THE ROLE OF CYTOPLASMIC POLYADENYLATION ELEMENT SEQUENCE ON MRNA ABUNDANCE IN PORCINE EMBRYOGENESIS

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KYLE BRADLEY DOBBS

Dr. Randall Prather, Thesis Supervisor

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The undersigned, appointed by the Dean of the Graduate School, have examined the thesis entitled

THE ROLE OF CYTOPLASMIC POLYADENYLATION ELEMENT SEQUENCE ON MRNA ABUNDANCE IN PORCINE EMBRYOGENESIS

Presented by Kyle Bradley Dobbs

A candidate for the degree of Master of Science

and hereby certify that in their opinion it is worthy of acceptance.

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Dr. Randall Prather

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Dr. Rocío Rivera

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Dr. David Setzer

This is dedicated to my Mom and Dad. They never guessed this is where I would be and where I am headed, but they supported me nonetheless and for that, I am forever grateful

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Nomenclature

2CF	2-cell In Vitro Fertilized
2CF C	2-cell In Vitro Fertilized Treated with Cordycepin
3′-dA	3′-deoxyadenosine
4CF	4-cell In Vitro Fertilized
4CF C	4-cell In Vitro Fertilized Treated with Cordycepin
ANOVA	Analysis of Variance
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
cDNA	Complementary Deoxyribonucleic acid
CDK2	Cyclin-dependent Kinase 2
CHO	Chinese Hamster Ovary
CPE	Cytoplasmic Polyadenylation Element
CPEB	Cytoplasmic Polyadenylation Binding Protein
CPSF	Cleavage and Polyadenylation Specificity Factor
CSF	Cytostatic Factor
CTD	Carboxyl Terminal Domain
DAN	Deadenylating Nuclease
DEPC	Diethyl Pyrocarbonate
DNA	Deoxyribonucleic Acid
eGFP	Enhanced Green Fluorescent Protein
eIF4F	Elongation Factor 4 F complex
EST	Expressed Sequence Tag

GLM	General Linear Model
GMP	Guanosine Monophosphate
GTP	Guanosine Triphosphate
GVBD	Germinal Vesicle Breakdown
GVO	Germinal Vesicle Oocyte
HSP	Heat Shock Protein
IVF	In Vitro Fertilization
ICC	Immunocytochemistry
LH	Luteinizing Hormone
MII	Second metaphase of Meiosis
MII C	Second metaphase of Meiosis Treated with Cordycepin
MAP	Mitogen-activated Protein
MPF	Maturation Promoting Factor
mRNA	Messenger Ribonucleic acid
mTBM	modified Tris-buffered medium
NTC	No Template Control
OPN	Osteopontin
PABP	Polyadenylation Binding Protein
PARN	Poly (A)-specific ribonucleases
PAT	Poly (A) tail Polymerase Chain Reaction
PCR	Polymerase Chain Reaction
Poly (A)	Polyadenylate
PVA	Polyvinyl Alcohol

PZM3	Porcine Zygote Medium-3
qPCR	Quantitative Polymerase Chain Reaction
RBP	Ribonucleic Acid Binding Protein
RNAi	Ribonucleic Acid Interference
rRNA	Ribosomal Ribonucleic Acid
siRNA	Small Interfering Ribonucleic Acid
snRNP	Small nuclear Ribonucleoproteins
Spt	Suppressor of Ty homolog
TBP	TATA Binding Protein
TCM-199	Tissue Culture Medium-199
TFII	Transcription Factor II
tRNA	Transfer Ribonucleic Acid
UTR	Untranslated Region
YWHAG	Tyrosine 3-monooxygenase/tryptophan 5-monoxygenase-activation protein 14-3-3 gamma
ZGA	Zygote to Embryo Activation

Chapter I

Literature Review

Introduction

"*Ex ovo omnia*" or everything comes from the egg, was the description that William Harvey stated in 1615 (Stitzel and Seydoux, 2007). This statement was revolutionary in the 1600's and still holds true today. Prior to fertilization, many important events occur that are crucial for the initial stages of development. During the first few rounds of cleavage, the embryo relies completely on stored maternal mRNA and proteins, which justifies Harvey's statement that everything starts and comes from the egg. The mechanisms that control the availability of mRNA has been investigated thoroughly throughout smaller species, but not to the same extent in farm animals. In chapter I, focus is directed at the involvement of cytoplasmic polyadenylation during oocyte/embryo development and how the different factors incorporate themselves into this process. To further the background on cytoplasmic polyadenylation, topics such as transcription and translation are covered due to their importance in the production and processing of polyadenylated mRNA. Oocyte/embryo development and transitional periods within their development will also be covered as they provide the most visible points where cytoplasmic polyadenylation have their greatest effect. Following discussion of translation, a few of the important experimental techniques are discussed detailing their history and purpose for this study. The main objective

for this study is reported in chapter II and determines if a cytoplasmic polyadenylation element (CPE) consensus site could be correlated with poly (A) tail production throughout four different stages of development in porcine oocyte/embryos. It is our thesis that these CPE sites can be classified into groups based on their effects on polyadenylation of transcripts during a transcriptionally quiescent time.

Embryo Development

Meiotic Maturation

Meiotic maturation is the process of forming the gametes into reproducing units, which, for this review, will focus strictly on the female gamete within *Xenopus* and *Sus scrofa*: the oocyte. The overall goal of meiotic maturation is to take the oocyte through normal meiosis by being able to reach MII-arrest and be released following fertilization. The oocyte and sperm (gametes) are different from other cells in that they contain half the amount of chromosomes (haploids). Within meiosis, there are two rounds of cell division with only one round of DNA replication. In *Xenopus*, the primary oocyte enters the meiotic cell cycle and undergoes a round of DNA replication while recombination is occurring (Ferrell, 1999). Following this, the oocyte enlarges at the G2 cell stage until it is fully grown and stays in meiotic arrest at the diplotene stage of meiotic prophase (Ferrell, 1999). At this point, the *Xenopus* oocyte is considered a stage VI oocyte and vitellogenesis, the increase in yolk material, occurs (Danilchik and Gerhart, 1987; Kanki and Donoghue, 1991). Following the release of progesterone from

follicle cells due to the LH surge, the oocyte transitions out of meiotic arrest at the diplotene stage and proceeds to metaphase II arrest with the help of meiotic promoting factor (MPF) (Kanki and Donoghue, 1991). In order to drive the germinal vesicle oocyte through metaphase I, MPF is required (Lohka et al., 1988; Masui and Markert, 1971). Following the joining of the p34 and cyclin B1 subunits, MPF works by being phosphorylated by cascades initiated by the progesterone surge that include the gene *c-MOS*, which is discussed later in this review (Gautier et al., 1990). Within six hours of progesterone acting on the oocyte, germinal vesicle breakdown (GVBD) begins and is characterized by the breakdown of the nuclear membrane and migration of the condensed chromosomes to the animal pole (Huchon et al., 1981). As maturation progresses, a second block occurs: meiotic maturation second metaphase or MII arrest. This occurs due to the presence of cytostatic factor (CSF) and the presence of cyclin-dependent kinase 2, cdk2, which interacts with the proto-oncogene *c-MOS's* product $p39^{mos}$ to halt the oocyte in preparation for fertilization (Gabrielli et al., 1993; Lorca et al., 1991). As the sperm fuses with the membrane, it induces a calcium flux that flows across the egg and binds with calmodulin to promote the degradation of cyclin thus allowing the egg to exit the second metaphase (Lorca et al., 1991).

In pigs, the process of meiotic maturation is similiar to that in *Xenopus* and humans. As the porcine oocyte develops prior to the LH surge, it increases in size, which displays its meiotic competence (Hunter, 2000). During meiotic maturation in pigs, the oocyte enters meiotic arrest at the first dictyate stage, just

like the diplotene stage in *Xenopus*, but unlike *Xenopus*, it does not enter vitellogenesis. MAP kinase can substitute for cdc2 and still induce GVBD even though MAP kinase is not phosphorylated prior to but soon after GVBD (Liang et al., 2005; Motlik et al., 1998). Porcine oocytes take 36-40 hours to complete the first stage of meiosis after exiting the G2 cell cycle stage, before entering the metaphase II block (Hunter, 2000). Pigs, just like other mammals, require cyclin B1 and the production of MAP kinase from cumulus cells to progress out of the diplotene block and transition from the G2 to M phase of the cell cycle followed by metaphase II arrest (Dekel, 1996; Liang et al., 2005; Varga et al., 2008). In conjunction with MPF and MAP kinase, the ubiquitin-proteasomal pathway is a critical component during meiotic maturation as an inhibitor of the complex did not allow GVBD (Sun et al., 2004). Once the oocyte enters the metaphase II block, it requires the reduction of CSF with the increase of calcium, just as in *Xenopus*, which occurs at the time of fertilization (Swann and Ozil, 1994).

As meiotic maturation occurs, many structural changes are also happening. In pigs, microtubules are not detected in the oocyte until after GVBD when the microtubule aster is formed and elongated during metaphase I to cover the chromatin (Kim et al., 1996). During metaphase I, the movement of microtubules and microfilaments are associated with MPF. At metaphase II, the microtubules are mainly associated around the meiotic spindle following movement of the spindle to the peripheral of the oocyte (Kim et al., 1996).

Maternal to Zygote Transition

During development, following fertilization, the embryo goes through a transition phase in which the maternal RNA is slowly degraded as the zygote begins to produce its own mRNA. Over the course of time in mammalian embryo development, a “clock” is initiated following fertilization before the zygote begins transcription and transcriptional regulation is based on the time after fertilization (Schultz, 1993). In pigs, this maternal transition occurs during the 4-cell stage (40-48 hours after fertilization in vitro), while in bovine it occurs between the 8-cell to 16-cell stage, at the mid-blastula stage in *Xenopus* and at the 14th interphase in *Drosophila* (Lequarre et al., 2003; Prather, 1993; Schier, 2007; Schoenbeck et al., 1992). At the 4-cell stage in pigs, resumption in the uptake of both uracil and methionine occurs, signaling the processes of transcription and translation have resumed (Jarrell et al., 1991; Schoenbeck et al., 1992). Several characteristics occur at this transition in pigs in vivo, RNA synthesis restarts and there is a distinct short G1 phase followed by a longer than normal G2 phase while in vitro, a culture block will occur if incorrect culture media is used (Schoenbeck et al., 1992). This stage in the pig is also when the embryo enters the uterus (Schoenbeck et al., 1992). Certain genes are expressed in different amounts during this transition period. In pigs, cyclin B1, which promotes meiotic entry, is fully degraded by the end of 4-cell stage and no new cyclin B1 is produced (Anderson et al., 1999).

