH treatment, although these differences were not significant ($P>0.10$). COCs cultured for 30 min after removal from follicles had less cAMP than those measured immediately

after recovery (P<0.06).

Table 2.3. Main effect least squares means for cAMP levels per 5 cumulus-oocyte complexes adjusted to 1 cumulus-oocyte complex.

Treatment	fmol cAMP (±SE)	cows/trt
Negative Control	4.95 (0.70)	8
6 h post GnRH	3.98 (0.06)	8
24 h post GnRH	4.12 (0.36)	8
Positive Control	7.41 (2.03)	8
0 min post aspiration	6.18[a] (1.04)	16
30 min post aspiration	4.04[b] (0.44)	16

[ab] Values without common superscripts differ (<0.06)

Experiment 3: mRNA Concentrations

Means for epiregulin concentration in granulosa cells are presented in table 2.4. Epiregulin concentration in granulosa cells increased greatly after cows were injected with GnRH (P<0.06). Identity of the individual cow had no effect on mRNA concentration (P>0.10).

Table 2.4. Means for epiregulin mRNA concentration in granulosa cells.

Treatment	[EREG] M/[actin] M (±SE)	oocytes/trt
Negative Control	0.065[c] (0.01)	9
6 h post GnRH	0.741[a] (0.15)	9
12 h post GnRH	0.427[b] (0.09)	9

[ab] Values without common superscripts differ (P<0.10)

Means for phosphodiesterase-3A concentration are presented in table 2.5. Phosphodiesterase-3A concentration did not differ between treatments (P>0.10). Neither cow nor oocyte source affected phosphodiesterase-3A concentration (P>0.10).

Table 2.5. Means for PDE-3A mRNA concentration in oocytes.

Treatment	[PDE-3A] M/[H2A.1] M (±SE)	oocytes/trt
Negative Control	0.36 (0.05)	9
6 h post GnRH	0.35 (0.06)	9
12 h post GnRH	0.42 (0.07)	9

No significant difference (P>0.10)

Discussion and Conclusions

In a preliminary study, treatment of superstimulated cows with GnRH before aspiration produced metaphase I oocytes regardless of when oocytes were aspirated after GnRH (0-24 h post GnRH). Recovered cumulus-oocyte complexes were successfully matured after an additional 23 h over the 23 h post GnRH aspiration time for maturation to occur. However, administering prostaglandin F2α, then GnRH 36 h later, and aspirating cumulus-oocyte complexes 23 h after GnRH yielded greater than 80% metaphase II oocytes. These results suggested the presence of some inhibitory system in place to block the action of GnRH though LH when luteal levels of progesterone were present. The current experiments were designed to study blocking oocyte maturation systematically in the presence of progesterone. Studies in sheep have shown that administration of GnRH during the luteal stage stimulates an LH surge similar to the preovulatory LH surge (Amiridis et al., 2005). Analysis of LH levels in blood from our experimental cows revealed that GnRH administration indeed stimulated a surge of LH ($P<0.0001$), but it was intermediate to cows treated with prostaglandin F2α prior to GnRH treatment (No GnRH: 0.84 ng/mL; GnRH: 9.45 ng/mL; In Vivo: 93.86 ng/mL,; average of samples collected 30 min, 1 h, and 2 h post GnRH). Perhaps the LH surge was delayed in cows with an intact corpus luteum was simply not as large as that seen in cows treated with PGF2α. Whether or not the LH surge observed in the GnRH treated group was large enough to stimulate oocyte maturation is unclear. Little is known about the level of LH needed to induce oocyte maturation. There is possibly a threshold level of LH needed to induce changes in cAMP and eventual germinal vesicle breakdown. If so, the insufficient release of LH could explain the failure of maturation instead of the

presence of progesterone. Perry and Perry (2009) showed that heifers treated with GnRH during the luteal phase of the estrous cycle had smaller LH responses when progesterone levels were high. They also showed that some heifers failed to ovulate in response to GnRH when GnRH was given between days 7 and 18 of the estrous cycle, although it is unclear what happens to the oocytes within these follicles.

Although the LH surge measured in cows treated with GnRH in the presence of progesterone was intermediate to levels seen in cows treated with prostaglandin F2α prior to GnRH, epiregulin mRNA levels increased markedly within 12 h over cows not exposed to GnRH. Studies in mares (Lindbloom et al., 2008) have shown that EGF-like factors such as epiregulin have an increased mRNA concentration following the LH surge. Park et al. (2004) showed that EGF-like factors bind EGF receptors on murine cumulus granulosa cells, likely mediating the LH stimulatory signal on oocyte maturation. In the present experiment, cattle had increased levels of epiregulin mRNA concentration upon treatment with GnRH to stimulate the LH surge. Our results produced a profile similar to that seen in horses (Lindbloom et al., 2008), where levels peaked within 6 h and remain elevated after 12 h. These data indicate that the LH surge in treated cattle was sufficient to induce epiregulin signaling. Thus, the block during the luteal phase of the estrous cycle is likely occurring further downstream in the maturation process. However, a pathway currently unidentified could be blocking oocyte maturation in the presence of high progesterone, even with stimulation of LH and eventual epiregulin upregulation.

An important aspect of oocyte maturation is the decrease in cAMP observed upon oocyte activation. cAMP regulation appears to be driven by receptors within the oocyte

itself. Mehlmann et al. (2002) showed that murine oocytes contain a G-protein coupled receptor that regulates cAMP levels within the oocyte. Knocking out this receptor resulted in uninhibited maturation of oocytes. Mehlmann et al. (2002) named this surface G-protein coupled receptor GPR3. The pathway linked to GPR3 could be responsible for our results, where oocytes recovered from superstimulated cows during the luteal phase of the estrus cycle were prevented from maturing. Measurement of cAMP in oocytes from the present study showed that cAMP levels in cumulus-oocyte complexes were similar regardless of the time frame allowed for oocytes to respond to the LH surge before aspiration. These results suggest that either inhibition has not been removed from the follicular environment in the presence of a stimulatory LH surge or the cumulus-oocyte complexes were incapable of degrading cAMP. This was corroborated by failure of GVBD in these oocytes. Culture of the cumulus-oocyte complexes in vitro for 30 min resulted in decreased levels of cAMP. These results show that the oocyte is capable of undergoing spontaneous maturation, with a decrease in cAMP as the first step.

Analysis of phosphodiesterase 3A mRNA, the oocyte isoform of phosphodiesterase, indicated that mRNA levels for oocytes collected from superstimulated cows were similar whether oocytes were collected with or without the influence of progesterone. We cultured oocytes for 30 min in vitro to attempt to determine if phosphodiesterase 3A activity differed between any of the treatment groups. However, our results did not show any differences that would suggest that oocytes collected after LH stimulation had more active phosphodiesterase 3A than those collected without LH stimulation as no significant treatment interactions were observed. Analysis

of protein levels might reveal differences between treatments. However, such analyses are impractical due to limited material.

Recovering in vivo-matured oocytes via transvaginal aspiration yields more competent oocytes than those matured in vitro. This phenomenon likely occurs due to differences in the maturation environment combined with differences in follicle size oocytes originate from. Current understanding of the exact combination of events occurring in vivo is insufficient to determine what step is most critical. With in vitro-matured oocytes undergoing spontaneous maturation, advances will likely occur by gaining an understanding of the sequence of events occurring between the LH surge and resumption of meiosis during in vivo oocyte maturation. The method and timing of the switch from inhibition to stimulation is likely of critical importance. Attempts to slow down spontaneous maturation to date have been though chemical regulation of meiotic resumption (Franz et al. 2003). A more natural method may be discovered though continued study of oocyte maturation in vivo.

Chapter III

Comparison of bovine in vivo- and in vitro-oocyte maturation mRNA profiles for CUG-BP, PARN, eIF-4E, and PAP-1

Abstract

Mammalian oocytes stockpile mRNA during folliculogenesis for use during oocyte maturation and early embryonic development. This maternal pool of mRNA is responsible for driving developmental processes until activation of the embryonic genome. Regulation of the maternal pool of mRNA is controlled primarily by specific protein interactions with mRNA molecules. Differences between developmental competence of in vitro- and in vivo-matured oocytes may be due to improper expression of genes involved in processing the maternal pool of mRNA. This study was designed to examine mRNA profiles of four genes involved in mRNA processing to determine what differences exist between in vitro- and in vivo-matured bovine oocytes. We also determined if changes expression levels during the first 6 h of maturation. eIF-4E and PARN mRNA concentrations increased over time in both in vitro- and in vivo-matured bovine oocytes ($P<0.05$). In vivo matured-bovine oocytes contained more eIF-4E mRNA molecules than in vitro-matured bovine oocytes ($P<0.10$). CUG-BP and PAP-1 concentrations remained stable over the first 6 h of maturation and were similar in the in vivo-matured and in vitro-matured oocytes.

Introduction

Follicle size can have profound effects on the success of embryo production. In cattle, oocytes obtained from follicles smaller than 2 mm yielded no blastocysts, whereas

oocytes from 2-4 mm follicles resulted in 21% blastocysts and oocytes from 4-8 mm follicles resulted in 29% blastocysts (Pavlok et al. 1992). As bovine antral follicles increase in size up to 3 mm in diameter, oocytes also increase in diameter to their full size of about 110 μm (not including the zona pelucida) (Fair et al., 1995). Differences in developmental competence between oocytes from follicles less than 3 mm and those larger than 3 mm are likely due in part to differences in mRNA content. As the oocyte increases in diameter, a maternal pool of mRNA is transcribed and stored for use during oocyte maturation and early embryonic development. Transcription levels decline markedly once oocytes become meiotically competent, at a diameter of about 110 μm (Moore and Lintern-Moore, 1978). Oocytes recovered before the decrease in transcription do not successfully complete meiosis I, while isolation of oocytes from ≥3 mm follicles results in oocytes that are capable of resuming meiosis and producing a blastocysts (Lonergan et al., 2004).

However, mRNA transcription is only part of the equation for oocyte competence. How the maternal pool is regulated via storage or degradation mechanisms is likely just as important as specific transcription events. mRNA molecules contain a 3' structure consisting of a series of adenosine residues known as the poly(A) tail. Lequarre et al. (2004) measured the pool of mRNA containing poly(A) tails in oocytes before and after maturation. They found that the total amount of poly A RNA decreased greatly over the maturation period. Research has proven that the overall amounts of mRNA and poly(A) tail length decrease until the maternal-zygotic transition, yet studies focusing on individual genes have revealed a more complex regulation. Some genes undergo polyadenylation during maturation while others undergo deadenylation (Brevini-Gandolfi

et al., 1999; Brevini et al., 2002). Lengthening the poly(A) tail stimulates mechanisms for storage or even translation while shortening stimulates degradation.

Poly(A) tail length is regulated by interactions between proteins and the 3' UTR. In mice, Tay et al. (2000) disrupted binding of proteins involved in polyadenylation to the 3' UTR, which resulted in delayed oocyte maturation. Further evidence for involvement of the 3' UTR in posttranscriptional regulation has been reported in Xenopus embryos. Richter (1999) proved that EDEN-BP targets A rich elements (AREs) and CUG elements in the 3' UTR to direct deadenylation of specific genes after fertilization. Although EDEN-BP bound targets were increasingly deadenylated in Xenopus embryos, it is unclear how EDEN-BP directs deadenylation. Recent research by Moraes et al. (2006) demonstrated that CUG-BP, the human homologue of EDEN-BP, actually interacts with poly A ribonuclease (PARN), suggesting that EDEN-BP targets PARN to deadenylate specific messages during development.

Polyadenylation status is a key component of maternal mRNA regulation. Molecular maturation most likely includes transcription and translation of genes involved in the processes of posttranscriptional modification and translation. One possible explanation for the inefficiencies observed with in vitro-maturation compared to in vivo-maturation could arise from altered expression of these genes. Recently, Corcoran et al. (2006) used microarray analysis to identify differentially expressed genes in in vitro- and in vivo-cultured bovine embryos. They showed that 10 out of 24 genes expressed at lower levels in in vitro-produced embryos were involved in transcription and translation. Smith et al. (2006 personal communication) also identified 6 differentially expressed genes involved in RNA processing though the use of microarray analysis. Both studies

provide evidence that in vitro- and in vivo-matured oocytes exhibit different patterns of gene expression for genes involved in posttranscriptional modification of RNA. Recovery of oocytes before the LH surge and maturing them in vitro may lead to incomplete molecular maturation, specifically altering the expression of genes involved in RNA regulation. This may in turn lead to altered regulation of the maternal pool of mRNA and subsequent decreases in developmental competence.

The purpose of this study was to measure mRNA concentrations for four specific genes that are involved in regulating mRNA posttranscriptionally to determine if they are expressed at different levels in in vitro- and in vivo-matured bovine oocytes. Gene expression profiles were determined for eukaryotic initiation factor-4E (eIF-4E), poly(A) polymerase (PAP), embryo deadenylation element-binding protein (EDEN-BP), and poly(A) ribonuclease (PARN) in bovine oocytes collected before or after the preovulatory LH surge.

Materials and Methods

Cloning of Bovine Homologues of CUG-BP, PARN, eIF-4E, and PAP-1

Human and mouse DNA sequences were blasted against the bovine genome to identify potential conserved regions for primer design for the genes of interest. Intron spanning primers were designed using the Invitrogen OligoPerfect^{TM} primer design tool. mRNA was isolated from bovine oocytes using an Arcturus Pico Pure RNA isolation kit following manufacturer's instructions and used for reverse transcription PCR using temperature gradients of 50˚C-64˚C for annealing of primers as described later for specific transcripts. Reverse transcription was performed using the Qiagen Sensiscript Reverse Transcriptase according to manufacturer's instructions. Resulting PCR products

were cloned into pGEM-T easy vectors following the manufacturer's instructions. DH5α cells were transformed with the ligated pGEM-T easy vectors and screened for gene incorporation via PCR. Colonies that tested positive via PCR using previously established conditions were grown overnight and the plasmids recovered using Qiagen maxi prep kits. The plasmids were sequenced by Macromolecular Resources at Colorado State University to ensure proper product amplification. Upon cloning of the bovine homologues of genes of interest, new intron spanning primers were designed. These new primers were designed to melt at similar temperatures for each gene and to amplify between 100-150 base pairs for real time PCR. Figure 3.1 shows the sequences of mRNA for each gene of interest and the location and sequence of the primers used for real time PCR.