Creation and Structure of mRNA

Mechanisms of Transcription

In the process of transcription, many different mechanisms and factors work together in transcribing mRNA from DNA. For this review, only some of more crucial parts will be discussed. In the 1960's, a polymerase was discovered that was capable of DNA-dependent RNA polymerization in prokaryotes that was uniquely different from DNA-dependent DNA polymerases (Cox, 2008). One of the key features of transcription within eukaryotes is the use of three DNA-dependent RNA polymerases labeled I, II and III (Young, 1991). RNA polymerase I is used specifically for production of rRNA while polymerase III is used for the production of both tRNA and rRNA (Seither et al., 1998; Willis, 1993). Polymerase II is the main component during transcription of DNA into mRNA and will be the focus polymerase for the remainder of this review (Kornberg, 1999).

Since being a key enzyme in the regulation of RNA production, polymerase II is highly conserved across most living organisms both eukaryotic and prokaryotic (Ebright, 2000; Sims et al., 2004). RNA polymerase II contains 12 subunits ranging from RBP1 (being the largest) to RBP12 (Cox, 2008). RBP1 contains a unique seven amino acid sequence in the carboxyl terminal domain, which varies in quantity between species, with mammals using up to 52 different heptad sets (Palancade and Bensaude, 2003). Carboxyl terminal domain (CTD) is phosphorylated to promote RNA polymerase II into beginning transcription. In order for polymerase II to successfully start the transcription process, it requires multiple basal factors to join together at the promoter region on the DNA. These basal factors include: TFIIB, TFIID, TFIIE, TFIIF and TFIIH, all of which are

subunits termed polymerase II transcription factors (Reese, 2003). Another important component in the process of transcription is the TATA box binding protein (TBP). The TBP is responsible for binding to the TATAA sequence in the 5' region of DNA (Cox, 2008). TFIID is required for recognition of the promoter sequence for polymerase II (Burley and Roeder, 1996). After TFIID and its subunit TBP bind to the promoter region, they induce a bend in the DNA sequence leaving space for TFIIB to join in and stabilize TFIID (Reese, 2003). TFIIB thus induces TFIIF, which is bound to the polymerase II enzyme, to bind to the sequence which is followed by the unwinding of DNA into an open complex by TFIIH helicase activity (Reese, 2003). Once the open complex is formed, polymerase II begins transcription, but requires breaking itself free from the promoter region (Saunders et al., 2006). During this process, there is the possibility of aborting the initiation process (Saunders et al., 2006). After the polymerase II has escaped the promoter region and following regulation in the pause phase, the entire transcription complex enters the elongation phase of transcription (Saunders et al., 2006). Within the pause phase, there are many different effectors, such as kinase inhibitor and negative elongation factors, which regulate transcription overall (Saunders et al., 2006). It has been hypothesized that the releasing protein that allows the complex to leave the paused phase of transcription due to phosphorylation of the CTD, is the positive transcription elongation factor-b (Price, 2000). During the elongation process, Spt4, 5 and 6 are shown to interact and be present. Studies have shown more specifically that Spt6, when introduced in vitro, causes an increase in elongation activity (Ardehali

et al., 2009; Hartzog et al., 1998; Winston, 2001). As elongation continues, TFIIE and TFIIH are released and returned for use in the next transcriptional initiation (Cox, 2008). Following elongation, the transcription process is terminated with the addition of the poly (A) tail sequence site (Connelly and Manley, 1989).

5' Cap

In order for RNA to sustain stability from ribonucleases after transcription, a 5' cap is attached to the front of the transcript. The 5' cap is also responsible for anchoring the ribosome to the RNA for translation. Research has shown that a longer 5' UTR region affects translational efficiency (Mendez and Richter, 2001; Preiss and Hentze, 1999). During the early part of transcription, the 5' cap, or 7-methylguanosine, is formed on all polymerase II products (Nevins, 1983). As polymerase II begins transcription, Guanylyl tranferase adds GMP to the 5' portion of the mRNA (Cox, 2008). Following the addition of the GMP, methyl transferases add methyl groups to the 7-nitrogen of the guainine and to the ribose sugars of the two penultimate nucleotides (Groner et al., 1978). The 5' cap insures that the above requirements are fulfilled and allows for successful communication between the 5' and 3' end of mRNA, thus increasing the rate of translational efficiency (Gallie, 1991).

Introns

DNA contains regions that, following the processing of mRNA, are removed from the transcript. One class of these regions is called introns. Introns

were classified in 1993 by Phillip Sharp and Richard Roberts but were first termed in 1978 by W. Gilbert (Gilbert, 1978; Moore and Sharp, 1993). Introns were once thought of as the “waste” portion of a DNA sequence and thus having no real function, but this view has changed within the recent decade as introns have been shown to influence post transcriptional modifications, RNA metabolism and overall message abundance (Le Hir et al., 2003). One example is during immune cell development, introns affect the arrangement of the V(D)J sequence by altering the binding sites for B-cell transcription factors (Sleckman et al., 1996). Introns are divided into four classes based on their splicing capabilities. Group 1 contains self-splicing introns that requires the intron region to assist in the splicing reaction (Cech, 1990). This occurs by two transesterfication reactions that happen consecutively starting at the 5' side of the intron and ending with the splicing of the 3' side through a nucleophilic attack (Cech, 1990). Group 2 introns are similar to Group 1 in that they are self-splicing, but differ due to their formation of a lariat, structure and further more are not found in animal cells (Michel and Ferat, 1995). The lariat structure forms through the 2'-OH on an adenosine located within the intronic region attacking the 5' side of the intron forming the lariat followed by a similar attack as in Group 1 (Michel and Ferat, 1995). The third Group is composed of introns that are spliced by splicosomes and snRNP. Inactive spliceosomes are constructed with U1 and U2 snRNPs followed by the binding of U4, U5 and U6 snRNPs (Legrain et al., 1988). U4 snRNP and occasionally U1, leaves the group of snRNPs and allows the mature spliceosome complex to begin splicing, following similar steps

of Group 2 splicing (Legrain et al., 1988). The final group of introns are associated with certain tRNAs that were first described in 1983 (Kaine et al., 1983). tRNA intron processing uses endonucleases to cleave at specific sites near the U8 and C56 residues in bacteria and within the 3' anticodon range in *Xenopus* (Kleman-Leyer et al., 1997). Cleavage at both sides occurs and linking of the DNA fragment occurs using RNA ligase (Kleman-Leyer et al., 1997).

History of Polyadenylation

Polyadenylation polymerase was first discovered in 1959 as an enzyme that catalyzed ATP to link adenine nucleotides together, but was not labeled as such until the 1970's (Edmonds and Abrams, 1960; Jackson and Standart, 1990; Wahle and Keller, 1992). In 1971, the term poly (A) tail was first coined by Darnell and colleagues when they noticed a linking of adenosines ranging from 150 to 250 nucleotides long in eukaryotic cells (Darnell et al., 1971). A mystery still existed into the early 1980's as to what the actual function of this tail was and continued to be discussed only at that point as a possible control for transcript availability (Brawerman, 1981). Brawerman and colleagues knew that once the mRNA started to be degraded, that the poly (A) tail was also beginning to shorten itself in eukaryotes (Brawerman, 1981). In the mid 1980's, a greater understanding of the polyadenylation signal was brought into the spotlight. During this time, Fitzgerald and Shenk showed that there was a specific sequence of nucleotides, AAUAAA, that were located 10 to 30 bases in front of the poly (A) tail (Wickens and Stephenson, 1984). When mutations were

inserted into this hexanucleotide, the poly (A) tail was not properly made, thus verifying its importance to the polyadenylation process. Most mRNA that is viable contains this hexanucleotide sequence in them with only a few exceptions, such as human cyclin D and histones, which are not polyadenylated (Wahle and Keller, 1992; Zhao et al., 1999). In 1991, polyadenylation was shown to be a key feature in the regulation of transcripts and their ability to be expressed efficiently in CHO cells in conjugation with the 5' cap (Gallie, 1991). Chou and colleagues showed that the downstream element, another important part of the 3' end processing of mRNA, contained a highly uracil enriched site that if modified, lowered the efficiency of the processing (Chou et al., 1994). The final piece to the mammalian puzzle of polyadenylation was the poly (A) site itself (Zhao et al., 1999). By 1999, it was well known that the hexanucleotide was important for both cleavage of the 3' UTR and polyadenylation (Zhao et al., 1999).

Most mammalian polyadenylation is similar to other species in origin but also different. Yeast, for example, are different from mammals as their polyadenylation sequences are not as highly conserved as mammals, but more complicated (Graber et al., 1999). The evolution of the poly (A) tail for eukaryotes has been traced by mass genome comparison which shows that the RNA physiological components make up 3 to 11% of all proteins (Anantharaman et al., 2002). Tracing the effects of poly (A) changes on proteins is of key importance. It has been shown that most extracellular proteins have one polyadenylation site in its mRNA while most intra-cellular proteins have multiple sites in their mRNA (Tian et al., 2005). Many of the components of

polyadenylation are mentioned below in the “Cytoplasmic polyadenylation” portion of this review.

Certain inhibitors to the polyadenylation process exist in nature and can be chemically synthesized in a laboratory setting. One of these chemicals is cordycepin, which incorporates itself in the process of adding adenosine during polyadenylation and inhibits further tail elongation (Krischek and Meinecke, 2002; Siev et al., 1969). Cordycepin accomplishes this by not containing a 3'-OH on its structure and is therefore called a 3'-deoxyadenosine (Siev et al., 1969). Originally extracted from the *Cordyceps* fungi and still used for medicinal purposes as a anti-fungal component, most all cordycepin for laboratory usage is now produced synthetically (Kuo et al., 1994).

Deadenylating nuclease

When eukaryotic mRNA is destined for destruction, one of the first steps is deadenylation through the deadenylation endonuclease (Korner et al., 1998). This degradation process was examined by inducing large quantities of the gene *c-fos’s* transcript, and watching the poly (A) tail length during this overexpression for deadenylation (Wilson and Treisman, 1988). This was done while identifying a portion of the 3' sequence that is AU rich and helps in controlling this loss of poly (A) tail. In addition to the shortening of the poly (A) tail, the removal of the 5' cap has been observed in yeast by a de-capping enzyme from the gene *DCP1*, which is associated with the length of the poly (A) tail of some mRNAs (Beelman et al., 1996). Once the deadenylating enzyme has shortened the tail, this leaves

the mRNA open for 5' to 3' or 3' to 5' endonucleases to begin degradation (Anderson and Parker, 1998). A component of the polyadenylation process that inhibits the interaction of a deadenylating enzyme on a Poly (A) tail is PABP, which in yeast is due to the stabilizing properties of PABP on the poly (A) tail (Korner et al., 1998). In cows and *Xenopus*, the enzyme responsible for deadenylation is deadenylating nuclease (DAN), a 74 kDa polypeptide that has been shown to distinctly search out the poly (A) tail for degradation when induced while in humans, deadenylation is caused by PARN (Henriksson et al., 2010; Korner and Wahle, 1997). Some poly (A) tails that are targeted for deadenylation by the above-mentioned enzymes are originally targets of micro RNAs (Giraldez et al., 2006).