Figure 3.1. mRNA sequences for EDEN, PARN, eIF-4E, and PAP-1 along with primer location and sequence used for real time PCR.

PARN mRNA NM002568: 114 bps amplified TGCAGCCTAAACGTGATCACGTCCTCCACGTGACATTCCCCAAAGAATGGAA AACCAGCGACCTTTACCAGCTTTTCAGTGCCTTTGGTAACATTCAGATATCTT GGATTGATGACACATCAGCATTTGTTTCTCTCAGCCAACCTGAAC
EDEN mRNA NM001025596: 134 bps amplified GCCATCCAGTCCATGAACGGCTTTCAGATCGGCATGAAGCGGCTTAAAGTGC AGCTCAAACGTTCGAAGAACGACAGCAAACCCTACTGAGCGTGCTCCCCTCT GAGACTGGAGTGAGAGGGTCTTCTGATTCCTGCCGTTTGTTCATCGTTGTGCC TAAAGCATGTCGATGTGGCGTCAAGTACATCGTCCAAATCCCTGCA
eIF-4E mRNA NM174310: 100 bps amplified TATGATGTATGTGGAGCTGTTGTTAATGTTAGAGCTAAAGGTGATAAAATAG CAATATGGACTACTGAATGTGAAAACAGAGAAGCTGTTACACATATAGGGAG GGTATACAAGGAAAGGTTAGGACTTCCTCCAAAGATAGTGATTGGTTATCAG TCCCATGCAGACACAGCTACTAAGAGCGGCTCCACACTAAAAAT
PAP-1 mRNA NM032632: 110 bps amplified GGATGAAACAAGTGAAGATGCTAACTGTCTTGCTTTGAGTGGACATGATAAA ACAGAAACAAAGGAACAACTTGATACAGAGACAAGTACAACTCAATCAGAA ACCATTCAGACAGCGACTTCTCTGTTGGCCTCTC
H2A-1 mRNA XM602557: 113 bps amplified GTCGTGGCAAGCAAGGAGGTAAGGCGCGAGCAAAGGCCAAGTCTCGTTCTTC GCGGGCGGGGCTCCAGTTCCCGGTGGGTAGAGTCCATCGTCTACTACGTAAG GGCAATTACGCCGAGCGTGTAGGCGCTGGGGCCCCGGTGTATCTGGCGGCGG TGTTGGAGTACCTAACGGCCGAGAT

Follicular Aspirations

Oocytes matured spontaneously were aspirated from 3-8 mm diameter follicles of slaughterhouse-derived ovaries. Ovaries were transported to the laboratory in 0.15 M NaCl saline at 22 to 27˚C. Upon arrival, oocytes were aspirated from follicles with vacuum pressure of about 50 mm Hg using and an 18-gauge needle. Cumulus-oocyte complexes were isolated in 20 mM hepes buffered chemically defined maturation medium (H-CDM-M) (De La Torre-Sanchez et al. 2006). Fifty oocytes with intact cumulus cells and evenly granulated cytoplasm were matured in 1 mL CDM-M (CDM +

15 ng/mL NIH-FSH-S, 1 μg/mL bovine LH-USDA, 50 ng/mL EGF, 0.1 mM cysteamine, and 1.0 g/mL estradiol 17-β) (De La Torre-Sanchez et al., 2006) at 39°C, 5% CO_2 in humidified air.

Induced oocytes were collected from cows superstimulated with 6 doses of 50 mg FSH (Follistatin, Bioniche Life Sciences, Atlanta, GA). Cows were subjected to FSH treatment initiated between days 8 and 13 of their estrous cycles. FSH injections were given i.m. at half-day intervals. For time zero, oocytes were recovered from follicles greater than 8 mm 48 h after the last FSH injection with a 5-mHz ultrasound-guided transvaginal needle. Induced groups were recovered 0, 3, and 6 hours after 100 μg GnRH, injected i.m. 48 h after the final FSH injection. A 2 inch 18 gauge hub lock needle attached to a needle guide was inserted into the transvaginal probe handle and follicles were punctured. Follicular fluid and cumulus-oocyte complexes were aspirated into 50 mL conical tubes using a foot activated vacuum pump set at 80 mg/mL Hg of pressure.

Oocyte Selection and Preparation

Tubes containing aspirate were emptied and rinsed into 22 μm filters. Filters were rinsed with modified Dulbecco's PBS (Tervit et al., 1972) supplemented with 0.25% Fraction V BSA and 10 units/mL heparin. Cumulus-oocyte complexes with intact cumulus cell layers and evenly granulated cytoplasm were recovered from 22 μm filters and isolated using a stereomicroscope. Once recovered, oocytes were vortexed for 5 min in modified Dulbecco's PBS supplemented with 0.2% PVA to remove cumulus cells. Thee oocytes from each of thee different cows were isolated from each treatment and individually loaded into 1.5 mL Falcon tubes in 240 uL of protein-free modified

Dulbecco's PBS supplemented with 0.2% polyvinyl alcohol (PVA). Once loaded into tubes, oocytes were snap frozen in liquid nitrogen and stored at -80˚C until RNA isolation.

Isolation of mRNA from Individual Bovine Oocytes

Total RNA isolation from individual oocytes was done using the PicoPure™ RNA Isolation Kit (Arcturus, Sunnyvale, CA). Samples for RNA isolation were treated with DNAse (Qiagen Inc, Valencia CA) and stored at -80°C in 10 µL RNase-free water. Complementary DNA (cDNA) was generated using the Sensiscript® Reverse Transcription Kit (Qiagen Inc, Valencia CA) with random primers.

Real Time Reverse Transcription Polymerase Chain Reaction

Eight point standard curves (10^{-16} to 10^{-23} moles) were generated for the control and genes of interest by serial dilutions of the calculated molarity of the plasmids. Real time PCR was performed with the LightCycler® 480 Real Time PCR System and LightCycler® 480 SYBR Green I Master detection reagents (Roche Applied Science, Indianapolis IN). Standard curve dilutions for each gene, including the housekeeping gene H2A.1 were analyzed in duplicate while mRNA levels for each gene of interest were analyzed in triplicate in 10 µL reactions. The PCR amplification included dissociation for 10 min at 95°C, 45 PCR cycles [5 sec at 95°C, annealing for 5 sec at 57˚C, and 20 sec at 72°C]. PCR products were analyzed on a 2% agarose gel with ethidium bromide to confirm specificity. The data were analyzed by The LightCycler® 480 Relative Quantification Software (Roche Applied Science, Indianapolis IN), and normalized to the housekeeping gene (H2A.1). Melting curves were determined for each sample to validate specificity of amplification. At least 3 oocytes/cow or ovary collection

date were evaluated individually for all 4 genes X 3 cows or ovary collection dates for each of the 3 treatments.

Experimental Design

Oocytes were aspirated from 3-5 mm follicles from slaughterhouse-derived bovine ovaries and by ultrasound-guided transvaginal procedures from >8mm follicles of FSH-treated cows injected with GnRH.

Table 3.1: Experimental Design

Induced	Hours after LH surge: Replicates are cows	0	3	6
		9 oocytes (3 reps of 3)	9 oocytes (3 reps of 3)	9 oocytes (3 reps of 3)
Spontaneous	Hours after aspiration: Replicates are batches of ovaries:	0	3	6
		9 oocytes (3 reps of 3)	9 oocytes (3 reps of 3)	3 oocytes (3 reps of 3)

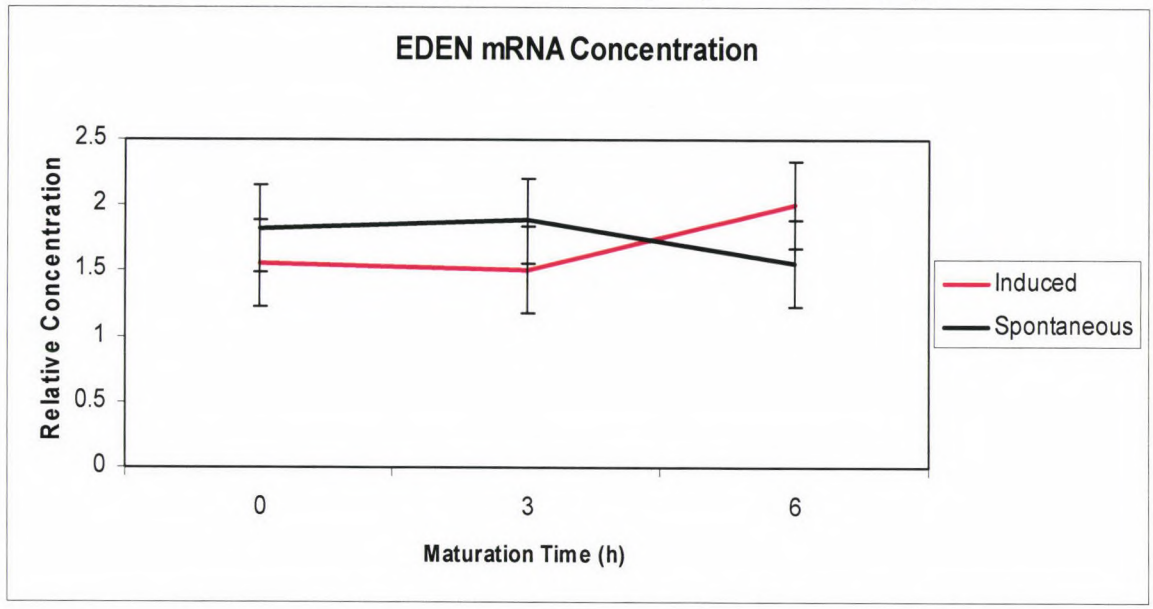
Statistical Analyses

Differences in mRNA were transformed with log10 to achieve homogeneity of variance and analyzed by ANOVA. Differences were compared using Tukey's hsd method for comparisons. Oocytes were aspirated from 3 FSH-treated cows for each treatment of each experiment. RT-RT-PCR was performed on at least 3 individual oocytes per cow or ovary delivery dates per treatment. At least 3 oocytes were recovered from each group and oocytes were analyzed in triplicate real time reactions. Real time data were normalized to the housekeeping gene (H2A.1) before analysis to correct for differences in efficiencies of RNA isolation, reverse transcription, and PCR

amplification. Vigneault et al. (2007) showed that H2A.1 levels remained stable throughout the early stages of oocyte development.

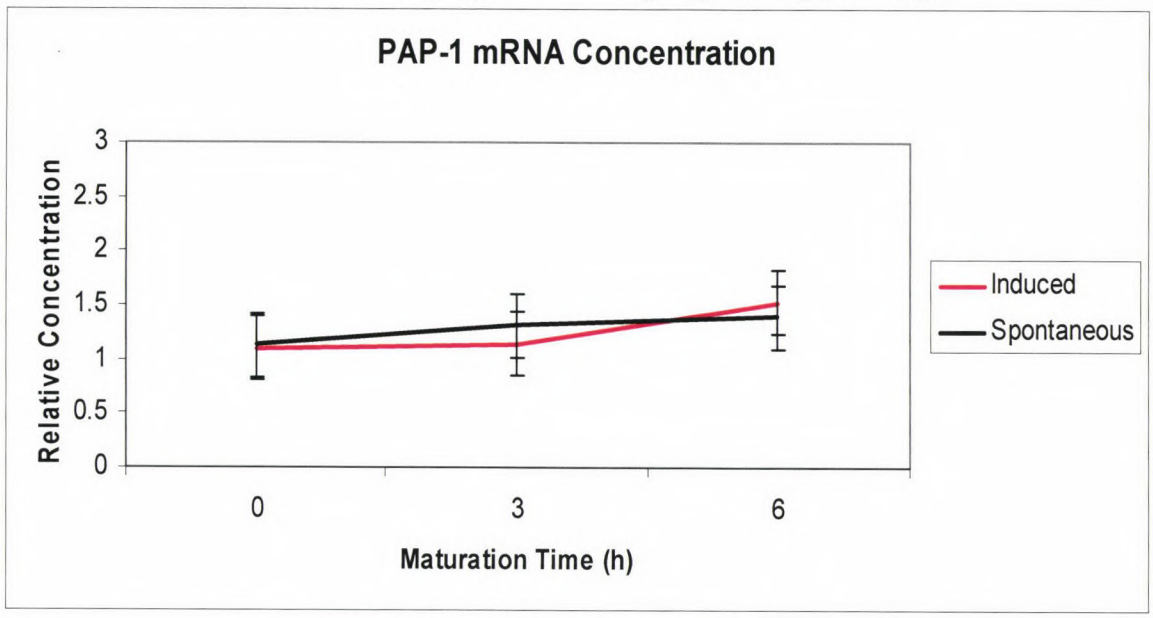
Results

Figure 3.2. Relative EDEN mRNA concentrations during the first 6 h of in vivo- or in vitro-maturation. Values displayed are 1+log10([EDEN]/[H2a.1]).



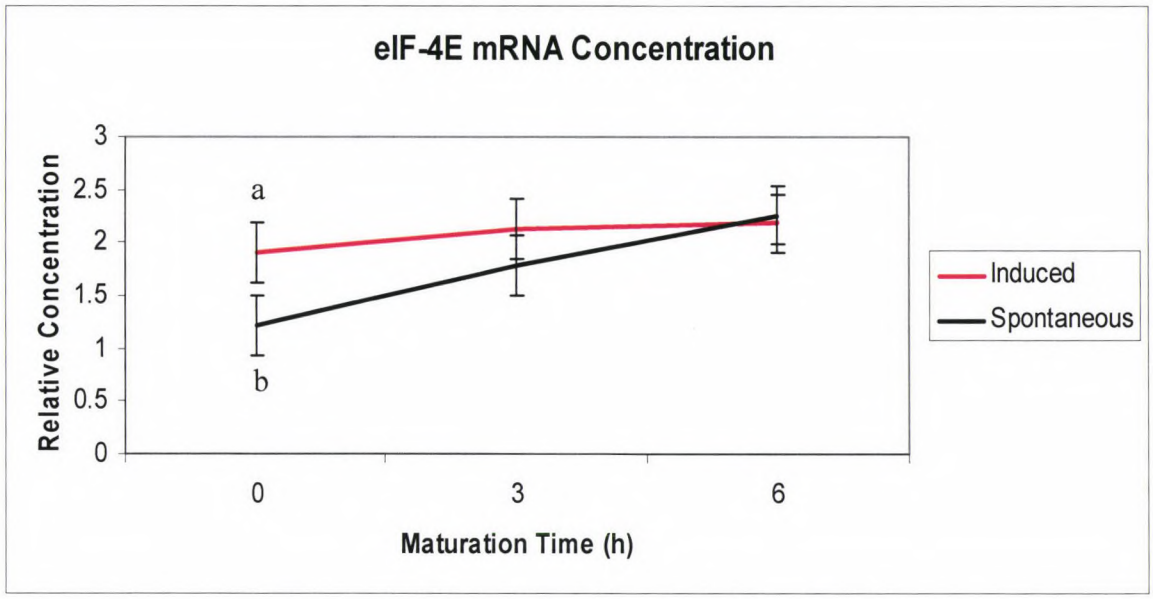
No significant difference (P>0.10)

Figure 3.3. Relative PAP-1 mRNA concentrations during the first 6 h of in vivo- or in vitro-maturation. Values displayed are 1+log10([PAP-1]/[H2a.1]).