Cytoplasmic Polyadenylation Element

CPE Background

Polyadenylation that occurs during the transition between germinal vesicle stage and metaphase II has been shown to be crucial for the production of transcripts at appropriate times. The cytoplasmic polyadenylation element (CPE) in the pig is a seven-nucleotide sequence that is located in the 3' untranslated region (UTR) portion of mRNA. The CPE is responsible for the polyadenylation that occurs during the transcriptionally silent stages of early oocyte development (Richter, 1999; Verrotti and Strickland, 1997). Mammalian mRNA must contain two sites in their 3' UTR in order for proper polyadenylation to occur; adenylation control element or a CPE site and a hexanucleotide (Richter, 1999). Without

these two sites, the process of polyadenylation will not happen correctly. Further explanation of the CPE components and the mechanism of activating cytoplasmic polyadenylation are described below.

Aurora-Kinase

The Auroras are a family of kinases that are involved in many cellular functions such as cell division, chromatin segregation and cytokinesis and were first described in *Xenopus* and *Drosophila* (Bolanos-Garcia, 2005; Crane et al., 2004). Within the family, there are three members: Aurora A, B and C, all of which are highly conserved in different eukaryotic species (Bolanos-Garcia, 2005; Keen and Taylor, 2004). Aurora-A is initiated by the Aurora-A/Ajuba complex, which is recruited to the centrosome in order for mitosis to begin (Hirota et al., 2003). After initiation of Aurora-A and binding to the centrosome by the TPX2 protein, Aurora-A goes through a conformational change in shape that allows for better substrate binding (Bolanos-Garcia, 2005). In human cells, when Aurora-A is repressed through RNAi, the cells enter mitosis more slowly, while overexpression of Aurora-A causes the cell to speed through the spindle checkpoint creating the possibility for tumor formation (Bolanos-Garcia, 2005; Keen and Taylor, 2004). Aurora-B kinase is very similar to Aurora-A in that it has a role in chromosome segregation and cytokinesis (Bolanos-Garcia, 2005). As the cell progresses through mitosis, Aurora-B accumulates in the midzone of microtubules (Murata-Hori and Wang, 2002). When Aurora-B is repressed, problems exist with spindle checkpoints, prometaphase chromosomal

condensation and microtubule motor control (Murata-Hori and Wang, 2002). Very little is known about Aurora-C even though it has been shown to localize during mitosis to the spindle poles and has a role during spermatogenesis (Bolanos-Garcia, 2005; Keen and Taylor, 2004). High levels of Aurora-C are associated with multiple types of cancers in humans while silencing of Aurora-C through siRNA has been shown to induce multinucleated cells (Bolanos-Garcia, 2005). Certain medical applications have been designed using the information of Aurora-A kinase. Since Aurora-A overexpression is found in tumor progression, it has been thought of as a good marker for cancer prognosis, and inhibition of Aurora-A kinases is being reviewed as anti-tumor treatment (Bolanos-Garcia, 2005). In *Xenopus* soon after progesterone binds to its cell surface receptor, there is a decrease in cAMP, which leads to a subsequent increase in Eg2 kinase (same family as Aurora kinases) activity (Richter, 1999). Aurora kinases phosphorylate cytoplasmic polyadenylation element binding proteins only in vertebrates, which then initiates the process of cytoplasmic polyadenylation (Andresson and Ruderman, 1998; Mendez and Richter, 2001; Richter, 1999).

Components of Cytoplasmic Polyadenylation

In order for cytoplasmic polyadenylation to occur, many different components are required. These components include but are not limited to: Maskin, CPEB, PABP, CPSF, eIF4F complex, and the 40S ribosome. Maskin, first discovered in *Xenopus*, is a 931 amino acid protein that is a member of the transforming acidic coiled-coil family (Meijer et al., 2007). It is responsible for

repressing polyadenylation by interacting with the cytoplasmic polyadenylation element binding protein (Cao et al., 2006; Meijer et al., 2007). Maskin functions by being phosphorylated by the Aurora-A kinase (as in *Drosophilia*) or CDK1 (as in *Xenopus*), thus causing the eIF4F complex to be released (Cao et al., 2006). Since its discovery, Maskin has been studied in conjunction with research describing *c-MOS* and cyclin B1 function, where a lack of phosphorylation of Maskin causes a repression of those genes, but has otherwise not been identified in pigs (Meijer et al., 2007). The CPSF, a multi subunit protein whose largest subunits are 100 kDa in cattle and 160 kDa in humans, is responsible for cleaving mRNA downstream of the polyadenylation signal (Dickson et al., 1999; Jenny et al., 1994; Murthy and Manley, 1995). CPSF utilizes cleavage stimulatory factor for binding to the mRNA at a much higher affinity then by itself and once bound, aids with other factors in the polyadenylation of a transcript (Colgan and Manley, 1997; Murthy and Manley, 1995). CPSF interacts with the 70 kDa (in humans) PABP to insure maximum polyadenylation (Groneberg et al., 2003). When PAPB is knocked out, poly (A) is inhibited in yeast but does not affect cleavage by CPSF and, as mentioned later, PAPB interacts with eIF4G to form the PAPB-eIF4G complex (Mendez and Richter, 2001; Minvielle-Sebastia et al., 1997). CPSF and Maskin also interact with CPEB, in which CPEB helps to stabilize CPSF during its binding to the polyadenylation signal sequence while Maskin binds to CPEB (Mendez and Richter, 2001). CPEB interacts with the mRNA sequence UUUUUAAU in *Xenopus* where antibodies to the protein cause a great reduction in polyadenylation, and supplementation, which CPEB caused

complete reversal of the loss of polyadenylation (Hake and Richter, 1994). After the oocyte has matured, CPEB is reduced by 90%, but remains in contact with the spindles and centrosomes (Mendez and Richter, 2001).

After initiation of polyadenylation has occurred, translational factors begin to bind to the transcript and of those factors, the 40S ribosome and eukaryotic initiation factor 4 (eIF4F) complex are the most critical. The eIF4F complex includes eIF4E, which binds to the m^7GpppN cap, eIF4A and allows the 40S ribosome and other factors to bind the mRNA and eIF4G thus making a scaffold protein for the complex (Gebauer and Hentze, 2004). Interaction between eIF4E, PABP and eIF4G mediate communication between the 5' cap and the 3' UTR to aid the translational control through 3' polyadenylation (Wells et al., 1998). Following phosphorylation of Maskin, the eIF4F complex is allowed to begin the movement towards translation by binding to the 5' cap methyl group (Richter, 1999). The recruitment of the 43S ribosome complex composed of the initiator $tRNA_{met}$, eIF2 and GTP is responsible for scanning the mRNA after it is unwound by eIF4A, and begin translation at the start codon, AUG (Gebauer and Hentze, 2004; Gray and Hentze, 1994). As the 43S ribosome scans the mRNA, another ribosome subunit, 60S, is required to actually start translating. The 60S ribosome subunit is recruited to the 43S complex after GTP is hydrolyzed by eIF5 (Gebauer and Hentze, 2004).

Certain CPE sites have been indentified through studies using *Xenopus* and their effect on polyadenylation using *Mus musculus*. In *Xenopus*, the sequence UUUUUAU located in the 3' UTR region of mRNA, was only

polyadenylated when progesterone was present (Fox et al., 1989). This consensus sequence can have slight variation, with the sequence having an additional adenosine (Fox et al., 1989). Another *Xenopus* CPE, UUUUUUAUAAAG, is located in an mRNA termed G10 and requires the binding of a protein in order to initiate polyadenylation (McGrew and Richter, 1990; Simon et al., 1992). In *Mus musculus* an example of how CPE length controls polyadenylation is the very abundant *Spin* gene in the developing egg, which has differing transcript levels when the 3' UTR is modified (Oh et al., 2000). When different variants of the 3' UTR of the *Spin* gene are inserted into an mRNA encoding a visible marker and microinjected, there was a difference in the amount of the protein that is generated. The 3' UTR containing a CPE in the *Spin* gene determines the length of the polyadenylation that results when the transcript is translated and for how long the protein exists (Oh et al., 2000). To accomplish this, the three differing lengths of poly (A) tails were examined, and the transcripts that were not degraded as fast as the others had CPE sites associated and a longer poly (A) tail (Oh et al., 2000). Also in *Mus musculus*, $U_3AU_7A_3$ and $AUAU_5A$ consensus CPEs control polyadenylation in the gene *c-MOS* and consensus AU_4A_2U control polyadenylation for tissue plasminogen activator and allow for normal message pattern of expression (Gebauer et al., 1994). Multiple potential CPEs exist in *Mus musculus* such as UUUUUAAU in β-actin and UUUUUAU, UUUUAAU in *Melk*, which do not affect polyadenylation or UUUUAU in β-catenin which causes a change in polyadenylation in the ovulated

egg/zygote, but they are not homologous with other CPE consensus sequences for the same genes in other species (Oh et al., 2000).

c-MOS

GVBD occurs when the cytoplasmic presence of the mitosis-promoting factors (MPF) appear following the release of progesterone in *Xenopus* and LH in mammals (Hunter, 2000; Sagata et al., 1989). One of the genes that is directly affected by this is the proto-oncogene *c-MOS*, which produces a protein-kinase to phosphorylate the MPF in *Xenopus* (Nebreda and Hunt, 1993). *c-MOS* is not detectable in the fertilized egg, it is degraded at activation and when *c-MOS* mRNA is injected into *Xenopus* 2-cell stage embryos, a metaphase arrest is induced (Sagata et al., 1989). In mammals, *c-MOS* has a slightly similar effect on the growing oocyte. When *c-MOS* is disrupted, it has no effect on metaphase I initiation, but does affect metaphase II arrest, thus showing that *c-MOS* is required in mammalian early development (Newman and Dai, 1996). Pig studies have shown that *c-MOS* is a tissue-specific gene that is required for development and metaphase II arrest, but is not the only factor (Newman and Dai, 1996). *c-MOS* is an essential gene for this study because it has previously been shown to have no change in its level of poly (A) tail length through the first 44 hours of in vitro maturation in pigs (Zhang et al., 2009).

Techniques utilized during the Study

Real-time PCR

Real-time PCR or quantitative qPCR was invented in 1986 to quantitatively measure DNA and mRNA levels (Freeman et al., 1999; VanGuilder et al., 2008). From 1977 to 1986, most RNA analysis was done using northern blots in which a gel was used to quantitatively measure RNA levels, but this technique lacked the ability to accurately measure small amounts like fluorophores could in a qPCR (VanGuilder et al., 2008). Quantitative PCR began being used for measuring mRNA in 1989 when levels of lymphokines from human macrophages were determined using small initial samples (Wang et al., 1989). Real time PCR works by using a fluorescent probe or dye which binds to the cDNA and measures its fluorescing levels through roughly 40 cycles (VanGuilder et al., 2008). There are many different types of fluorescing agents that are used, such as TaqMan probes (Applied Biosystems), Lightcycler (Roche), LUX (Invitrogen) and Molecular Beacons (Sigma-Aldrich), but for this review, only the dye SYBR green will be discussed. As mentioned above, SYBR green binds quantitatively to double strand DNA following PCR replication and thus emits a light that increases in intensity as the amount of PCR product increases and can then be read by a plate reader (VanGuilder et al., 2008; Zipper et al., 2004). Some disadvantages to using such a dye are that it binds to any double stranded DNA product that is involved in the reaction and only emits one type of escence. Some advantages are it is sold by multiple companies and is easier to use than the other mentioned products (VanGuilder et al., 2008). Output values from a real-time thermal cycler give the threshold cycle at which time the PCR product is greater than the “threshold” set by being about 10 times

brighter than the standard deviation of the base line (Heid et al., 1996). The higher the threshold cycle number, the lower the amount of sample that was originally present. The graph for that specific gene reaches a plateau when the fluorophore is exhausted as the amount of PCR product increases exponentially on a logarithmic scale (Heid et al., 1996). Most analysis following this involve a technique called the relative standard curve method in which the threshold cycle values are compared to a curve that was generated using previously known concentration of samples that are diluted in 10-fold increments (Heid et al., 1996).

YWHAG or tyrosine 3-monooxygenase/tryptophan 5-monoxygenase-activation protein 14-3-3 gamma, is utilized as a control in the real-time PCR process for studies that want to examine early development in pigs due to its consistent level of transcript during this time (Horie et al., 1999; Whitworth et al., 2005). It was first discovered in bovine brain tissue and since then, has been described in both invertebrates and vertebrates (Aitken et al., 1992). Within the 14-3-3 family of proteins, there are nine isoforms with seven of them coming from unique genomic regions while the other two are phosphorylated versions of those seven distinct isoforms (Horie et al., 1999). YWHAG is affected by growth factors and is involved in mitosis and cellular proliferation (Whitworth et al., 2005).

Express Sequence Tags Sequencing

ESTs or Express Sequence Tags sequencing is a process that is used to analyze the amount and type of mRNAs that is present within a distinct type of tissue or sample. To generate an EST, a tissue to be examined has its RNA extracted and reverse transcribed (Bouck and Vision, 2007). Following the reverse transcription, the cDNA is then cloned into a vector for rapid replication and sequenced from either the 5' or 3' end (Bouck and Vision, 2007). For this review, focus will be directed toward the 3' processing since this gives information on the 3' UTR. The short sequences from clones that are generated from 3' general primers, range from 200 to 500 bases and can be compared against a known database to provide annotation and sequences with similar annotation can be grouped together to estimate gene expression levels. To verify mRNA levels that the genes found during the EST process signify actual mRNA levels, it is important to verify using a more stringent approach, such as qPCR. Once a gene sequence has been determined, it can be entered into the genebank library. Some concerns with ESTs include not being able to measure mRNAs that are extremely low to begin with and a mis-representation of total transcripts during the PCR process, but regardless of these hazards, ESTs remain one of the easier ways to determine gene expression on a large scale (Bouck and Vision, 2007).

Poly (A) tail PCR

PAT Assay or Poly (A) tail polymerase chain reaction is one method that is used to measure the length of the Poly (A) tail of specific genes of interest.

Developed by Sallé and Strickland, a PAT assay begins by extracting RNA from tissue followed by reverse transcription (Salles and Strickland, 1999). The reverse transcription for a PAT assay is different from a standard reverse transcription because it uses a T4 ligase to link oligo dT's together capped by an anchor primer that has a specific sequence that "hangs" off the 3' end of the poly (A) tail, thus reproducing all poly (A) tails into poly (T) tails (Salles and Strickland, 1999). Following the reverse transcription, a gene specific primer is utilized during the normal PCR process along with the PAT assay anchor primer to generate PCR products. These PCR products can then be used to determine the length of a the Poly (A) tail to within 50 bases on an agarose gel and almost precisely using sequencing or enzyme digestion. PAT assays have been used extensively since its creation for measuring poly (A) tails of specific transcripts (Benoit et al., 2005; Zhang et al., 2009).

Conclusion

During porcine early development, there are many changes that materialize, specifically from germinal vesicle to and past the 4-cell stage. Within this time, there is little or no transcription occurring thus making the developing oocyte dependent on the maternal contributions of mRNA. Control of mRNA during this period is critical to maintaining developmental competence and is thought to be under the influence of the poly (A) tail being either polyadenylated or deadenylated. Although most of the research with this topic has been done in *Xenopus* and in mice, much has been left without a description in terms of

cytoplasmic polyadenylation in pigs and cows. Thus, our thesis is that cytoplasmic polyadenylation is under control of the CPE; a 7-nucleotide consensus sequence located in the 3' UTR, which controls when a transcript is express and that classification these CPE consensus sequences will enable us to better understand early development.

CHAPTER II

The Role of Cytoplasmic Polyadenylation Element Sequence on mRNA Abundance in Porcine Embryogenesis

Abstract

Development of a porcine germinal vesicle oocyte (GVO) to a 4-cell stage embryo occurs during a transcriptionally silent period when the oocyte/embryo relies on maternally derived mRNA to encode proteins required for development. Regulation of translation and degradation of maternal mRNA is thought to be partially dependent upon cytoplasmic polyadenylation elements (CPEs) within the 3′ untranslated region of the mRNA. The goal of this study was to determine how CPE sites affect the abundance of mRNA during embryogenesis and parthenogenetic development, and how cordycepin, a 3′-deooxyadenosine (3′-dA) which inhibits poly (A) tail formation, affects polyadenylation and transcript abundance. Expressed sequence tags (ESTs) from oocytes and 4-cell stage embryos were scanned for the presence of five consensus CPEs. Nineteen different transcripts containing one to three putative CPEs were selected and transcript abundance was determined in GVO, metaphase II, 2-cell and 4-cell stage embryos via real-time PCR; and the length of the poly (A) tail was determined by using a Poly (A) tail PCR (PAT) assay. Real time PCR was performed on three biological and two technical replicates for each stage. There

was no direct correlation between poly (A) tail length, transcript abundance and the CPE. In addition, the abundance of some messages was different if the embryo was the result of parthenogenetic activation. Cordycepin prevented polyadenylation of transcripts that normally undergo noticeable polyadenylation. Thus CPEs may not be the only factors that regulate message stability, and parthenogenetic activation does not result in changes in transcript abundance that mimic in vitro fertilization.

Introduction

From the first meiotic prophase or the meiotic arrest to the 4-cell stage when zygotic genome activation (ZGA) occurs, there is little or no transcription in the pig (Jarrell et al., 1991; Schoenbeck et al., 1992). The beginning of zygotic transcription during the 4-cell stage in pig is similar to the time of transcription initiation in the human (4 to 8-cell stage), while transcription initiation begins at the mid-blastula transition in *Xenopus* and during the fourteenth interphase in *Drosophila* (Gandolfi and Gandolfi, 2001). Proteins required post-fertilization to genome activation are derived from translationally repressed maternal mRNAs stored in the growing oocyte with short poly (A) tails of 20 to 40 nucleotides (Radford et al., 2008). The translation and degradation of these preexisting messages is thought to be carefully controlled to occur at the appropriate time during this transcriptional quiescence. Cytoplasmic polyadenylation is one mechanism that regulates translation and degradation (Gebauer and Richter, 1996; Gebauer et al., 1994; McGrew and Richter, 1990; Richter, 1999; Simon et

al., 1992). Cytoplasmic polyadenylation is the addition of a poly (A) to the 3′ tail of all non-histone eukaryotic mRNA in the cytoplasm and is highly conserved (Barabino and Keller, 1999). Polyadenylation stabilizes the mRNA transcript ensuring that it will be more stable than when not polyadenylated, thus assuring that the mRNA will be available for translation (Guhaniyogi and Brewer, 2001). Polyadenylation is a well-orchestrated event that is responsible for the release of mRNAs for translation. It is thought that an important part of polyadenylation control is the cytoplasmic polyadenylation element (CPE) (McGrew and Richter, 1990). The CPE is generally thought of as a seven-nucleotide sequence that is located in the 3′ untranslated region (UTR) portion of mRNA. Most CPE sites have been well characterized in *Xenopus* where they are not limited to seven nucleotides (Fox et al., 1989). For example, the consensus UUUUUAU has been shown to be an extensively used CPE site in *Xenopus* during oocyte maturation and lies within 15 base pairs of the highly conserved cleavage recognition hexanucleotide sequence (Barnard et al., 2004; Simon et al., 1992; Stebbins-Boaz et al., 1996). Another example of a *Xenopus* CPE is UUUUUUAUAAAG located in an mRNA termed G10, which requires the binding of a protein in order to initiate polyadenylation in the *Xenopus* egg (McGrew and Richter, 1990; Simon et al., 1992). CPE consensus sequences have also been examined in mice and pigs where the transcript for c-MOS is believed to be under control of CPEs that are enriched in uridine (Dai et al., 2005; Gebauer et al., 1994). c-MOS is a proto-oncogene whose product is part of the crucial maturation promoting factor (MPF) maintenance gene that has been shown in *Xenopus* to be activated

after progesterone treatment during meiotic maturation. Its inhibition prevents germinal vesicle breakdown (Gandolfi and Gandolfi, 2001; Gebauer et al., 1994). What makes c-MOS an attractive message to examine is that it is polyadenylated during oocyte maturation and is degraded during the maternal to zygotic transition.