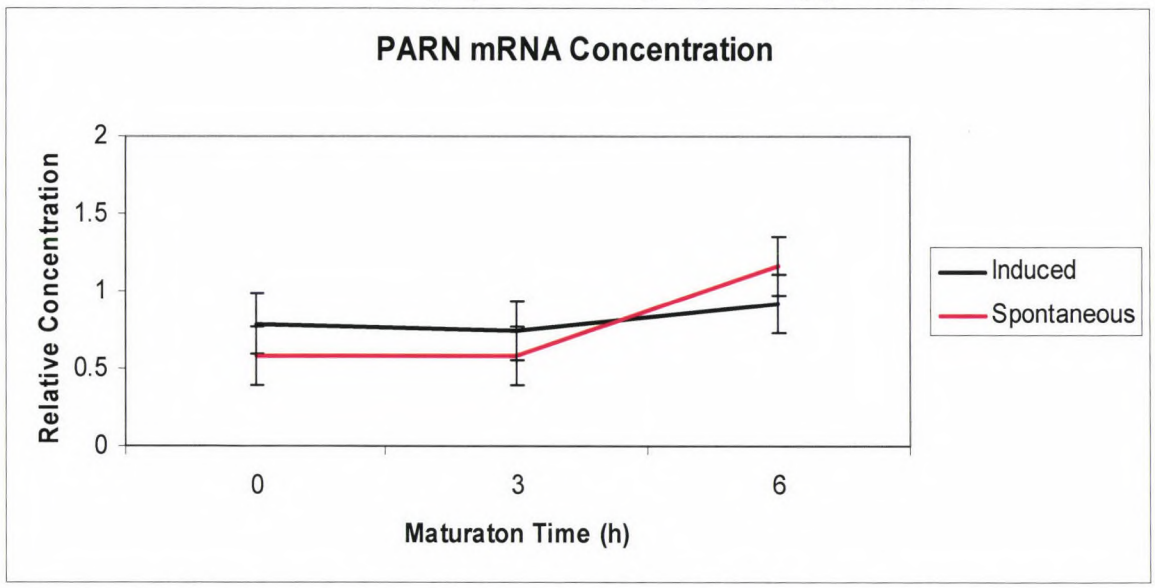
No significant difference (P>0.10)

Figure 3.4. Relative eIF-4E mRNA concentrations during the first 6 h of in vivo- or in vitro-maturation. Values displayed are 1+log10([eIF-4E]/[H2a.1]).



[ab]Values with differ (P<0.10).

Figure 3.5. Relative PARN mRNA concentrations during the first 6 h of in vivo- or in vitro-maturation. Values displayed are 1+log10([PARN]/[H2a.1]).

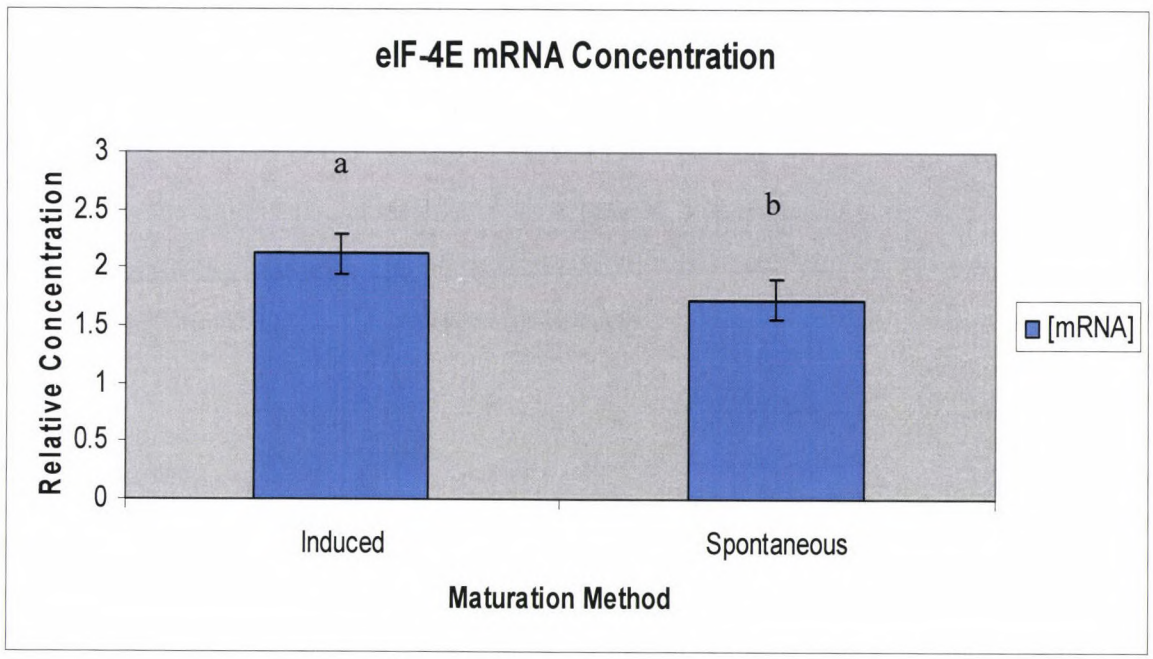


No significant difference (P>0.10)

Relative mRNA concentrations for EDEN, eIF-4E, PAP-1, and PARN during in vitro- or in vivo-maturation are shown in figure 3.2, 3.3, 3.4, and 3.5. Measuring mRNA concentrations from individual oocytes showed that individual animal affected mRNA

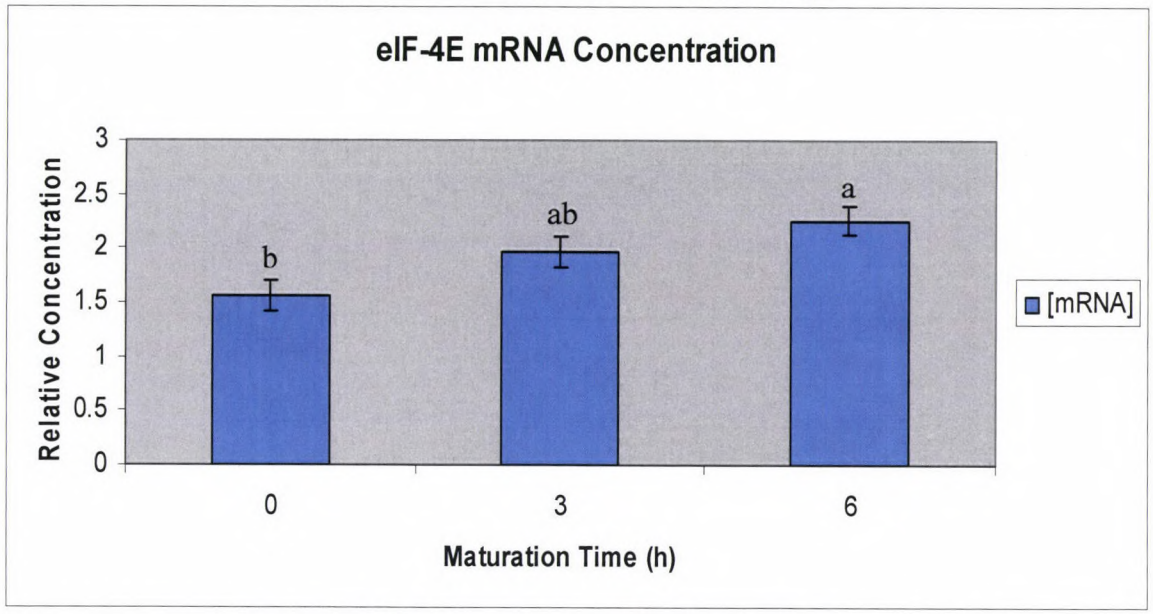
levels for PAP-1, eIF-4E, and EDEN ($p<0.10$). There also were oocyte within cow effects on PARN mRNA concentration ($p<0.10$). eIF-4E concentration was also affected by maturation type ($p<0.10$) and time ($p<0.05$). In vivo-matured oocytes (Figure 3.6) contained more eIF-4e mRNA than in vitro-matured oocytes averaged over time ($p<0.10$). Averaged over maturation method, eIF-4E concentration increased over 6 h of maturation (Figure 3.7) ($p<0.05$). Figure 3.8 shows that PARN mRNA concentration was also affected by time of oocyte maturation, with oocytes matured for 6 h containing higher levels of PARN transcripts ($p<0.10$).

Figure 3.6: eIF-4E mRNA concentration for main effect in vitro- and in vivo-matured oocytes. Values displayed are 1+log10([eIF-4E]/[H2a.1]).



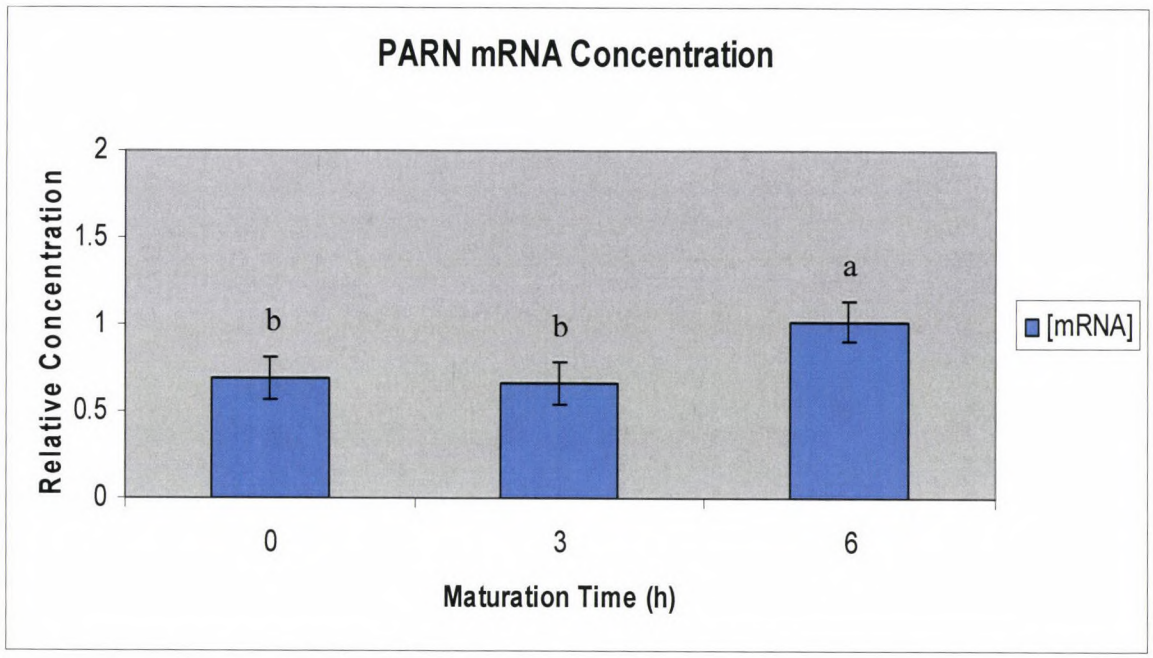
[ab]Values with differ P<0.10.

Figure 3.7: eIF-4E mRNA main effect concentration changes over time of maturation. Values displayed are 1+log10([eIF-4E]/[H2a.1]).



[ab]Values with differ P<0.05.

Figure 3.8. PARN mRNA concentration main effect changes over time of maturation. Values displayed are 1+log10([PARN]/[H2a.1]).



[ab]Values with differ P<0.10.

Discussion and Conclusions

Oocytes build up maternal mRNA during the growth phase of oogenesis. Once oocytes reach preovulatory size, transcription decreases to minimal levels (Moore and Lintern-Moore, 1978). The oocyte and embryo must use the maternal pool of mRNA during oocyte maturation and early embryonic development until activation of the embryonic genome. The majority of the material needed for these early stages is accumulated and stored over time. However, production of specific transcripts clearly continues throughout the initial phases of oocyte maturation and early embryonic development (Fair et al., 2007). We chose to examine the mRNA concentrations in early oocyte maturation of four genes involved in mRNA processing.

eIF-4E is an initiator of translation that binds the 5'-methyl cap of mRNA and targets the RNA for ribosomal translation. It also plays a role in regulating RNA storage by interacting with maskin to block ribosomal protein binding via steric interactions (Barnard et al., 2005). We chose this gene for its integral role in mRNA processing. mRNA concentration for eIF-4E increased over time and was higher in in vivo- than in vitro-matured oocytes. As oocyte maturation progresses, more and more processes are activated; therefore, more and more proteins are needed. Increased production of eIF-4E mRNA likely corresponds with an increasing need for eIF-4E protein to be involved in translation or even storage. eIF-4E mRNA concentration may be indicative of developmental competence where more competent oocytes contain more eIF-4E; alternatively one response of oocytes to the LH surge could be production of eIF-4E mRNA. In the present study, in vivo-matured oocytes were recovered from cows treated with GnRH to stimulate the LH surge. In vivo-matured oocytes had higher

concentrations of eIF-4E mRNA upon aspiration from the follicle than in vitro-matured oocytes. This suggests that the in vivo-matured oocytes simply start producing eIF-4E earlier than in vitro-matured oocyte. Overall, the levels of eIF-4E increased over the time points chosen here for early maturation. eIF-4E appears to be one gene whose transcription does not cease upon completion of the oocytes growth during oogenesis.