Parthenogenetic activation is used extensively to study the process of fertilization, as well as for assisting some reproductive technologies such as intracytoplasmic sperm injection (Tesarik et al., 1994; Tesarik and Testart, 1994) and somatic cell nuclear transfer (Whitworth et al., 2009). Electrical poration in the presence of calcium has been a standard method to induce artificial activation of the oocyte (Kure-Bayashi et al., 2000). Oocytes that are parthenogenetically activated can develop past day 25 of gestation (Jolliff and Prather, 1997; Kure-Bayashi et al., 2000), and when used in conjunction with somatic cell nuclear transfer can develop to term and produce viable offspring (Prather et al., 2008). So the quality of activation, in some cases, is compatible with term development, but many embryos die in utero. In the case of parthenogenetic development the cause of the death has generally been attributed to inappropriate genomic imprinting or homozygosity, and in the case of somatic cell nuclear transfer inadequate nuclear reprogramming. Here we present evidence that the type of oocyte activation may also be a contributing factor.

In swine, very little has been published on the exact type of CPE consensus sequences that exist and what they control. While CPEs in swine

have not been widely studied, their sequences have been used in the building of constructs and are conserved through different variants of genes such as mPer1 and mPer2 (Yamamoto et al., 2005). Here we show that there appears to be little correlation between the consensus CPE sequence or the type of nucleotide arrangement within the CPE to length of the poly (A) tail and the transcript abundance; i.e. two transcripts with the same CPE don't have the same pattern in change of abundance or poly (A) tail length over the germinal vesicle oocyte (GVO) stage to the 4-cell stage. In addition, for a specific CPE the transcript abundance in parthenogenetically developing embryos does not mimic that seen in those derived by in vitro fertilization. Overall, we conclude that the CPE is not the only factor controlling transcript abundance during porcine embryogenesis.

Materials and Methods

Unless indicated otherwise, media and components used in this study were purchased from Sigma (St. Louis, MO, USA).

Oocyte and Embryo Collection

GVOs were aspirated from slaughterhouse-derived ovaries washed three times with TL-HEPES and those that had three or more layers of cumulus cells and an uniform appearance were matured in vitro (Lai and Prather, 2003). GVOs were matured and vortexed in an oocyte-denuding medium containing hyaluronidase (see below) (Lai and Prather, 2003) to remove the cumulus cells, and those oocytes with a polar body were used for in vitro fertilization. Oocytes were then co-incubated with freshly capacitated boar's sperm for four hours in

modified Tris-buffered medium (mTBM) containing 0.01 mg/ml osteopontin (OPN), covered with mineral oil (Lai and Prather, 2003). After fertilization (greater than four hours of co-incubation), the embryos were removed and washed four times in porcine zygote medium-3 (PZM3) (Lai and Prather, 2003). After four washes, the embryos were placed into a 500 μl of PZM3 in a four-well plate for culture and placed in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5°C.

Parthenogenetic Activation

Some MII oocytes were used for parthenogenetic activation. 1-2 μl of tissue culture medium-199 (TCM-199) was added into the normal activation medium. Twenty MII oocytes at a time were washed in activation medium that was warmed to 39 °C (Lai and Prather, 2003). Activation medium contained 0.3 M mannitol, 1.0 mM $CaCl_2 \cdot 2H_2O$, 0.1 mM $MgCl_2$, $\cdot 6H_2O$ and 0.5 mM TL-HEPES all at a pH between7.0 and 7.4 (Lai and Prather, 2003). After the wash, those 20 oocytes were placed in a dish containing activation medium between two wires with a gap length of 1 mm connected to a BTX Electro Cell Manipulator 200 and a BTX optimizer graphic pulse analyzer (BTX, San Diego, CA) and exposed to 2 DC pulses with a 1-second interval of 1.2 kV / cm for 30 μs each. After activation, the oocytes were washed in manipulation medium followed by washes in PZM3 before additional culture.

Cordycepin treatment

3′-Deoxyadenosine (cordycepin) dissolved in RNase free water (Applied Biosystems, Foster City, CA, USA) was added to the treatment groups in the maturation medium or PZM3 at a concentration of 40 μM. A control group, which development levels are normal, of no cordycepin treatment was used in tandem with the treatment groups.

Oocyte/Embryo Processing

Cumulus cells were removed from oocytes after 4 minutes of vortexing in 0.03% hyaluronidase. Oocytes and embryos were washed three times in TL-HEPES and placed for one minute into droplets of 2% pronase diluted in TL-HEPES, to remove the zona pellucida. The intact oocytes or embryos were then placed into PBS with 0.1% BSA to stop the proteinase from reacting further with the embryo. Three washes were then done in 50 μl droplets of 0.1% polyvinyl alcohol (PVA) diluted in Dulbecco's phosphate buffered saline (PBS) and the 30 or 60 oocytes or embryos in 5 μl pools were snap frozen in liquid nitrogen in a 500 μl micro centrifuge tube and stored at -80 °C.

CPE transcript selection and identification

cDNA transcripts were selected from a pool of ESTs based on their CPE consensus sites. Basic local alignment search tool (BLAST) was used for each transcript through the National Center for Biotechnology Information's (NCBI) nucleotide collection to identify Genbank accession numbers. Genebank accession numbers are located in (Table 1). CPE consensus sites were

determined by scanning the adenylation control element sequence from the ESTs for seven nucleotide AU rich regions.

RNA Isolation/Reverse Transcription

RNA was isolated from the pools of oocytes/embryos using Qiagen's RNeasy plus mini kit (Qiagen, Germantown, MD, USA). RNA was reconstituted in 35 μl of RNase and DNase free water giving a concentration of .85 oocytes/embryos per μl. This was followed by Invitrogen's Superscript III First-Strand Reverse Transcription kit using 20-mer oligo dT's (Invitrogen, Carlsbad, CA, USA). cDNA was then filtered through Bio-Rad's Micro Bio-spin P-30 Tris RNase free columns giving a concentration of .19 oocytes/embryos per μl and stored at -20 °C for later use in real-time and regular PCR applications (Bio-Rad, Hercules, CA, USA).

Real-Time PCR

Real-time PCR primers were designed by using IDT's PrimerQuest (IDT, Coralville, IA, USA). Verification of primers was done using GoTaq green master mix from Promega (Promega, Madison, WI, USA). PCR setup was as follows: 95 °C for 3 min, 35 cycles of 95 °C for 30 seconds, 59 °C for 30 seconds, 72 °C for 45 seconds ended by a holding temperature of 4 °C. Samples were then analyzed on a 2% agarose gel with ethidium bromide and a DNA ladder from New England Biolabs (New England Biolabs, Ipswich, MA, USA). Real-time PCR was done using Bio-Rad's MyiQ Single-Color Real-Time PCR Detection

System (Bio-Rad, Hercules, CA, USA). cDNA samples were run in technical duplicate and in biological triplicate for each primer pair. On each plate, Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide (YWHAG) was used as the reference gene and water as a no-template control, for each primer pair (Magnani and Cabot, 2008). The standard curve method was utilized to generate the fold differences from the real-time data, which operates by using a previously known amount of cDNA from a reference RNA pool. This reference RNA contains reproductive and non-reproductive porcine tissue RNA and the concentration of cDNA was diluted into 100, 10, 1.0, 0.1 or 0.01 ng/μl (Nanodrop ND1000 spectrophotometer, Wilmington, DE, USA) (Whitworth et al., 2005). The reference cDNA was used on every plate to generate the standard curve. These concentrations were then used as standards within the Bio-Rad MyiQ single color real-time PCR detection system software for each primer used (Bio-Rad, Hercules, CA, USA). YWHAG was used on every plate in technical duplicates and biological triplicates. Plate setup for real-time PCR included 12.5 μl of Bio-Rad iQ SYBR Green Supermix, 9.5 μl of nuclease-free water, corresponding forward and reverse primer at 10 nM concentration and 1 μl of cDNA (.19 oocytes/embryos) sample in each well (Bio-Rad, Hercules, CA, USA). A negative template control of DEPC water was used to ensure that there was no nucleotide contamination. Bio-Rad's software automatically calculated the starting quantity of each sample that is run in comparison with the known amount of YWHAG cDNA utilized in the standard curve (Bio-Rad, Hercules, CA, USA). Visual verification was also accomplished

by examining the melt-curve that is produced for each sample, which gives the dissociation of double-stranded DNA against temperature.

Statistical Analysis for Real-time PCR

The starting quantities were averaged for each stage of development within each of the primers that were used, which generated the averages of the two technical replicates for each of the three biological replicates. For each stage (GVO, MII, 2-cell and 4-cell), the starting quantity averages were then compared with the YWHAG averages to standardize them. Following this, the standardized-to-YWHAG samples were then all standardized to the GVO stage for their corresponding primers, generating the fold-change against GVO. One-way ANOVA was performed on the starting quantities that were generated from the Bio-Rad software following standardization to YWHAG on SAS (SAS Institute, Cary, NC, USA). Within the program SAS, a proc GLM with LSmeans and proc power was used to generate overall p-values and p-values from comparisons. A proc GLM uses the method of least squares to give a general linear model of the data while a proc power shows how many replicates are needed to show a statistical difference if one exist. P-values from the one-way ANOVA table were then analyzed at the $p<0.05$ level of significance. All figures contain appropriate comparisons with the following nomenclature: Rep (technical repetition of the sample), Transcript (one of two cDNA transcripts that were used), Act (type of activation used, whether it was IVF or parthenogenetic), and Stage (stage of development). Statistics were done on the parthenogenetic data

with SAS using the proc GLM and followed the guidelines that were used in the IVF statistical analysis. To differentiate from the IVF statistical analysis, the parthenogenetic embryos were compared at each stage of development to their IVF counterparts using a fertilization type by stage of development interaction. LSmeans were constructed for the previously mentioned interaction only if the fertilization type and the stage of development were significantly different ($p<0.05$). Interactions within the LSmeans were examined only for parthenogenetic and IVF stage comparisons, for example, parthenogenetic MII were compared to IVF MII. In graphs that contained a parthenogenetically activated and IVF sample for the same transcript, their corresponding GVO and MII stage levels were combined, as there are no biological differences between the two at these stages. Thus, also at GVO and MII stage, there are 4 technical replicates for each bar in Figure 1A through 1E. Only at 2-cell and 4-cell stage are the effects of activation type taken into account, as these stages are the only stages that should show any effect. This also produces standard error bars from the mean, which are different from the 2-cell and 4-cell stage analysis. Standard error bars were used from the model that included the three-way interaction of transcript by activation by stage of development.