Another gene involved in translation is poly(A) polymerase-1 (PAP-1). PAP-1 increases the length of the poly(A) tail. Increased poly(A) tail length results in unmasking mRNA, allowing it to be translated after specific proteins bind the mRNA molecules. PAP-1 mRNA concentration showed little change over time and was expressed at similar levels in the in vitro- and in vivo-matured oocytes. PAP-1 mRNA production did not increase over the period of oocyte maturation examined in the present study. The oocyte appears to produce PAP-1 mRNA early and rely on this stockpile for early maturation and embryonic development. PAP-1 has additional roles in maintaining mRNA stability in addition to translation as will be discussed later.

The final two genes we chose to analyze are involved in mRNA degradation. PARN is responsible for degrading the poly(A) tail, eventually shortening it to the point where the 3' end of the mRNA is exposed to ribonuclease degradation. PARN mRNA concentration changed little over the early stages of oocyte maturation with a sharp increase in mRNA levels after 6 h of maturation ($P<0.10$). PARN is potentially involved in all thee processes of mRNA processing; mRNA stability, translation, and degradation. Kim and Richter (2006) showed that mRNA storage is controlled though constant shortening and lengthening of the poly(A) tail driven by PAP-1 and PARN. If this mechanism is truly the major driver of mRNA stability in mammalian oocytes, both PAP-

1 and PARN mRNA would be produced early in oocyte development, with stable levels of transcript and protein being needed throughout early embryonic development. The increase in PARN mRNA concentration observed in oocytes 6 h after maturation may correspond to an increased need for PARN to aid in degradation of maternal RNA transcripts after their use for triggering oocyte maturation.

The other transcript studied involved in RNA degradation in the present study was first identified in xenopus embryos. Richter (1999) found that EDEN targets transcripts containing specific sequences for degradation after fertilization. Their research showed that EDEN could play a role in controlled degradation events. Later, Morales et al. (2006) showed that EDEN plays a role in targeting PARN to the poly(A) tail of mRNA transcripts. EDEN's role in driving degradation was via recruiting a deadenylase to expose the 3' end of mRNA to degradation the exosome. We chose to study mRNA concentrations of EDEN during oocyte maturation due to its cooperative role with PARN in driving RNA degradation. However, there was no change in the concentration of EDEN mRNA over the duration of the experiment. Like PARN, EDEN concentrations were accumulated before maturation and did not change upon initiation of maturation by removal from the follicle or exposure to the LH surge.

Lequarre et al. (2004) showed that the total amount of polyadenylated mRNA decreases during early embryogenesis. However, our data clearly show that some transcripts actually increase in concentration during early maturation as also shown by (Fair et al., 2007). Likely, multiple genes are transcribed during these early stages of development, and these transcripts may play a role in regulating the maternal pool. The four genes chosen in the present study were selected due to their role in RNA regulation.

Identifying specific genes responsible for regulating the storage, translation, and degradation of the maternal pool may provide clues about control of mRNA in competent oocytes, and more importantly, protein composition. The possibility exists that changes in concentration may actually be occurring at the protein level rather than the mRNA pool; also changes may occur later in maturation or even in early stages of embryonic development.

Chapter IV

Timing of deadenylation of cyclin B1 and GDF-9 3' UTR constructs in bovine oocytes

Abstract

The objective of this study was to determine how poly-(A) tail length of transcripts is processed in bovine oocytes by time of in vitro maturation. Cyclin B1 and GDF-9 3' untranslated regions (UTRs) were cloned into modified p-GEM plasmids containing a poly-(A) tract of 60 or 0 adenosines (A60 or A0, respectively). Each 3' UTR was transcribed in vitro with or without a poly-(A) tail to generate UTP^{32} labeled RNA. Transcriptions producing at least 200,000 counts per min per mL were used for subsequent injections into denuded bovine oocytes. Cumulus-oocyte complexes (n=576) recovered from slaughterhouse-derived ovaries were vortexed to remove cumulus cells immediately after aspiration, after 3 h of in vitro maturation, or after 19 h of maturation in a chemically defined medium. Denuded oocytes were injected and snap frozen, or cultured in vitro for 1 or 3 h. Eight oocytes were injected with ~0.5 nL labeled RNA at each time point in four replicates. Total RNA was isolated from injected oocyte pools and loaded onto a 5% denaturing acrylamide gel for size separation. Radiolabeled A0 was used as a control point of reference for deadenylation. Cyclin B1 3' UTR A60 began shortening by 1 h and was mostly degraded by 3 h regardless of cell cycle stage (0, 3, or 19 h of maturation). GDF-9 3' UTR A60 remained stable in oocytes injected immediately or 3 h after aspiration. Oocytes matured 19 h maintained stable GDF-9 3'UTR A60 until 1 h after injection, after which GDF-9 3' UTR A60 degraded rapidly. Thus, the polyadenylated GDF-9 3' UTR is regulated differently than the Cyclin B1

polyadenylated 3' UTR during oocyte maturation. Furthermore, this research shows that RNA produced in vitro and injected into bovine oocytes can be recovered with sufficient sensitivity to test hypotheses in a physiologically relevant system

Introduction

Understanding how mammalian oocytes process the maternal pool of mRNA may provide insight into the deficiencies of in vitro-maturation. Current protocols for producing embryos in vitro yield approximately half the number of blastocyst stage embryos compared with in vivo-production (Rizos et al. 2002). Rizos et al. (2002) showed successful embryo production depended on maturation environment. The process of oocyte maturation is complex and is orchestrated by coordination between signal transduction, gene expression/transcription, and mRNA translation. Although these processes are complex and not yet fully understood, we know that the oocyte is a unique cell in that it builds up a store of mRNA and then arrests most transcription and translation until activation by the LH surge or aspiration from its inhibitory environment (Rodriguez and Farin, 2004). Maturation and early embryonic development are supported by this maternal pool of mRNA. Regulation of the pool of mRNA via degradation, storage, and translation drives embryonic development.

Fate for most RNA molecules is controlled by the poly(A) tail. Many studies have been performed in cells where specific elements of the 3' untranslated region (UTR) are mutated to determine the role of each region in RNA stability (Sokoloski et al., 2008). Most of these studies were performed with cell extracts in test tubes. However, mammalian oocytes are large cells that can be manipulated intact and then cultured. This provides an opportunity to use an intact mammalian cell to observe RNA stability. Two

genes that have been extensively studied in mammalian oocytes are Cyclin B1 and GDF-9. The extent to which they have been characterized makes them ideal for use in developing a technique for tracking poly(A) tail changes in a living cell.

Cyclin B1 is a key component of MPF. Expression and regulation of cyclin B1 expression has been studied extensively. Cyclin B1 mRNA levels remain relatively constant throughout bovine oocyte maturation, but poly(A) tail length shortens over time (Lequarre et al., 2004). Use of cyclin B1 specific hexamer 3' primers showed that levels of cyclin B1 during maturation increase while use of oligo (dT) 3' primers revealed a decrease in cyclin B1 levels during maturation (Lequarre et al., 2004). The authors state that the poly(A) tail of cyclin B1 must shorten during maturation, inhibiting oligo dT's ability to bind the poly(A) tail for reverse transcription. Although the cyclin B1 poly(A) tail shortens throughout maturation, Tremblay et al. (2005) showed that cyclin B1 actually undergoes polyadenylation during the first 10 h of maturation, coinciding with the appearance of cyclin B1 protein in the bovine oocyte. Research on cyclin B1 has shown that genes are regulated in a timely manner and do not necessarily follow the overall pattern of mRNA decreases observed by Lequarre et al. (2004).

GDF-9 is involved in initiating folliculogenesis, and as such is often linked with early follicular development (Dong et al., 1996). Recent studies on maternal RNA expression show that GDF-9 also is expressed at high levels in oocytes undergoing in vitro maturation (Dalbies-Tran and Mermillod, 2003; Donnison and Pfeffer, 2004; Vallee et al., 2005). In addition to being identified as a transcript expressed in maturing oocytes, Lonergan et al. (2003) showed that GDF-9 mRNA was expressed at significantly higher levels in oocytes matured in vitro than those matured in vivo. However, the role of GDF-

9 in early embryogenesis is unknown. The presence of GDF-9 during oocyte maturation may be critical for cumulus cell expansion (Eppig, 2001). Unlike Cyclin B1, little is known about regulation of GDF-9 during oocyte maturation, (Lonergan et al., 2003) except that GDF-9 expression levels in oocytes matured in vitro or in vivo differ. The fact that lower transcription levels exist during oocyte maturation points to RNA regulation as a key factor in altering expression of GDF-9, making it an interesting candidate for studying differences in RNA regulation for in vitro- and in vivo-matured bovine oocytes.

Materials and Methods

Cloning of Bovine Homologues of GDF-9 and Cyclin B1 3' UTRs

Bovine sequences for the 3' UTRs of Cyclin B1 and GDF-9 were used to design primers for RT-PCR using Invitrogen's OligoPerfect^{TM} primer design tool. mRNA was isolated from bovine ovaries using the Trizol method following manufacturer's directions. Resulting PCR products were cloned into pGEM-T easy vectors following the manufacturer's instructions. DH5α cells were transformed with the ligated pGEM-T easy vectors and screened for gene incorporation via PCR. Colonies that tested positive via PCR using previously established conditions were grown overnight and the plasmids recovered using Qiagen's maxi prep kit. The plasmids were sequenced by Macromolecular Resources at Colorado State University to ensure proper product amplification. GDF-9 and cyclin B1 3' UTRs were then cloned into modified p-GEM plasmids (Stratagene).

In Vitro Transcription of Radiolabeled 3' UTR Constructs

Plasmids containing a 60-nucleotide or 0-nucleotide poly(A) tract (gem A60 or A0, respectively) were cloned into the multiple cloning site. After positive identification of genes of interest, plasmids were digested with EcoR1 and Pst1. Digestion products were loaded into a 6% acrylamide gel to separate 3' UTRs of Cyclin B1 and GDF-9 from the rest of the plasmid. Bands corresponding to each product were dissected from the gel and products were eluted. Eluted cDNA was loaded into a transcription reaction with 32p labeled UTP. SP6 RNA polymerase was added to the reaction to create radiolabelled, polyadenylated 3' UTR regions. After 3 h of transcription at 37°C, radiolabelled RNA was loaded onto a 5% denaturing polyaccrylamide gel. Bands corresponding to products of interest were recovered from the gels and the products were re-eluted. RNA was then exposed to phenol:chloroform for purification and eventually precipitated in 5 µl of 0.15 M KCL; 1 µl was counted on a liquid scentillation counter to determine radioactivity. Only reactions that yielded at least 100,000 cpm/µl were used for injections.

Collection of Bovine Oocytes

Cumulus-oocyte complexes were aspirated 3-8 mm follicles of slaughterhouse-derived ovaries. Ovaries were transported to the laboratory in 0.15 M NaCl saline at 22 to 27°C. Upon arrival, oocytes were aspirated from follicles with vacuum pressure of ~50 mm Hg and an 18-gauge needle. Cumulus-oocyte complexes were isolated in 20 mM hepes- buffered chemically defined maturation medium (H-CDM-M) (De La Torre et al., 2006). 50 oocytes with intact cumulus cells and evenly granulated cytoplasm were matured in 1 mL CDM-M (CDM + 15 ng/mL ovine FSH-NIH, 1 µg/mL bovine LH-

USDA, 50 ng/mL EGF, 0.1 mM cysteamine, and 1.0 g/mL estradiol 17-β) at 39°C, 5% CO_2 in humidified air (De La Torre-Sanchez et al., 2006) for 0, 5, or 19 h. Once matured, oocytes were vortexed in H-CDM-M for 3 min to remove cumulus cells.

Injection of radiolabeled RNA

Denuded oocytes (n=24) were loaded into 50 μL H-CDM-M under filtered mineral oil. Microinjection was performed using Nareshigi micromanipulators and a Nikon inverted microscope. RNA was injected into the oocyte though handmade micropipettes. Approximately 0.5 nL of radiolabelled RNA was injected into each oocyte at each time point. After injection, oocytes were loaded into 140 μL PBS+0.2% PVA. Eight oocytes/treatment were then snap frozen in 1.5 mL snap cap tubes and stored at -20°C until RNA isolation.

Isolation of RNA from Pools of Bovine Oocytes

Oocytes were thawed, and RNA was recovered using Qiagen's viral micro isolation kit following the manufacturer's instructions. RNA was further purified using phenol: chloroform and then resuspended in RNA loading dye. A 5% denaturing acrylamide gel was then used to separate different sizes of constructs.

Analysis of Results

Gels were dried and examined on a phosphoimager. Cyclin B1 and GDF-9 products with a poly(A) tail of 60 or 0 adenosines not injected into oocytes were used as controls. Analysis of changes in construct size compared to the controls indicates how the cells responded to each 3' UTR with poly(A) tails.

Experimental Design

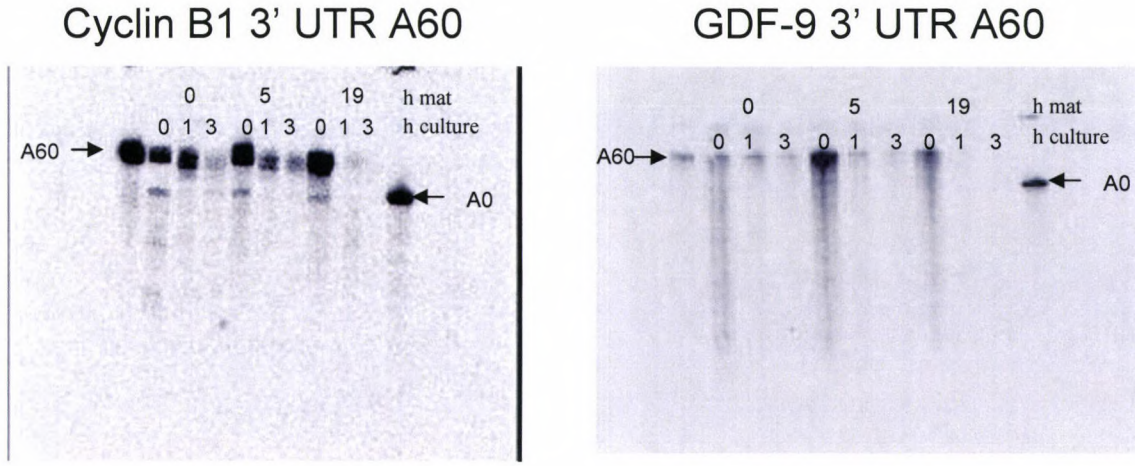
Cumulus oocyte complexes (n=72) were matured for 0, 5, or 19 h after aspiration in each of four replicates. After each maturation time point, oocytes (n=24) were vortexed and injected with Cyclin B1 or GDF-9 3' UTR with a poly(A) tail of 60 adenosines. Only one gene was injected in each trial. After injection, 8 oocytes were snap frozen immediately (time 0). The additional 16 oocytes were cultured in CDM-M, half for 1 h and half for 3 h. At the appropriate time, each group was frozen in 15 uL of RNase free water.