Polyadenylation Tail length PCR

The analysis of polyadenylation tail lengths using PCR was accomplished using an assay created by Salles and Strickland (1999). Two µl of the pool of 60 oocytes/embryos RNA (.58 oocytes/embryos per µl) for each stage of

development collected from IVF was placed into a new RNase-free tube with 3 µl of RNase-free DEPC water that had been exposed to ultraviolet radiation. Two µl of Invitrogen's 12-18-mer length oligo dT's at 10 ng/µl were then added to each of the RNA samples (Invitrogen, Carlsbad, CA, USA). Following this, the RNA was denatured at 65 ºC for 5 minutes before being transferred to a 42 ºC water bath. While the RNA was being denatured at 65 ºC, a mix was made up of the following and placed into the 42 ºC bath when completed: 4 µl (800 U) of Superscript III buffer (Invitrogen, Carlsbad, CA, USA), 2 µl of 0.1 M DTT (Invitrogen, Carlsbad, CA, USA), 1 µl of 10 mM ATP from the T4 ligase kit (New England BioLabs, Ipswich, MA, USA), 4 µl of DEPC water and 1 µl (2000 cohesive end units) of T4 ligase (New England BioLabs, Ipswich, MA, USA). The RNA was incubated in the water bath and the mix was pipetted slowly into the individual tubes followed by for 30 minutes at 42 ºC. Following the incubation, the anchor primer-labeled PAT Assay primer was added to the mix at a concentration of 10 mM and incubated at 12 ºC for 2 hours then moved to 42 ºC for 2 minutes. After this, 1 µl (2 U) of superscript III RNase H was added and continued at an incubation of 42 ºC for 1 hour. Finally, incubating the ligase for 20 minutes at 65 ºC terminated the reaction.

PCR was used to determine the length of the Poly (A) tail using the following a standard protocol; Twelve and a half µl of GoTaq green master mix (containing 400µM dATP, 400µM dGTP, 400µM dTTP, 400µM dCTP, 3mM $MgCl_2$ and *Taq* polymerase), 9.5 µl of DEPC water, 1 µl of cDNA (.13 oocytes/embryos) template, 1 µl of 10nM reverse primer for the transcript of

interest and 1 µl of the 10nM anchor primer were all mixed and added to individual tubes (Promega, Madison, WI, USA). The samples were then subjected to the following thermal cycler program: 93 ºC for 5 minutes, 35 cycles of 93 ºC for 30 seconds, 60 ºC for 1 minute and 72 ºC for 3 minutes followed by a termination of 72 ºC for 7 minutes and holding at 4 ºC (Eppendorf, Hamburg, Germany). Samples were then run on a 5% polyacrylamide gel (Bio Rad, Hercules, CA, USA) for 45 minutes and visualized using ethidium bromide. Confirmation of PAT assay activity was verified by comparing c-MOS to previous studies, which indicated similar polyadenylation activity (Zhang et al., 2009).

Results

Unless otherwise stated, significance was considered when $p<0.05$.

CPEs were postulated from sequence analysis in GVO and metaphase II (MII) expressed sequence tags (ESTs) using bioinformatics software. From the ESTs, two cDNA transcripts for each of five CPE consensus sequences as well as five pairs of transcripts with two or more consensus sequences were used (Table 1) and primers were designed (Table 2) to measure transcript abundance and poly (A) tail length. Real time PCR was used to determine transcript abundance and followed up by using a poly (A) tail PCR (PAT) assay to measure the length of the transcript's poly (A) tail. Results described below are displayed based on the CPE consensus sequence.

Transcript Abundance (Real Time PCR)

The relative abundance of one of the two transcripts that contained the consensus CPE UUAAAAU increased from GVO to 4-cell stage for in vitro fertilization (IVF) while the other transcript decreased from GVO to 4-cell stage (Figure 1A 6E_07 vs. 4E_07, Table 3). There was a significant interaction when comparing the two UUAAAAU CPE transcripts to the stage of development (Table 3). Parthenogenetically activated embryos showed no difference when compared to the IVF embryos and there was no difference between the type of activation and the stage of development (Table 3).

For the consensus sequence UUAAAAA (Figure 1B), there was a significant difference between the two cDNA transcripts, between the two types of activation, between the different stages and between the three different types of interactions (Table 3). Transcript 10c06 decreased from GVO to 4-cell stage for both parthenogenetically activated and IVF activated embryos while the other transcript (E12) only increased for the parthenogenetically activated embryos (Figure 1B).

CPE consensus sequence AUUAAAA (Figure 1C) showed that there was no significant difference between the two transcripts but that there was significant difference between the two types of activation and activation by stage interaction, but there was no transcript by stage interaction (Table 3). Figure 1C shows that for both transcripts, the parthenogenetically activated 4-cell stage has increased and is much higher than the 4-cell IVF counterpart. The transcript abundance for the IVF embryos peaked at MII stage and decreased steadily through the 4-cell stage (Figure 1C).

Both transcripts for the CPE consensus sequence UUUAAAA (Figure 1D) either decreased or stayed at the same level from GVO to 4-cell stage. There were significant differences between the transcript and between the different stages (Table 3). There were no significant differences between the two types of activation methods, but transcript c-MOS showed a decrease in IVF activated embryos to 4-cell stage, while parthenogenetically activated embryos have no significant difference from their GVO stage levels for the same transcript.

Consensus CPE sequence AUAAAAA (Figure 1E) showed that transcript F01 decreased from GVO to 4-cell stage for both types of activation, unlike for the other transcript E08, which stayed at similar levels throughout. This is evident by the significant differences between the two transcripts and between the stages (Table 3). There was no difference when comparing the two types of activation to each other (Table 3). Transcript F01 was almost completely undetectable by 4-cell stage for IVF activated embryos.

No difference was detected between transcripts, or transcript by stage of development interaction for the consensus sequence of UUAAAAA, AUAAAAA and UUUAAAA (Figure 1F, Table 3). These two transcripts both increased from GVO to MII stage where they both peaked and then decreased through the 4-cell stage (Figure 1F). Parthenogenetically activated embryos were not evaluated for the double and triple CPE consensus sequences.

Transcripts with the double CPE consensus sequence site of UUUAAAA and UUAAAAU (Figure 1G) had a significant difference between the two types of transcripts but not between the different stages (Table 3). Between the two

transcripts, major differences existed at 2-cell stage of development. Transcript A03 increased from GVO to 2-cell stage but was reduced at the 4-cell stage (Figure 1G).

For the transcripts with the consensus CPE sequence AUUAAAA, UUAAAAA and UUUAAAA (Figure 1H), there was no difference between the transcripts but there was a significant difference between the stages of development (Table 3). Only transcript B09 increased from GVO to MII stage before joining transcript E05 at the 2-cell stage. There was no significant interaction between the transcript and the stage of development.

Transcripts with the consensus CPE sequence AUUAAAA and UUAAAAA (Figure 1I) showed no significant difference between the two transcripts or between the stages of development (Table 3). Only transcript B06 increased from GVO to MII stage before decreasing in abundance at the 4-cell stage.

Transcript Abundance (Real Time PCR) Cordycepin Treatment

To examine the effects of inhibiting polyadenylation on transcript abundance, oocytes and embryos were treated with cordycepin. Cordycepin, or 3'-deoxyadenosine, is used instead of regular adenosine by the poly (A) polymerase during polyadenylation causing any further polyadenylation to halt. Cordycepin should cause a decrease in the ability of the poly (A) tail to lengthen and may reduce transcript abundance during the treated stages. Real time PCR on consensus CPE AUAAAAA showed there was a significant difference between treated samples and non-treated samples for the transcript E08 at the

MII and 4-cell stages (Figure 1J). For F01, the other transcript containing the same consensus CPE as E08, there was no significant difference between treated and non-treated samples (Figure 1J). For the other two samples that were used for real time PCR (E05 and B05), there were no significant differences between treated and non-treated samples at each stage (Figures 1K-L).

Poly (A) Tail length (PAT Assay)

In order to measure the length of the poly (A) tail, we used a PAT assay. Transcript 6E_07 with CPE sequence UUAAAAU does not have a clear change in poly (A) tail length from GVO to 4-cell (Figure 2A: the companion CPE transcript 4E_07 was not amplified by the PAT assay). While the abundance of message for 6E_07 increases as development proceeds to the 4-cell stage (Figure 1A). There is no noticeable change between the 4-cell IVF and 4-cell parthenogenetically activated tail length (data not shown).

Transcript B05 with CPE consensus sequence UUUAAAA had an increase in poly (A) tail length from GVO to MII stage followed by a decrease to GVO levels at 2-cell and 4-cell stage (Figure 2B). c-MOS contained the same CPE sequence but the PAT assay did not show any change of poly (A) tail length (Figure 2C). There was no difference in tail length when comparing IVF 2-cell and 4-cell to parthenogenetically activated 2-cell and 4-cell stage embryos (data not shown). Transcript abundance for transcript B05 was numerically higher at the MII stage than at GVO or 2-cell stage, but not significantly (Figure 1D).

Transcript E08 with CPE sequence AUAAAAA showed an increase in poly (A) tail length at the MII stage followed by a decrease at the 2-cell stage (Figure 2D) unlike transcript F01 with the same CPE sequence (Figure 2E). There was no change between parthenogenetically activated and IVF poly (A) tail length (Figure 2F and supplemental Figure 1 for E08 and F01 is not shown). When compared to Figure 1E, there are no obvious correlations between polyadenylation and transcript abundance.

Transcript H02 with consensus CPE sequence UUAAAAA, AUAAAA and UUUAAAA showed an increase in poly (A) tail length between GVO and MII stage with a decrease in tail length to GVO levels for both 2-cell and 4-cell stage (Figure 2G). Transcript C10 contained the same consensus sequence but showed no poly (A) tail changes at 2-cell and 4-cell stage for both parthenogenetically activated and IVF embryos (data not shown).

Transcript G06 with CPE sequence UUUAAAA and UUAAAAU did not change in poly (A) tail length (Figure 2H), while the other transcript with the same consensus, A03, did have a change in poly (A) tail with an increase from GVO to MII stage (Figure 2I). These two transcripts did not have any change of poly (A) tail length between parthenogenetically activated and IVF activated embryos (data not shown).

Transcript E05 (Figure 2K, L) with CPE sequence AUUAAAA, UUAAAAA and UUUAAAA, did not have changing levels of polyadenylation from GVO to 4-cell stage while the other transcript with the same CPEs, B09, did have a change in poly (A) tail length at the MII stage (Figure 2J). This correlates with transcript

abundance for both transcripts (Figure 1H) in that there is a greater abundance of message for B09 when the poly (A) tail is longer. Parthenogenetically activated 2-cell and 4-cell embryos compared to IVF 2-cell and 4-cell embryos do not show a difference in poly (A) tail when compared to each other (data not shown).

Transcript B06 with consensus CPE sequence AUUAAAA and UUAAAAA showed a decrease in poly (A) tail length between MII and 4-cell stage (Figure 2M). Transcript G10 continued the same consensus sequence but only showed poly (A) tail existence at the 2-cell and 4-cell stage for both parthenogenetically activated and IVF embryos (data not shown).

Sequencing utilizing the PAT assay anchor primer was used to confirm that the appropriate PCR product was being amplified using the PAT assay procedure. Results from sequencing confirmed that the product that was amplified was indeed part of the clone.

Poly (A) Tail length (PAT Assay) Cordycepin treatment

MII, 2-cell and 4-cell embryos that were treated with cordycepin, a 3′-deoxyadenosine (3′-dA), only affected polyadenylation for transcript B05 and E08 that changed in length (Figure 3A,B). For transcript E05 and F01 that did not have any change in the length of the poly (A) tail, cordycepin at 1 μg/ml did not have any effect (Figure 3C,D).

Overall, there were no significant differences between the technical and biological repetitions of the transcripts (Tables 3 and 4, replication column).

Discussion

It is thought that the type of CPE located in the 3′ UTR of pig mRNA regulates transcript abundance and poly (A) tail length during the transcriptional quiescence stages of development which, in the pig, exists from GVO to 4-cell stage (Jarrell et al., 1991; Schoenbeck et al., 1992). During this period, only stored maternal mRNA is used for translation until ZGA, when zygotic transcription begins. CPE sites are crucial in determining when polyadenylation occurs in several species thus determining when transcripts are available for translation (Dai et al., 2005; Gandolfi and Gandolfi, 2001; Gebauer and Richter, 1996; McGrew and Richter, 1990). In pigs, cyclin B1 mRNA decreases in abundance as the embryo progresses to the 4-cell stage and is not detectable after this stage (Anderson et al., 1999; Zhang et al., 2009). While the CPE site for cyclin B1 mRNA in pigs has not been examined, similar studies in bovine and *Xenopus* show that cyclin B1 mRNA is linked to a CPE and has possible implications for why cyclin B1 mRNA is polyadenylated differently (Tremblay et al., 2005). The gene c-MOS in pigs has an area in the 3' UTR that contains a region of U and A-rich nucleotides, which is very similar to the *Xenopus* CPE site (Newman and Dai, 1996). For this experiment, analysis of highly abundant transcripts that contained CPE sites that were previously known was done through real-time PCR, PAT assays and cordycepin treatment. Evidence is presented here which questions if the CPE is the only factor that regulates transcript abundance during early development in pig.

The data generated in this study show that transcripts with the same type and number of CPEs do not appear to degrade at similar rates and that multiple CPE sites have no specific control on when a transcript is made available for translation. This is shown through real-time PCR studies using transcripts with known CPE sites sequencing of those results. The relative abundance of one of the two transcripts that contained the consensus UUAAAAA decreased after MII, while the other did not change. One of the two transcripts that contained the consensus UUUAAAA decreased (c-MOS) after MII, while the other did not change. One of the two transcripts with the consensus AUAAAAA decreased after the GV stage while the other did not change. Of the two transcripts with the consensus UUAAAAU one increased after the 2-cell stage, while the other decreased after GV. The two transcripts that contained the AUUAAAA consensus did not change over the stages of embryos evaluated. Two of five transcripts with two CPEs showed no change during these stages, while all four transcripts with three CPEs decreased from GV through the 4-cell stage. Interestingly, there was a difference in the transcript levels between parthenogenetic activated and in vitro fertilized embryos that contained the same CPE consensus site at the 4-cell stage for five of the transcripts that were identified. This is likely due to differences in activation types, which could cause irregular patterns of poly (A) expression of certain transcripts. Electrical poration in the presence of calcium is a method of inducing parthenogenetic activation that results in development to term for somatic cell nuclear transfer embryos (Prather et al., 2008). Indeed, measurement of transcript abundance may

provide another useful tool to evaluate the adequacy of different strategies for oocyte activation.

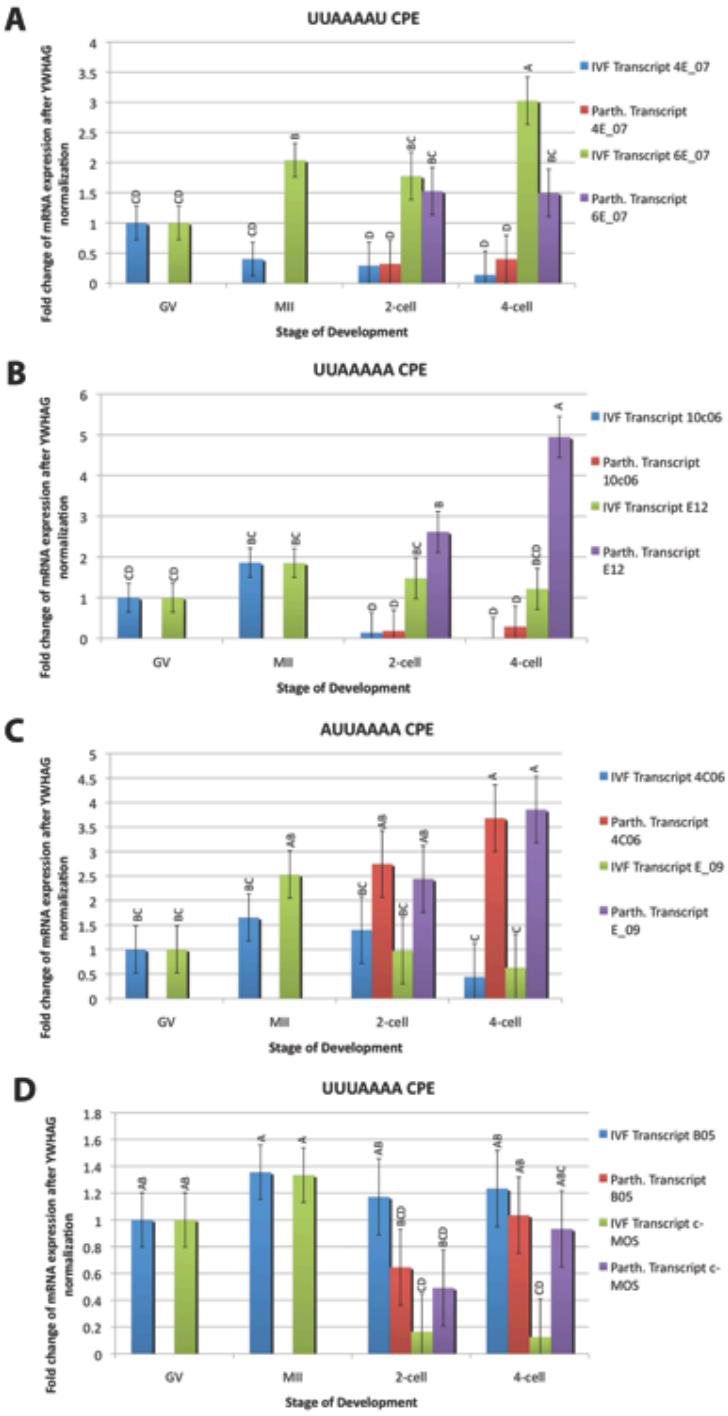
Although real-time PCR was used to confirm transcript abundance in the transcripts with known CPE consensus sites, PAT assays and sequencing of the PCR products were done to verify that the poly (A) tail was changing in respect to the decrease or increase in transcript levels. It is agreed that polyadenylation of a transcript increases the stability and chances of being translated into a protein (Guhaniyogi and Brewer, 2001). However, poly (A) tail length does not necessarily correlate with transcript abundance directly. Some transcript levels were shown to correspond directly to the poly (A) length while some were not. This is most likely due to other factors that are acting on the transcript such as 5′ cap regulation or the distance of the CPE from the hexanucleotide sequence (Richter, 1999). Interestingly, when comparing the CPE sequence of the two transcripts together for each separate CPE sequence, there was no consensus as to how that CPE sequence controls the lengthening or shortening of the poly (A) tail. Also, what was noted is that a majority of the transcripts have an increase of poly (A) tail at the MII stage, but regress at the 2-cell stage to levels that were seen at GVO. This is most likely explained by the increase in maternal mRNA that is lengthened in order to be more readily available for transcription.

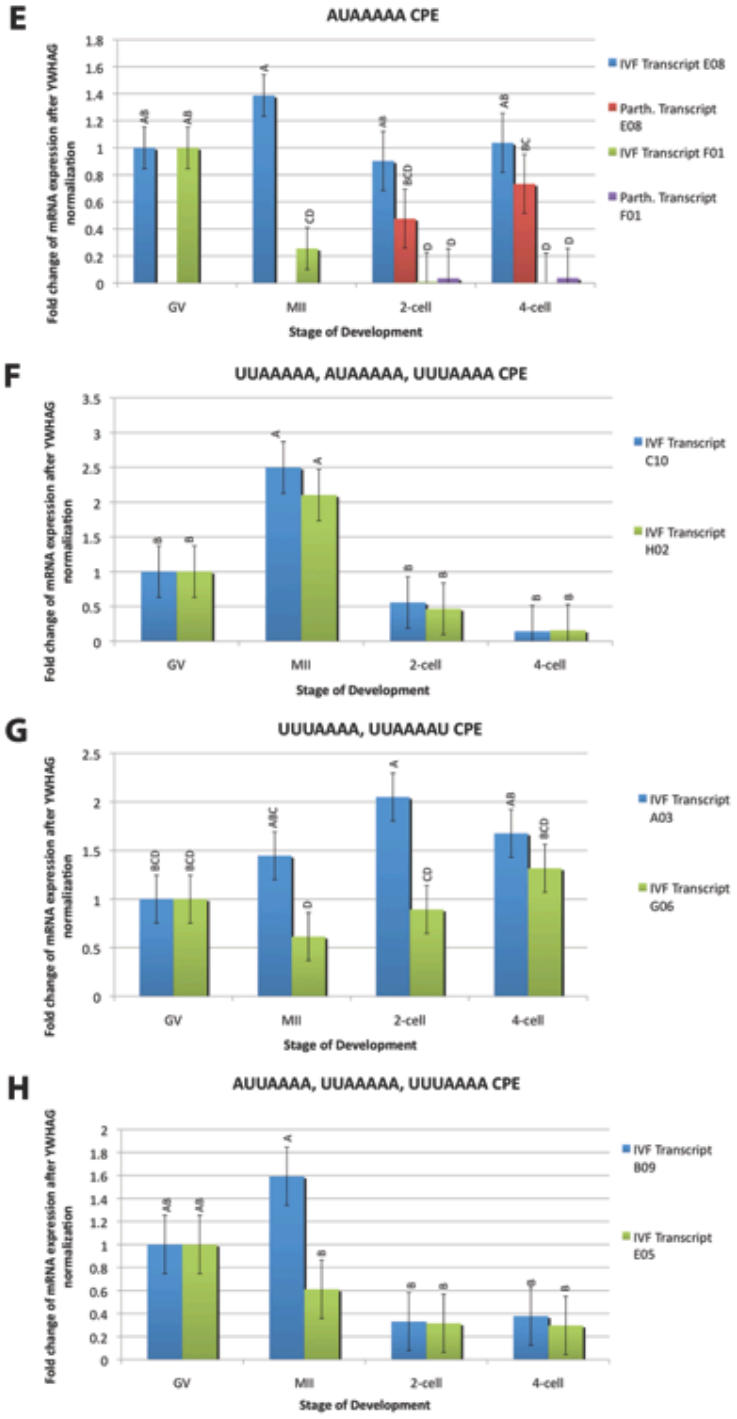
Treating embryos with cordycepin during development will inhibit poly (A) tail lengthening and halt development (Zhang et al. 2009). After treatment with cordycepin, this increase in poly (A) tail length was inhibited at the MII stage while transcripts that showed no change in poly (A) tail length originally, also