Results

Injection of A60 constructs

Upon injection into oocytes, 3'UTR regions for Cyclin B1 remained stable with degradation occurring after 1 h of culture, with most of the RNA being degraded after 3 h of culture in oocytes matured for 0 or 5 h. Oocytes matured for 19 h degraded Cyclin B1 3' UTR much more rapidly, with most being gone within 1 h (Figure 4.1). GDF-9 3' UTR A60 was degraded much more rapidly, with the majority of RNA being degraded immediately upon injection regardless of maturation timing (Figure 4.1). GDF-9 RNA also appeared to show more nonsense mediated decay compared to Cyclin B1 (Figure 4.1). GDF-9 maintained stability at A60 or was simply degraded. Cyclin B1 actually showed a band at A0, suggesting that the majority of degradation for Cyclin B1 was deadenylation driven (Figure 4.1). GDF-9 3'UTR constructs displayed no such A0 product.

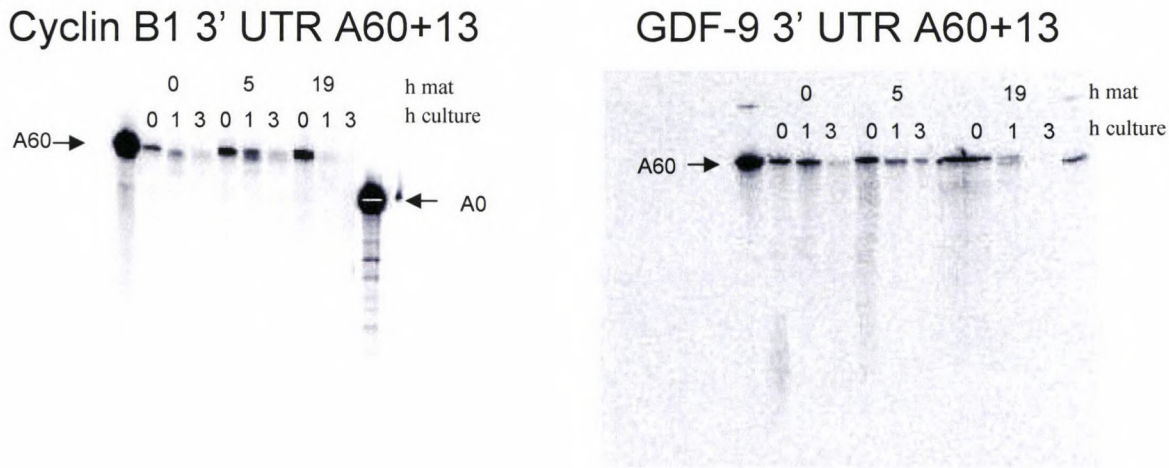
Figure 4.1: Cyclin B1 and GDF-9 3' UTR constructs with A60 poly(A) tail injected into bovine oocytes after 0, 5, or 19 h maturation and cultured for an additional 0, 1, or 3 h.



Injection of A60 constructs with 13 random nucleotides added

To determine if Cyclin B1 degradation was in fact deadenylation driven, 13 random nucleotides were added to end of the poly(A) tail. Simple addition of this random stretch of nucleotides to Cyclin B1 3' UTR produced a degradation pattern more like that seen in GDF-9 3' UTR A60 where RNA was fairly stable at A60 or was simply degraded randomly with no A0 intermediate (Figure 4.2).

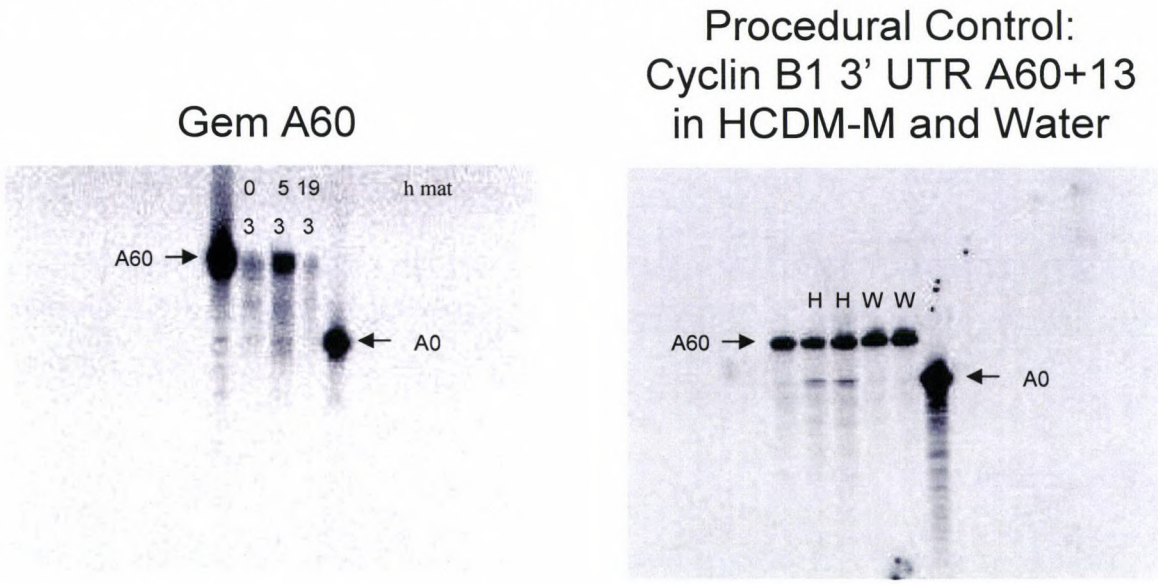
Figure 4.2: Injection of Cyclin B1 and GDF-9 3' UTR with A60 poly(A) tail plus 13 random nucleotides injected into bovine oocytes after 0, 5, or 19 h of maturation followed by an additional 0, 1, or 3 h of culture.



Injection Controls

Gem A60 plasmid inserts without the 3' UTR regions for each gene were injected into bovine oocytes to determine if the 3' UTR region had any influence on degradation or if the cell simply responded to introduction of foreign RNA with degradation. After 3 h of culture, Gem A60 was degraded in a pattern consistent with that of GDF-9 3'UTR A60 constructs, with no A0 intermediate being displayed. Injection of Cyclin B1 3' UTR A60+13 into RNase free water and HCDM-M provided an indicator of how much degradation was due to procedural techniques (Figure 4.3). Figure 4.3 shows that RNA handling, injection, and RNA recovery produced little degradation.

Figure 4.3: Injection of Gem A60 plasmid into bovine oocytes and Cyclin B1 3' UTR with A60 poly(A) tail and 13 random nucleotides into holding medium and water.



Discussion and Conclusions

The purpose of this experiment was to determine if in vitro transcribed radiolabelled RNA could be injected into bovine oocytes to track polyadenylation status. This allows use of intact living cells to perform experiments on 3' UTR driven regulation

of genes of interest. This experiment showed that RNA could indeed be injected into oocytes and recovered intact and evaluated.

Cyclin B1 was regulated though deadenylation as shown in Figure 4.1. The cyclin B1 A60 poly(A) tail was shortened over time until it reached A0. Afterwards, Cyclin B1 was degraded. GDF-9 displayed more of a nonsense mediated decay pattern with no A0 intermediate appearing. This suggests that the GDF-9 was either maintained stably at A60 or simply degraded rapidly. Differences in regulation could stem from differences in the role for each gene and may provide insight into how genes are regulated differently. GDF-9 is thought to participate in cumulus expansion in maturing bovine oocytes (Eppig, 2001), an event that is completed by 22 h of maturation in bovine oocytes. Further studies are needed to determine if endogenous GDF-9 mRNA follows a similar expression pattern in bovine oocytes and to determine which portion of GDF-9 3' UTR confers stability in bovine oocytes.

Simply examining the lanes and looking at smears of RNA does not prove that deadenylation dependent decay and nonsense mediated decay were both occurring. Cyclin B1 A0 could be an artifact. To prove that Cyclin B1 3' UTR A60 was indeed shortened to an A0 intermediate, 13 random nucleotides were added onto the end of the transcript. This would interrupt deadenylation dependent decay and stimulate nonsense mediated decay, forcing Cyclin B1 A60 to perform more like GDF-9 A60. Figure 2 showed that indeed, Cyclin B1 3'UTR A60 became more stable, persisting though most time points like GDF-9 3' UTR A60, with A0 intermediate no longer appearing. This proves that Cyclin B1 3' UTR A60 injected into bovine oocytes was undergoing deadenylation dependent decay.

However, the question still remained as to whether or not the decay observed in Figure 4.1 or 4.2 was actually a cell driven process or simply an artifact of RNA handling. Injection of gem A60 without the 3' UTR regions for GDF-9 and Cyclin B1 removed the elements that recruit RNA degradation proteins to RNA molecules. By doing this experiment, we were able to determine if the cell was simply responding to the introduction of foreign RNA by initiating degradation. The cell drove some random decay after gem A60 was injected, but gem A60 remained fairly stable over 3 hours of culture in bovine oocytes. Injection of Cyclin B1 3' UTR A60+13 into RNase free water and HCDM-M allowed us to account for degradation caused by RNA handling and processing. Very little decay was seen in this control, proving that our techniques were not causing most of the degradation seen when cells were injected with 3'UTRs.

This experiment proved that intact, living oocytes could be used to test deadenylation patterns of 3' UTR regions for genes of interest. Understanding how different 3' UTRs affect RNA degradation could allow RNA to be grouped into regulatory categories. Likely, multiple RNA molecules are regulated by a select group of proteins, and subtle differences in the 3' UTRs of each RNA regulate how that RNA is processed and when. The fact that GDF-9 and Cyclin B1 behaved differently in the current experiment may suggest that these two genes belong to different categories. Further examination of their 3' UTRs may provide insight into mechanisms of differential mRNA regulation. Possibly, the timing of the current study was not appropriate to track GDF-9. Endogenous RNA for each gene should be studied to determine if it behaves like our injected RNA.

Chapter V

Overall Conclusions

Embryonic development is profoundly affected by conditions of oocyte maturation. This initial step of embryo production is of critical importance for successful fertilization, implantation, and development. Current protocols for in vitro production of embryos rely too heavily on composition of follicular fluids and too little on understanding the sequence of events that lead up to oocyte maturation in vivo. Understanding what takes place after the LH surge in vivo will increase the chances that in vitro-production systems will be able to produce viable embryos efficiently.

The LH surge stimulates oocyte maturation in vivo by initiating a sequence of events that includes production of EGF-like factors that bind EGF receptors on the cumulus granulosa cells. Upon binding, these EGF-like factors induce phosphorylation events leading to gap junction closure and eventually a decrease in cAMP concentrations within the oocyte. However, little is know about how an unknown inhibitory molecule in follicular fluid is contravened by the LH surge. It is clear that this molecule or pathway exists, as removal of oocytes from the follicular fluid without LH induction results in a similar sequence of events present after the LH surge, with the final product being an oocyte arrested at metaphase II (Pincus and Enzmann, 1935).

Studies in our laboratory have identified a potential model for studying the interaction between the LH stimulatory pathway and an unidentified inhibitory pathway. Failure to regress the corpus luteum before stimulation of an LH surge blocks oocyte maturation. This finding suggests that the inhibitory pathway is stronger than the stimulatory ability of LH. By examining known steps of oocyte maturation in cows with

luteal levels of progesterone and cows exposed to PGF2α to regress the corpus luteum, we have attempted to identify where the disconnect in oocyte maturation occurs in the presence of progesterone. Our results showed that although the LH surge occurred and EGF like factor transcripts increased, cAMP levels remained stable across treatments, with in vivo-matured oocytes actually having increased levels of cAMP. Culture of oocytes for 30 min after removal from follicles yielded oocytes with lower levels of cAMP, suggesting that phosphodiesterase activity was normal and the oocytes were capable of undergoing maturation.

These findings suggest that cAMP production continues in the oocyte despite production of the stimulatory LH surge. With cAMP levels being maintained through the inhibitory signaling cascade, the LH surge is unable to drive oocyte maturation when progesterone is present. When progesterone is removed via regression of the corpus luteum by PGF2α, in vivo-matured oocytes resume meiosis and proceed to metaphase II after a decrease in cAMP that triggers oocyte maturation before recovery from the follicle. Their intracellular signaling pathways are more advanced in timing compared to the negative control or the groups exposed to the LH surge. Mehlmann et al. (2004) identified a g-protein coupled receptor (GPR-3) present in the oocyte that is connected to adenylate cyclase. Knocking out this receptor yields metaphase II oocytes in mice regardless of treatment. The ligand for this receptor is likely produced in thecal cells when the follicle is under the influence of luteal phase levels of progesterone. At this time, it is unclear if progesterone actually drives the production of this molecule or if some other molecule is responsible. Interestingly, the ability of this inhibitory molecule is overcome during the follicular phase of the estrous cycle. This suggests that something

being produced by the corpus luteum is involved in stimulating production of the GPR-3 ligand in thecal cells.

In addition to understanding external events driving in vivo-maturation, focus has shifted to gene profiling in in vitro- and in vivo-matured oocytes to compare concentrations of specific transcripts between these groups. However, mRNA concentrations are greatly affected by posttranscriptional mRNA processing. Likely, a handful of proteins actually control the fates of hundreds of transcripts stored in the maternal pool of mRNA. Variations in protein or transcript levels in these select genes could result in the aberrant mRNA profiles often seen when examining in vitro-matured oocytes. Microarray studies have identified gene groups involved in posttranscriptional regulation as major areas of difference between the gold standard in vivo-matured oocyte and the in vitro-matured variant (Corcoran et al., 2006).

Although results in the present study showed little change in mRNA levels for the selected genes, this does not rule their importance, nor does it rule out that the genes responsible for posttranscriptional regulation is of critical importance. The mRNA profiles for these genes need to be studied over a wider window of oocyte maturation and early embryonic development. More importantly, protein profiles need to be studied, although current technology does not allow this on such small samples.

More work is needed to determine the mechanisms for RNA regulation in the maternal pool of mRNA. The oocyte is unique in that it builds up a stockpile of messages and proteins to use during its early stages of development and does so in a step by step process that is interrupted during specific phases of arrest. This requires precise

regulation of the pool of mRNA and proteins to successfully develop into a competent metaphase II oocyte capable of being fertilized for development into a fetus.

Literature Cited

Aktas H., Wheeler M.B., First N.L., Leibfried-Rutledge M.L. Maintenance of meiotic arrest in vitro by elevating [cAMP]i have physiological relevance in bovine oocytes. J Reprod Fert 1995a; 105: 237-245.

Aktas H., Wheeler M.B., Rosenkrans C.F., First N.L., Leibfried-Rutledge M.L. Maintenance of bovine oocytes in prophase of meiosis I by high [cAMP]i. J Reprod Fert 1995b; 105: 227-235.

Ali A. and Sirard M.A. The effects of 17 beta-estradiol and protein supplement on the response to purified and recombinant follicle stimulating hormone in bovine oocytes. Zygote 2002; 10: 65-71.

Ali A. and Sirard M.A. Protein kinases influence bovine oocyte competence during short-term treatment with recombinant human follicle stimulating hormone. Reproduction 2005; 130: 303-310.

Algire J.E., Srikandakumar A., Guilbault L.A., Downey B.R. Preovulatory changes in follicular prostaglandins and their role in ovulation in cattle. Can J Vet Res 1992; 56: 67-69.

Amiridis G.S., Valasi I., Menegatos I., Rekkas C., Goulas P., Papanikolaou T., Deligiannis C. Luteal stage dependence of pituitary response to gonadotrophin-releasing hormone in cyclic dairy ewes subjected to synchronization of ovulation. Reprod Fert Dev 2005; 17: 769-774.

Atkins J.A., Busch D.C., Bader J.F., Keisler D.H., Patterson D.J., Lucy M.C., Smith M.F. Gonadotropin-releasing hormone-induced ovulation and luteinizing hormone release in beef heifers: effect of day of the cycle. J Anim Sci 2008; 86: 83-93.

Barnard D.C., Ryan K., Manley J.L., Richter J.D. Symplekin and xGld-2 are required for CPEB-mediated cytoplasmic polyadenylation. Cell 2004; 119: 641-651.

Barnard D.C., Cao Q., Richter J.D. Differential phosphorylation controls Maskin association with eukaryotic translation initiation factor 4E and localization on the mitotic apparatus. Mol Cell Biol 2005; 25: 7605-7615.

Bilodeau S., Fortier M.A., Sirard M.A. Effect of adenylate cyclase stimulation on meiotic resumption and cyclic AMP content of zona-free and cumulus-enclosed bovine oocytes in vitro. J Reprod Fert 1993; 97: 5-11.

Blondin P., Bousquet D., Twagiramungu H., Barnes F., Sirard M.A. Manipulation of follicular development to produce developmentally competent bovine oocytes. Biol Reprod 2002; 66: 38-43.

Bo G.A., Adams G.P., Nasser L.F., Pierson R.A., Mapletoft R.J. Effect of estradiol valerate on ovarian follicles, emergence of follicular waves and circulating gonadotropins in heifers. Theriogenology 1993; 40: 225-239.

Brevini T.A.L., Lonergan P., Cillo F., Franscisci C., Favetta L.A., Fair T., Gandolfi F. Evolution of mRNA polyadenylation between oocyte maturation and first embryonic cleavage in cattle and its relation with developmental competence. Mol Reprod Dev 2002; 63: 510-517.

Brevini-Gandolfi T.A.L., Favetta L.A., Mauri L., Luciano A.M., Cillo F., Gandolfi F. Changes in poly(A) tail length of maternal transcripts during in vitro maturation of bovine oocytes and their relation with developmental competence. Mol Reprod Dev 1999; 52: 427-433.

Burke C.R., Boland M.P., Macmillan K.L. Ovarian responses to progesterone and oestradiol benzoate administered intravaginally during diestrus in cattle. Anim Reprod Sci 1999; 55: 23-33.

Callesen H., Greve T., Hyttel P. Premature ovulations in superovulated cattle. Theriogenology 1986; 25: 71-86.

Cavalieri J., Heworth G., Parker K.I., Wright P.J., Macmillan K.L. Effect of treatment with progesterone and oestradiol when starting treatment with an intravaginal progesterone releasing insert on ovarian follicular development and hormonal concentrations in holstein cows. Anim Reprod Sci 2003; 76: 177-193.

Channing C.P., Schaerf F.W., Anderson L.D., Tsafriri A. Ovarian follicular and luteal physiology. Int Rev Physiol 1980; 22: 117-201.

Copeland P.R. and Wormington M. The mechanism and regulation of deadenylation: Identification and characterization of Xenopus PARN. RNA 2001; 7: 875-886.

Corcoran D., Fair T., Park S., Rizos D., Patel O.B., Smith G.W., Coussens P.M., Ireland J.J., Boland M.P., Evans A.C.O., Lonergan P. Suppressed expression of genes involved in transcription and translation in in vitro compared with in vivo cultured bovine embryos. Reproduction 2006; 131: 651-660.

Corden J.L., Cadena D.L., Ahearn J.M., Dahmus M.E. A unique structure at the carboxyl terminus of the largest subunit of RNA Polymerase II. Proc Natl Acad Sci 1985; 82: 7934-7938.

Crozet N., Kanka J., Motlik J., Fulka J. Nucleolar fine structure and RNA synthesis in bovine oocytes from antral follicles. Gamete Res 1986; 42: 437-442.

Cummings A. and Sommerville J. Protein kinase activity associated with stored messenger ribonucleoprotein particles of xenopus oocytes. J Cell Biol 1988; 107: 45-56.

Dalbies-Tran R., Mermillod P. Use of heterologous complementary DNA array screening to analyze bovine oocyte transcriptome and its evolution during in vitro maturation. Biol Reprod 2003; 68: 252-261.

Dahmus M.E. Structural relationship between the large subunits of calf thymus RNA Polymerase II. J Biol Chem 1983; 258: 3956-3960.

Dehlin E., Wormington M., Korner C.G., Wahle E. Cap-dependent deadenylation of mRNA. Embo J 2000; 19 (5): 1079-1086.

de Moor C.H. and Richter J.D. Translational control in vertebrate development. Int Rev Cytol 2001; 203: 567-608.

de Moor C.H., Meijer H., Lissenden S. Mechanisms of translational control by the 3' UTR in development and differentiation. Seminars in Cell Dev Biol 2005; 16: 49-58.

De La Torre-Sanchez JF, Preis KM, Seidel, G.E., Jr. Metabolic regulation of in vitro-produced bovine embryos. I. Effects of metabolic regulators at different glucose concentrations. Effects of metabolic regulators at different glucose concentrations with embryos produced by semen of different bulls. Reprod Fert Dev 2006; 18: 585-596.

Detivaud L., Pascreau G., Karaiskou A., Osborne H.B., Kubiak J.Z. Regulation of EDEN-dependent deadenylation of Aurora A/Eg2-derived mRNA via phosphorylation and dephosphorylation in Xenopus laevis egg extracts. J Cell Sci. 2003; 116: 2697-2705.

Dong J., Albertini D.F., Nishimori K., Kumar T.J., Lu N., Matzuk M.M. Growth differentiation factor-9 is required during early ovarian folliculogenesis. Nature 1996; 383: 531-535.

Donnison M. Pfeffer P.L. Isolation of genes associated with developmentally competent bovine oocytes and quantification of their levels during development. Biol Reprod 2004; 71: 1813-1821.

Elsden R.P., Hasler J.F., Seidel G.E. Jr. Non-surgical recovery of bovine eggs. Theriogenology 1976; 6: 523-532

Eppig J.J. and Downs S.M. Chemical signals regulate mammalian oocyte maturation. Biol Reprod 1984; 30: 1-11.

Eppig J.J. and Downs S.M. The effect of hypoxanthine on mouse oocyte growth and development in vitro: maintenance of meiotic arrest and gonadotropin-induced oocyte maturation. Dev Biol 1987; 119: 313-321.

Eppig J.J., O'Brien M.J., Pendola F.L., Watanabe S. Factors affecting the developmental competence of mouse oocytes grown in vitro: follicle-stimulating hormone and insulin. Biol Reprod 1998a; 59: 1445-1453.

Eppig J.J., Pendola F.L., Wigglesworth K. Mouse oocytes suppress cAMP-induced expression of LH receptor mRNA by granulosa cells in vitro. Mol Reprod Dev 1998b; 49: 327-332.

Eppig J.J. Oocyte control of ovarian follicular development and function in mammals. Reproduction 2001; 122: 829-938.

Eppig J.J., Vivieros M.M., Marin-Bivens C., De La Fuente R. Regulation of mammalian oocyte maturation. In The Ovary 2004; pp. 113-129. Eds PCK Leung & EY Adashi. Amsterdam: Elsevier Academic Press.

Fair T., Hyttel P., Greve T. Bovine oocyte diameter in relation to maturational competence and transcriptional activity. Mol Reprod Dev 1995; 42: 437-442.

Fair T., Carter F., Park S., Evans A.C.O., Lonergan P. Global gene expression analysis during bovine oocyte in vitro maturation. Theriogenology 2007; 68S: S91-S97

Fan H.Y., Su Q.Y. Involvement of mitogen-activated protein kinase cascade during oocyte maturation and fertilization in mammals. Biol Reprod 2004; 70: 535-547.

Fitz T.A., Mayan M.H., Sawyer H.R., Niswender G.D. Characterization of two steroidegenic cell types in the ovine corpus luteum. Biol Reprod 1982; 27: 703-711.

Fortune J.E. and Hansel W. Concentrations of steroids and gonadotropins in follicular fluid from normal heifers and heifers primed for superovulation. Biol Reprod 1985; 32: 1069-1079.

Franz L.C., Cho Y.H., Squires E.L., Seidel Jr G.E., Hinrichs K. Effects of roscovitine on maintenance of the germinal vesicle in horse oocytes, subsequent nuclear maturation, and cleavage rates after intracytoplasmic sperm injection. Reproduction 2003; 125: 693-700.

Fulka Jr. J., Leibfried-Rutledge M.L., First N.L. Effect of 6-dimethylaminopurine on germinal vesicle breakdown of bovine oocytes. Mol Reprod Dev 1991; 29: 379-384.

Garneau N.L., Wilusz J., Wilusz C.J. The highways and byways of mRNA decay. Nature Reviews 2007; 8: 113-126.

Gouveia Nogueira M.F., Barros B.J.P., Teixeira A.B., Trinca L.A., D'Occhio M.J., Barros C.M. Embryo recovery and pregnancy rates after the delay of ovulation and fixed time insemination in superstimulated beef cows. Theriogenology 2002; 57: 1625-1634.

Gu W., Tekur S., Reinbold R., Eppig J.J., Choi Y.C., Zheng J.Z., Murray M.T., Hecht N.B. Mammalian male and female germ cells express a germ-cell specific Y-box protein, MSY-2. Biol Reprod 1998; 59: 1266-1274.

Gurevich M., Harel-Markowitz E., Marcus S., Shore L.S., Shemesh M. Prostaglandin production by the oocyte cumulus complex around the time of fertilization and the effect of prostaglandin E on the development of the early bovine embryo. Reprod Fert Dev 1993; 5: 281-283.

Hendriksen P.J.M., Bos P.L.A.M. Steenweg W.N.M., Bevers M.M., Dieleman S.J. Bovine follicular development and its effect on the in vitro competence of oocytes. Theriogenology 2000; 53: 11-20.

Hinckley M., Vaccari S., Horner K., Chen R., Conti M. The G-protein-coupled receptors GPR3 and GPR12 are involved in cAMP signaling and maintenance of meiotic arrest in rodent oocytes. Dev Biol 2005; 287: 249-261.

Homa S.T. Effects of cyclic AMP on the spontaneous meiotic maturation of cumulus-free bovine oocytes cultured in chemically defined medium. J Exp Zool 1988; 248: 222-231

Hyttel P., Fair T., Callesen H., Greve T. Oocyte growth, capacitation and final maturation in cattle. Theriogenology 1997; 47: 23-32.

Izadyar F., Zeinstra E., Bevers M.M. Follicle-stimulating hormone and growth hormone act differently on nuclear maturation while both enhance developmental competence of in vitro matured bovine oocytes. Mol Reprod Dev 1998; 51: 339-345.

Johnson G., Kannan B., Shoyab M., Stromberg K. Amphiregulin induces tyrosine phosphorylation of the epidermal growth factor receptor and p185erbB2. Evidence that amphiregulin acts exclusively though the epidermal growth factor receptor at the surface of human epithelial cells. J Biol Chem 1993; 268: 2924-2931.

Karlach V. The effect of FSH, LH, oestradiol-17β, and progesterone on cytoplasmic maturation of bovine follicular oocytes in vitro. Folia Biologica 1982; 33: 268-265.

Kastrop P.M.M., Hulshof S.C.J., Bevers M.M., Destree O.H.J., Kruip Th.A.M. The effects of α-amanitin and cycloheximide on nuclear progression, protein synthesis and phosphorylation during bovine oocyte maturation in vitro. Mol Reprod Dev 1991a; 28: 249-254.

Kastrop M., Bevers M.M., Destree O.H.J., Kruip Th.A.M. Protein synthesis and phosphorylation patterns of bovine oocytes maturing in vivo. Mol Reprod Dev 1991b; 29:271-275.

Kalinowski R.R., Berlot C.H., Jones T.L., Ross L.F., Jaffe L.A., Mehlmann L.M. Maintenance of meiotic prophase arrest in vertebrate oocytes by a Gs protein-mediated pathway. Dev Biol 2004; 267: 1-13.

Kim J.H. and Richter J.D. Opposing polymerase-deadenylase activities regulate cytoplasmic polyadenylation. Molecular Cell 2006; 24: 173-183.

Kotani T., Yamashita M. Discrimination of the roles of MPF and MAP kinase in morphological changes that occur during oocyte maturation. Dev Biol 2002; 252: 271-286.

Kusov Y.Y., Shatirishvili G., Dzagurov G., Gauss-Muller V. A new G-tailing method for the determination of the poly(A) tail length applied to hepatitis A virus RNA. Nucleic Acids Res 2001; 29: E57-57.

Lane E.A., Austin E.J., Roche J.F., Crowe M.A. The effect of estradiol benzoate or a synthetic gonadotropin-releasing hormone used at the start of a progesterone treatment on estrous response in cattle. Theriogenology 2000; 56: 79-90.

Larimer F.W., Hsu C.L., Maupin M.K., Stevens A. Characterization of the XRN1 gene encoding a 5'-3' exoribonuclease: sequence data and analysis of disparate protein and mRNA levels of gene-disrupted yeast cells. Gene 1992; 120: 51-57.

Lindbloom S.M., Farmerie T.A., Clay C.M., Seidel Jr. G.E., Carnevale E.M. Potential involvement of EGF-like growth factors and phosphodiesterases in initiation of equine oocyte maturation. Anim Reprod Sci 2008; 103: 187-192.

Lequarre A.S., Traverso J.M., Marchandise J., Donnay I. Poly (A) RNA is reduced by half during bovine oocyte maturation but increases when meiotic arrest is maintained with CDK inhibitors. Biol Reprod 2004; 71: 425-431.

Lonergan P., Monaghan P., Rizos D., Boland M.P., and Gordon I. Effect of follicle size on bovine oocyte quality and developmental competence following maturation, fertilization, and culture in vitro. Mol Reprod Dev 1994; 37: 48-53.

Lonergan P., Dinnyes A., Fair T., Yang X., Boland M. Bovine oocyte and embryo development following meiotic inhibition with butyrolactone I. Mol Reprod Dev 2000; 57: 204-209.

Lonergan P., Gutierrez-Adan A., Rizos D., Pintado B., Fuente J.D.L., Boland M.P. Relative messenger RNA abundance in bovine oocytes collected in vitro or in vivo before and 20 h after the preovulatory luteinizing hormone surge. Mol Reprod Dev 2003; 66: 297-305.

Luciano A.M., Modina S., Vassena R., Milanesi E., Lauria A., Gandolfi F. Role of intracellular cyclin adenosine 3', 5'-monophosphate concentration and oocyte-cumulus cells communications on the acquisition of the developmental competence during in vitro maturation of bovine oocytes. Biol Reprod 2004; 70: 465-472.

Lynch S.S., Reay P., Bluck M. The extraction and purification of human pituitary follicle-stimulating hormone and luteinizing hormone. Acta Endocrinol Suppl 1988; 288: 12-19.

Marello K., LaRovere J., Sommerville J. Binding of Xenopus oocyte masking proteins to mRNA sequences. Nucleic Acids Res 1992; 20: 5593-6000.

Martinez M.F., Kastelic J.P., Bo G.A., Caccia M., Mapeltoft R.J. Effects of oestradiol and some of its esters on gonadotrophin release and ovarian follicular dynamics in CIDR-treated beef cattle. Anim Reprod Sci 2005; 86: 35-52.

Mayes M.A., Sirard M.A. Effect of type 3 and type 4 phosphodiesterase inhibitors on the maintenance of bovine oocytes in meiotic arrest. Biol Reprod 2002; 66: 180-184.

Mehlmann L.M., Jones T.L., Jaffe T.A. Meiotic arrest in the mouse follicle maintained by a Gs protein in the oocytes. Science 2002; 297: 1343-1345.

Mehlmann L.M., Yoshinaga S., Tanaka S., Brennan T.J., Evsikov A.V., Pendola F.L., Knowles B.B., Eppig J.J., Jaffe L.A. The Gs-linked receptor GPR3 maintains meiotic arrest in mammalian oocytes. Science 2004; 306: 1947-1950.

Memili E., Dominko T., First N.L. Onset of transcription in bovine oocytes and preimplantation embryos. Mol Reprod Dev 1998; 51: 36-41.

Memili E., First N.L. Developmental changes in RNA Polymerase II in bovine oocytes, early embryos, and effect of α-amanitin on embryo development. Mol Reprod Dev 1998; 51: 381-389.

Memili E., First N.L. Control of gene expression at the onset of bovine embryonic development. Biol Reprod 1999; 61: 1198-1207.

Moore G.P.M., Lintern-Moore S. Transcription of the mouse oocyte genome. Biol Reprod 1978; 17: 865-870.

Morales K.C.M., Wilusz C.J., Wilusz J. CUG-BP binds to RNA substrates and recruits PARN deadenylase. RNA 2006; 12: 1-8.

Park J.Y., Su Y.Q., Ariga M., Law E., Jin S.L.C., Conti M. EGF-like growth factors as mediators of LH action in the ovulatory follicle. Science 2004; 303: 682-684

Patel O.V., Suchyta S.P., Sipkovsky S.S., Yao J., Ireland J.J., Coussens P.M., Smith G.W. Validation and application of a high fidelity mRNA linear amplification procedure for profiling gene expression. Vet Immunol and Immunopathol 2005; 105: 331-342.

Pavlok A., Lucas-Hahn A., Niemann H. Fertilization and developmental competence of bovine oocytes derived from different categories of antral follicles. Mol Reprod Dev 1992; 31: 63-67.

Paynton B.V. RNA-binding proteins in mouse oocytes and embryos: expression of genes encoding y box, dead box RNA helicase, and poly A binding proteins. Dev Gen 1998; 23: 285-198.

Peng X.R., Hsueh A.J., La Polt P.S., Bjersing L., Ny T. Localization of luteinizing hormone receptor messenger ribonucleic acid expression in ovarian cell types during follicle development and ovulation. Endocrinology 1991; 129: 3200-3207.

Perry G.A., Perry B.L. Effect of the timing of controlled internal drug-releasing device insertion on the gonadotropin-releasing hormone-induced luteinizing hormone surge and ovulatory response. J Anim Sci 2009; 87: 3983-3990.

Pincus G., Enzmann E.V. The comparative behavior of mammalian eggs in vivo and in vitro: 1. The activation of ovarian eggs. J Exp Med 1935; 62: 665-675.

Richard F.J., Sirard M.A. Effects of follicular cells on oocyte maturation. II: Theca cell inhibition of bovine oocyte maturation in vitro. Biol Reprod 1996; 54: 22-28.

Richter J.D. Cytoplasmic polyadenylation in development and beyond. Microbiol. Mol. Biol. Rev. 1999; 63: 446-456.

Rinehart J. A Physician's View of Art Procedures and Legal Policy. http://www.thehumanfuture.org/commentaries/commentary_rinehart.html. 2005

Rizos D., Ward F., Duffy P., Boland M.P., Lonergan P., Consequences of bovine oocyte maturation, fertilization, or early embryo development in vitro versus in vivo: Implications for blastocyst yield and blastocyst quality. Mol Reprod Dev. 2002; 61: 234-248.

Robert C., Hue I., McGraw S., Gagne D., Sirard M.A. Quantification of cyclin B1 and p34cdc2 in bovine cumulus-oocyte complexes and expression mapping of genes involved in the cell cycle by complementary DNA macroarrays. Biol Reprod 2002; 67: 1456-1464.

Rodriguez K.F. and Farin C.E. Gene transcription and regulation of oocyte maturation. Reprod, Fert, and Dev 2004; 16: 55-67.

Sakurai H., Adams B.M., Adams T.E. Concentration of GnRH receptor and GnRH receptor mRNA in pituitary tissue of ovarectomized sheep: effect of oestradiol, progesterone, and progesterone withdrawal. J Endocr 1997; 152: 91-98.

Seli E., Lalioti M.D., Flaherty S.M., Sakka D., Terzi N., Steitz J.A. An embryonic poly (A)-binding protein is expressed in mouse oocytes and early preimplantation embryos. Proc. Nat. Acad. Sci. 2005; 102: 367-372.

Silva C.C., Knight P.G. Effects of androgens, progesterone and their antagonists on the developmental competence of in vitro matured bovine oocytes. J Reprod Fert 2000; 119: 261-269.

Simon M., Jilek F., Fulka Jr J., Effect of cycloheximide upon maturation of bovine oocytes. Reprod Nutr Dev 1989; 29: 533-540.

Sirard M.A., Florman H.M., Leibfried-Rutledge M.L., Barnes F.L., Sims M.L., First N.L. Timing of nuclear progression and protein synthesis necessary for meiotic maturation of bovine oocytes. Biol Reprod 1989; 40: 1257-1263.

Sirard M.A., Bilodeau S. Granulosa cells inhibit the resumption of meiosis in bovine oocytes in vitro. Bio Rep 1990; 43: 777-783.

Sirard M.A., Dufort I., Coenen K., Tremblay K., Massicotte L., Robert C. The use of genomics and proteomics to understand oocyte and early embryo functions in farm animals. Reproduction 2003; 61(Suppl): 117-129.

Sirois J. and Fortune J.E. Ovarian follicular dynamics during the estrous cycle in heifers monitored by real-time ultrasonography. Bio Reprod 1988; 39: 308-317.

Sirotkin A.V. Involvement of steroid hormones in bovine oocyte maturation in vitro. J Steroid Biochem 1992; 41: 855-858.

Smith M.S., Tenney D.Y. Effects of steroids on mouse oocyte maturation in vitro. J Reprod Fert 1980; 60: 331-338.

Smith S.L., Sung L.Y., Page R., Henderson B., Du F., Everts R.E., Nedambale T., Rodriguez-Zas S., Renard J.P., Lewin H.A., Yang X., Tian X.C. Expression profiling of single bovine embryos reveals significant effects of in vitro maturation, fertilization and culture. Reprod Fert Dev 2006; 18: 111 (Abstract)

Sokoloski K.J., Wilusz J., Wilusz C.J. The preparation and applications of cytoplasmic extracts from mammalian cells for studying aspects of mRNA decay. Methods Enzymol 2008; 448: 139-163

Stebbins-Boaz B., Cao Q., de Moor C.H., Mendez R., Richter J.D. Maskin is a CPEB-associated factor that transiently interacts with eIF-4E. Molecular Cell 1999; 4: 1017-1027.

Steiger M., Carr-Schmid A., Schwartz D.C., Kiledjan M., Parker R. Analysis of recombinant yeast decapping enzyme. RNA 2003; 9: 231-238.

Su Y.Q., Xia G.L, Byskov A.G., Fu G.D., Yang C.R. Protein kinase C and intracellular calcium are involved in follicle-stimulating hormone-mediated meiotic resumption of cumulus cell-enclosed porcine oocytes in hypoxanthine-supplemented medium. Mol Reprod Dev 1999; 53: 51-58.

Su Y.Q., Wigglesworth K., Pendola F., O'Brien M.H., Eppig J.J. Mitogen activated protein kinase activity in cumulus cells is essential for gonadotropin induced oocyte meiotic resumption and cumulus expansion in the mouse. Endocrinology 2002; 143: 2221-2232.

Summerville J. RNA-binding phosphoproteins and the regulation of maternal mRNA in Xenopus. J Reprod Fert 1990; 42(Suppl): 225-233.

Tay J., Hodgman R., Richter J.D. The control of cyclin B1 mRNA translation during mouse oocyte maturation. Dev Biol 2000; 221: 1-9.

Tay J., Hodgman R., Sarkissian M., and Richter J.D. Regulated CPEB phosphorylation during meiotic progression suggests a mechanism for temporal control of maternal mRNA translation. Genes and Dev 2003; 17: 1457-1462.

Tervit, H.R., Whittingham, D.C., and Rowson, L.E.A. Successful culture in vitro of sheep and cattle ova. J Reprod Fert 1972; 30: 493-497.

Thibier M. Transfers of both in vivo derived and in vitro produced embryos in cattle still on the rise and contrasted trends in other species in 2005. Embryo Transfer Newsletter 2006; 24 no. 4: 12-18.

Thomas R.E., Armstrong D.T., Gilchist R.B. Differential effects of specific phosphodiesterase isoenzyme inhibitors on bovine oocyte meiotic maturation. Dev Biol 2002; 244: 215-225.

Thomas R.E., Thompson J.G., Armstrong D.T., Gilchist R.B. Effect of specific phosphodiesterase isoenzyme inhibitors during in vitro maturation of bovine oocytes on meiotic and developmental capacity. Biol Reprod 2004; 71: 1142-1149.

Tremblay K., Vigneault C., McGraw S., Sirard M.A. Expression of cyclin B1 messenger RNA isoforms and initiation of cytoplasmic polyadenylation in the bovine oocyte. Biol Reprod 2005; 72: 1037-1044.

Walker D.J., De La Torre J.F., Seidel G.E. Effects of glucose concentration and presence of EGF and hormones on bovine oocyte maturation. J Dairy Sci 2003; 86(Suppl 1): 33 (abstract).

Weesner G.D., Harms P.G., McArthur N.H., Wilson J.M., Forrest D.W., Wu T.J., Pfaff D.W. Luteinizing hormone-releasing hormone gene expression in the bovine brain: anatomical localization and regulation by ovarian state. Biol Reprod 1993; 49: 431-436.

Wells S.E., Hillner P.E., Vale R.D., Sachs A.B. Circularization of mRNA by eukaryotic translation initiation factors. Mol Cell 1998; 2: 135-140.

Wilusz C.J., Wilusz J. Bringing the role of mRNA decay in the control of gene expression into focus. Genetics 2004; 20: 491-497.

Vallee M., Gravel C., Palin M.F., Reghenas H., Stothard P., Wishart D.S., Sirard M.A. Identification of novel and known oocyte-specific genes using complementary DNA subtraction and microarray analysis in thee different species. Biol Reprod 2005; 73: 63-71.

van de Leemput E.E., Vos P.L.A.M., Zeinstra E.C., Bevers M.M., van der Weijden G.C., Dieleman S.J. Improved in vitro embryo development using in vivo matured oocytes from heifers superovulated with a controlled preovulatory LH surge. Theriogenology 1999; 52: 335-349.

Viggiano J.M., Herrero M.B., Cebral E., Boquet M.G., de Gimeno M.F. Prostaglandin synthesis by cumulus-oocyte complexes: effects on in vitro fertilization in mice. Prostaglandins Leukotrines and Essential Fatty Acids 1995; 53: 261-265.

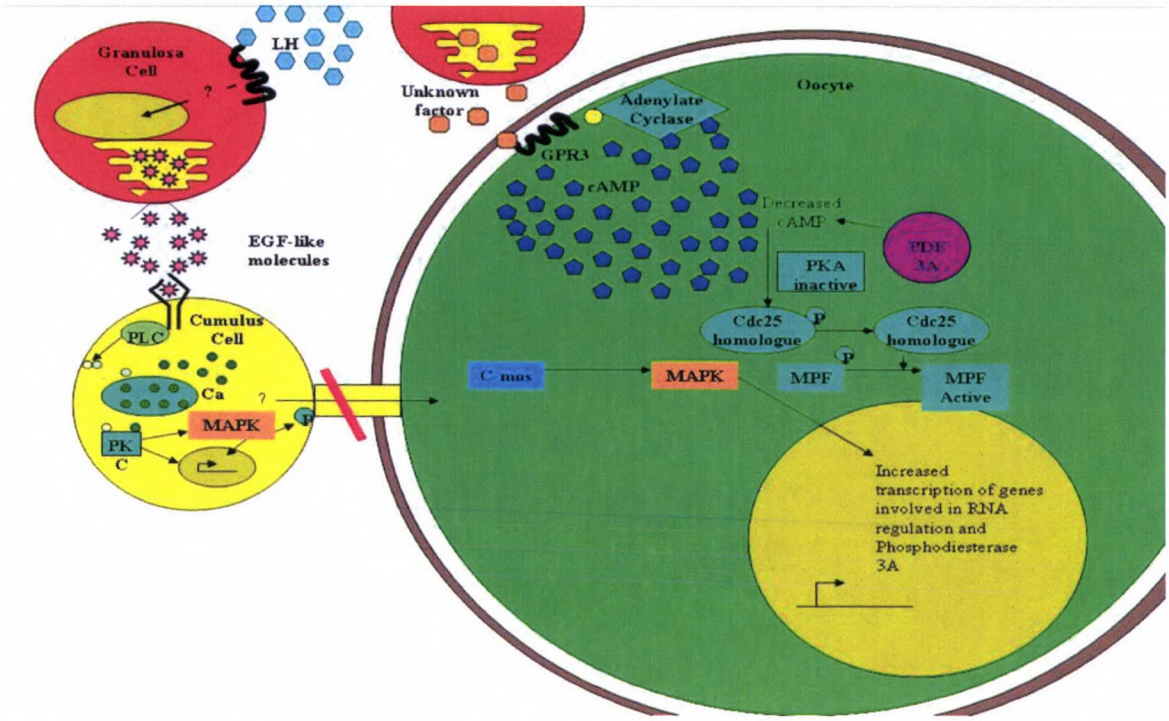
Vigneault C., Gilbert I., Sirard M.A., Robert C. Using the histone H2a transcript as an endogenous standard to study relative transcript abundance during bovine early development. Mol Reprod Dev 2007; 74: 703-715.

Vozzi C., Formenton A., Chanson A., Senn A., Sahli R., Shaw P., Nicod P., Germond M., Haefliger J.A. Involvement of connexin 43 in meiotic maturation of bovine oocytes. Reproduction 2001; 122: 619-628.

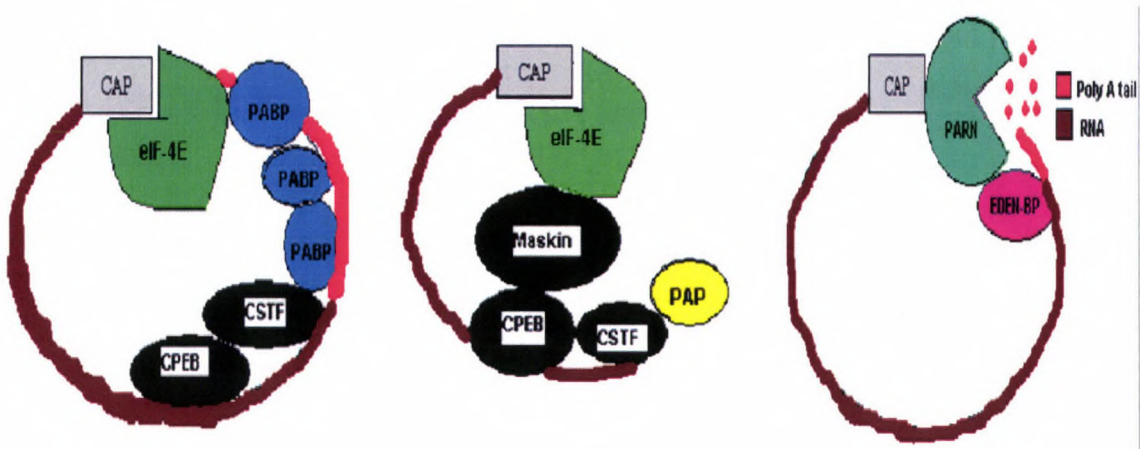
Yu J., Hecht N.B., Schultz R.M. RNA-binding properties and translation repression in vitro by germ-cell specific MSY2 protein. Biol Reprod 2002; 67: 1093-1098.

Appendix

A.1. Diagram of events occurring during early in vivo-oocyte maturation.



A.2. Diagram of interactions between poly(A) tail and proteins involved in translation, storage, or degradation.



B.1 ANOVA tables for Chapter II experiment.

aI. LH Concentration

Dependent Variable: MeanLH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	48338.21101	6042.27638	31.50	<.0001
Error	18	3453.22408	191.84578		
Corrected Total	26	51791.43509			

R-Square	Coeff Var	Root MSE	MeanLH Mean
0.933324	39.89600	13.85084	34.71737

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Trt	2	47549.52236	23774.76118	123.93	<.0001
Time	2	56.47715	28.23857	0.15	0.8642
Trt*Time	4	732.21150	183.05287	0.95	0.4561

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Trt	2	47549.52236	23774.76118	123.93	<.0001
Time	2	56.47715	28.23857	0.15	0.8642
Trt*Time	4	732.21150	183.05287	0.95	0.4561

bI. Germinal Vesicle Breakdown

Dependent Variable: Gvrate

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	30165.86350	7541.46588	16.60	<.0001
Error	35	15899.32625	454.26646		
Corrected Total	39	46065.18975			

R-Square	Coeff Var	Root MSE	Gvrate Mean
0.654852	37.06539	21.31353	57.50250

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	4	30165.86350	7541.46588	16.60	<.0001

cI. cAMP Concentration

Dependent Variable: Fmol

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	97.1388834	24.2847208	2.64	0.0555
Error	27	248.1954208	9.1924230		
Corrected Total	31	345.3343041			

R-Square	Coeff Var	Root MSE	Fmol Mean
0.281289	59.28576	3.031901	5.114046

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treat	3	60.67993016	20.22664339	2.20	0.1111
Time	1	36.45895319	36.45895319	3.97	0.0566

dI. Normalized transformed (log10) Epiregulin [mRNA]

Dependent Variable: tNorm

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	5.06456818	2.53228409	32.49	<.0001
Error	24	1.87081782	0.07795074		
Corrected Total	26	6.93538600			

R-Square	Coeff Var	Root MSE	tNorm Mean
0.730250	-43.91366	0.279197	-0.635785

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Trt	2	5.06456818	2.53228409	32.49	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Trt	2	5.06456818	2.53228409	32.49	<.0001

eI. Normalized transformed (log10) Actin [mRNA].

Dependent Variable: tNorm

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	0.41883060	0.20941530	3.47	0.0475
Error	24	1.44888648	0.06037027		
Corrected Total	26	1.86771708			

R-Square	Coeff Var	Root MSE	tNorm Mean
0.224247	-1.305875	0.245704	-18.81525

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Trt	2	0.41883060	0.20941530	3.47	0.0475

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Trt	2	0.41883060	0.20941530	3.47	0.0475

fI. Normalized transformed (log10) PDE-3A [mRNA].

Dependent Variable: tNorm

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	0.01826691	0.00913346	0.08	0.9233
Error	78	8.92052974	0.11436577		
Corrected Total	80	8.93879665			

R-Square	Coeff Var	Root MSE	tNorm Mean
0.002044	-61.59435	0.338180	-0.549044

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Trt	2	0.01826691	0.00913346	0.08	0.9233

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Trt	2	0.01826691	0.00913346	0.08	0.9233

gI. Normalized transformed (log10) H2a.1 [mRNA].

Dependent Variable: tNorm

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	1.96288550	0.98144275	5.84	0.0043
Error	78	13.10052887	0.16795550		
Corrected Total	80	15.06341437			

R-Square	Coeff Var	Root MSE	tNorm Mean
0.130308	-1.948555	0.409824	-21.03219

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Trt	2	1.96288550	0.98144275	5.84	0.0043

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Trt	2	1.96288550	0.98144275	5.84	0.0043

B.2. ANOVA tables for Chapter III experiment.

aII. Normalized transformed (log10) EDEN [mRNA].

Dependent Variable: normalt

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	21.93563542	3.13366220	7.61	<.0001
Error	43	17.70834436	0.41182196		
Corrected Total	50	39.64397978			

R-Square	Coeff Var	Root MSE	normalt Mean
0.553316	87.65690	0.641734	0.732097

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Cow	2	20.80883944	10.40441972	25.26	<.0001
Oocyte	2	1.06932543	0.53466271	1.30	0.2835
Mat	1	0.30078583	0.30078583	0.73	0.3975
Time	2	0.57484009	0.28742004	0.70	0.5032

Dependent Variable: normalt

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	1.91866259	0.38373252	0.46	0.8055
Error	45	37.72531719	0.83834038		
Corrected Total	50	39.64397978			

R-Square	Coeff Var	Root MSE	normalt Mean
0.048397	125.0667	0.915609	0.732097

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Trt	5	1.91866259	0.38373252	0.46	0.8055

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Trt	5	1.91866259	0.38373252	0.46	0.8055

bII. Normalized transformed (log10) eIF-4E [mRNA].

Dependent Variable: normalt

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	12.69323070	1.81331867	3.64	0.0037
Error	42	20.91149749	0.49789280		
Corrected Total	49	33.60472819			

R-Square	Coeff Var	Root MSE	normalt Mean
0.377722	79.47218	0.705615	0.887877

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Cow	2	5.93207177	2.96603588	5.96	0.0053
Oocyte	2	1.92759451	0.96379726	1.94	0.1570
Mat	1	1.85272604	1.85272604	3.72	0.0605
Time	2	3.93414562	1.96707281	3.95	0.0268

Dependent Variable: normalt

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	6.34054149	1.26810830	2.05	0.0906
Error	44	27.26418670	0.61964061		
Corrected Total	49	33.60472819			

R-Square	Coeff Var	Root MSE	normalt Mean
0.188680	88.65784	0.787173	0.887877

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Trt	5	6.34054149	1.26810830	2.05	0.0906

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Trt	5	6.34054149	1.26810830	2.05	0.0906

cII. Normalized transformed (log10) PAP-1 [mRNA].

Dependent Variable: normalt

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	15.24306775	2.17758111	6.06	<.0001
Error	42	15.09926387	0.35950628		
Corrected Total	49	30.34233162			

R-Square	Coeff Var	Root MSE	normalt Mean
0.502370	231.2380	0.599588	0.259295

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Cow	2	13.94597676	6.97298838	19.40	<.0001
Oocyte	2	0.49870248	0.24935124	0.69	0.5054
Mat	1	0.07911621	0.07911621	0.22	0.6414
Time	2	1.19949104	0.59974552	1.67	0.2008

Dependent Variable: normalt

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	1.17929484	0.23585897	0.36	0.8757
Error	44	29.16303678	0.66279629		
Corrected Total	49	30.34233162			

R-Square	Coeff Var	Root MSE	normalt Mean
0.038866	313.9757	0.814123	0.259295

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Trt	5	1.17929484	0.23585897	0.36	0.8757

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Trt	5	1.17929484	0.23585897	0.36	0.8757

dII. Normalized transformed (log10) PARN [mRNA].

Dependent Variable: normalt

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	3.51908898	0.50272700	1.96	0.0818
Error	45	11.53145505	0.25625456		
Corrected Total	52	15.05054402			

R-Square	Coeff Var	Root MSE	normalt Mean
0.233818	-240.3875	0.506216	-0.210583

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Cow	2	0.48192833	0.24096417	0.94	0.3980
Oocyte	2	1.54223282	0.77111641	3.01	0.0594
Mat	1	0.04026161	0.04026161	0.16	0.6937
Time	2	1.36685858	0.68342929	2.67	0.0804

Dependent Variable: normalt

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	2.05588938	0.41117788	1.49	0.2120
Error	47	12.99465465	0.27648201		
Corrected Total	52	15.05054402			

R-Square	Coeff Var	Root MSE	normalt Mean
0.136599	-249.6948	0.525816	-0.210583

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Trt	5	2.05588938	0.41117788	1.49	0.2120
Source	DF	Type III SS	Mean Square	F Value	Pr > F
Trt	5	2.05588938	0.41117788	1.49	0.2120

eII. Normalized transformed (log10) H2a.1 [mRNA].

Dependent Variable: tNorm

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	15.40589542	1.71176616	3.86	0.0012
Error	43	19.05504064	0.44314048		
Corrected Total	52	34.46093607			

R-Square	Coeff Var	Root MSE	tNorm Mean
0.447054	-3.166767	0.665688	-21.02106

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Oocyte	2	9.70883622	4.85441811	10.95	0.0001
Trt	5	1.45601571	0.29120314	0.66	0.6577
Cow	2	4.24104349	2.12052175	4.79	0.0133
Source	DF	Type III SS	Mean Square	F Value	Pr > F
Oocyte	2	9.44393967	4.72196984	10.66	0.0002
Trt	5	1.33835187	0.26767037	0.60	0.6971
Cow	2	4.24104349	2.12052175	4.79	0.0133

Dependent Variable: tNorm

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	1.89428521	0.37885704	0.55	0.7399
Error	47	32.56665085	0.69290746		
Corrected Total	52	34.46093607			

R-Square	Coeff Var	Root MSE	tNorm Mean
0.054969	-3.959890	0.832411	-21.02106

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Trt	5	1.89428521	0.37885704	0.55	0.7399
Source	DF	Type III SS	Mean Square	F Value	Pr > F
Trt	5	1.89428521	0.37885704	0.55	0.7399