showed no change in poly (A) length after cordycepin treatment. It is likely that this response is due to the actions of the 3′-dA interacting with the poly (A) polymerase causing inhibition of adenosine being added to the poly (A) tail. While the abundance of E08 was not significantly different at the MII stage as compared to the GVO ($p=0.44$) or the 2-cell stage ($p=0.32$) the trend is as expected for the poly (A) tail length and the ability to inhibit the lengthening. This shows that when the poly (A) tail is being adenylated the abundance of the message increases. Interestingly the CPE sequence alone was not a good indicator of change in abundance or poly (A) length.

In conclusion, it was determined that cytoplasmic polyadenylation elements are not entirely responsible for maintaining the stability of transcripts during pig embryogenesis and that transcript abundance after parthenogenetic activation does not necessarily mimic in vitro fertilization. Other possible mechanisms have been shown to control the rate of translation of mRNA such as miRNAs, processing bodies, 5′ capping, internal ribosomal entry sequences, upstream open reading frames, steric blockage, RNA-binding proteins and deficiency of amino acids required for protein production (Gebauer and Hentze, 2004). From these other mechanisms of control, they fall in either the category of local mRNA control or global mRNA control but all have can have control of transcript abundance and at the different stages of translation. Future studies should continue to examine other factors that may affect mRNA degradation and cytoplasmic polyadenylation, and their correlation with the CPE consensus sequences.

Figure 1A-L: Real-time PCR for each CPE site.




E
AUAAAAA CPE
Fold change of mRNA expression after YWHAG normalization
Stage of Development
GV
MII
2-cell
4-cell
IVF Transcript E08
Parth. Transcript E08
IVF Transcript F01
Parth. Transcript F01
F
UUAAAAA, AUAAAAA, UUUAAAA CPE
IVF Transcript C10
IVF Transcript H02
G
UUUAAAA, UUAAAAU CPE
IVF Transcript A03
IVF Transcript G06
H
AUUAAAA, UUAAAAA, UUUAAAA CPE
IVF Transcript B09
IVF Transcript E05

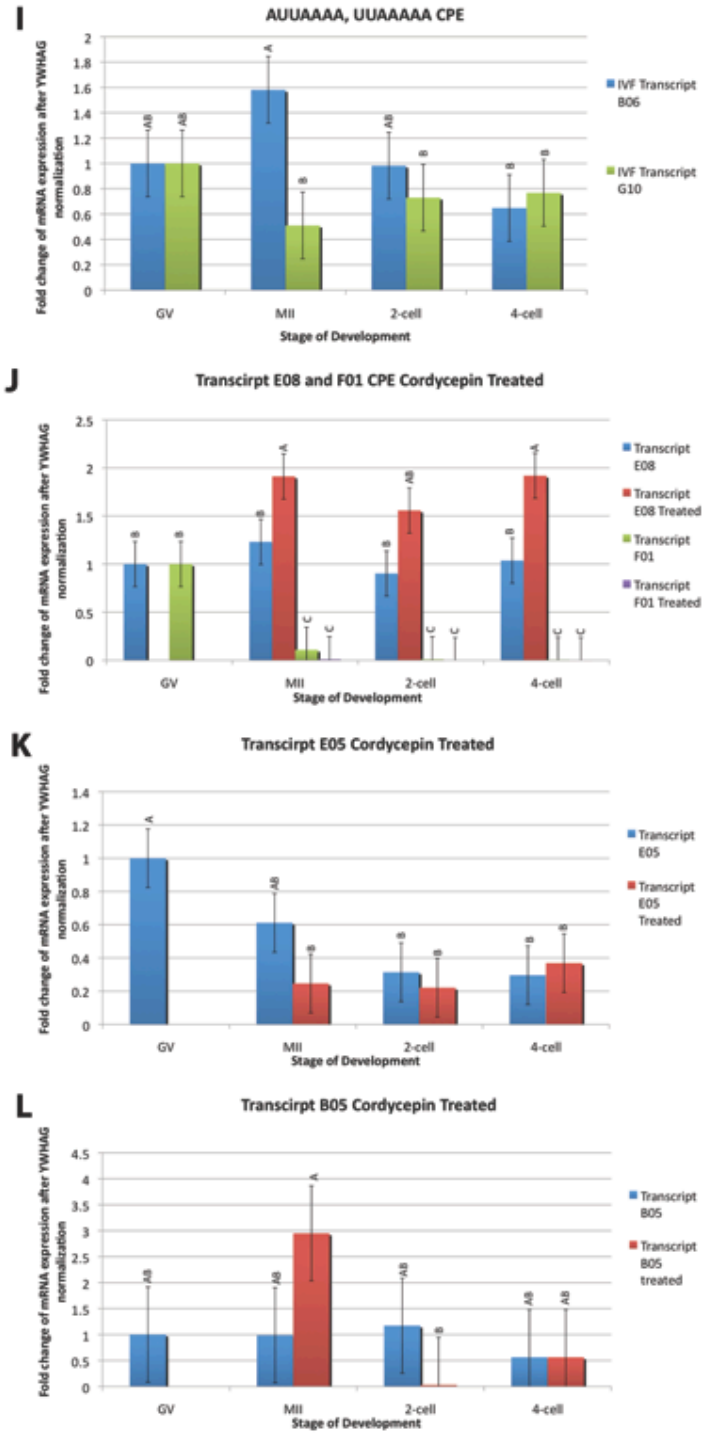

I
AUUAAAA, UUAAAAA CPE
Fold change of mRNA expression after YWHAG normalization
Stage of Development
GV
MII
2-cell
4-cell
IVF Transcript B06
IVF Transcript G10
J
Transcirpt E08 and F01 CPE Cordycepin Treated
Transcript E08
Transcript E08 Treated
Transcript F01
Transcript F01 Treated
K
Transcirpt E05 Cordycepin Treated
Transcript E05
Transcript E05 Treated
L
Transcirpt B05 Cordycepin Treated
Transcript B05
Transcript B05 treated

Figure 2A-M: PAT Assay for IVF oocyte/embryos on 2% Agarose Gel.

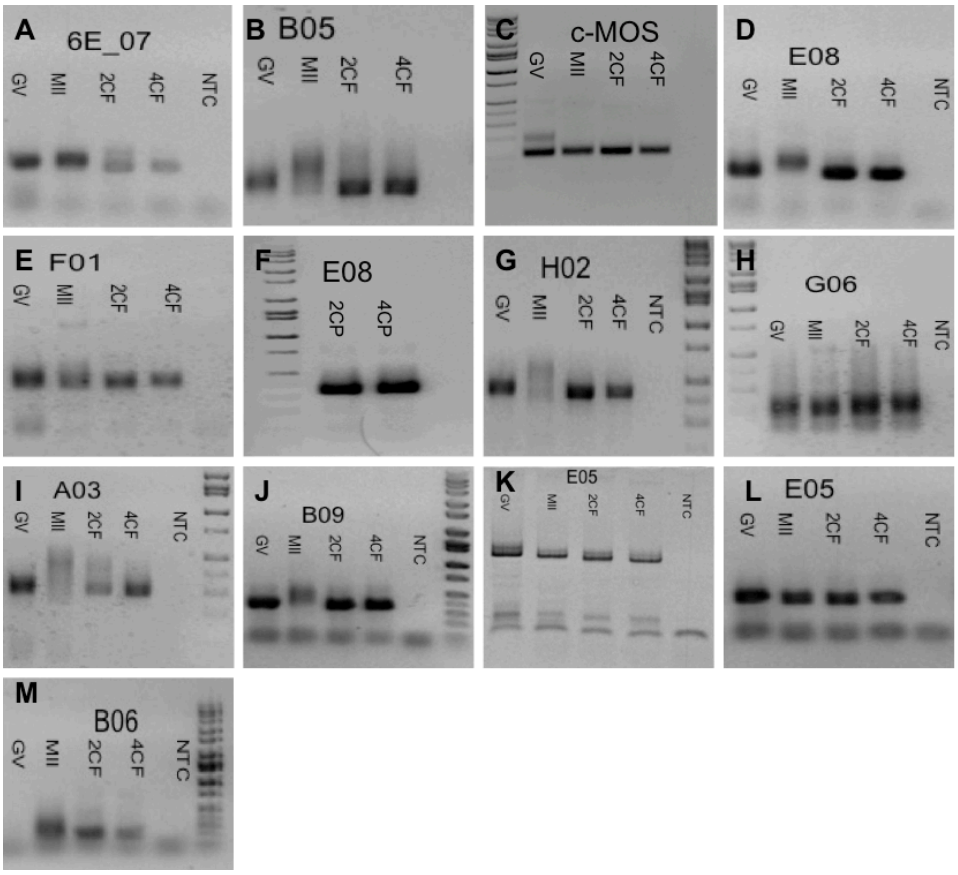


Figure 3: PAT assay for IVF Transcript E08, B05, E05 and F01 treated with Cordycepin on a 2% agarose.

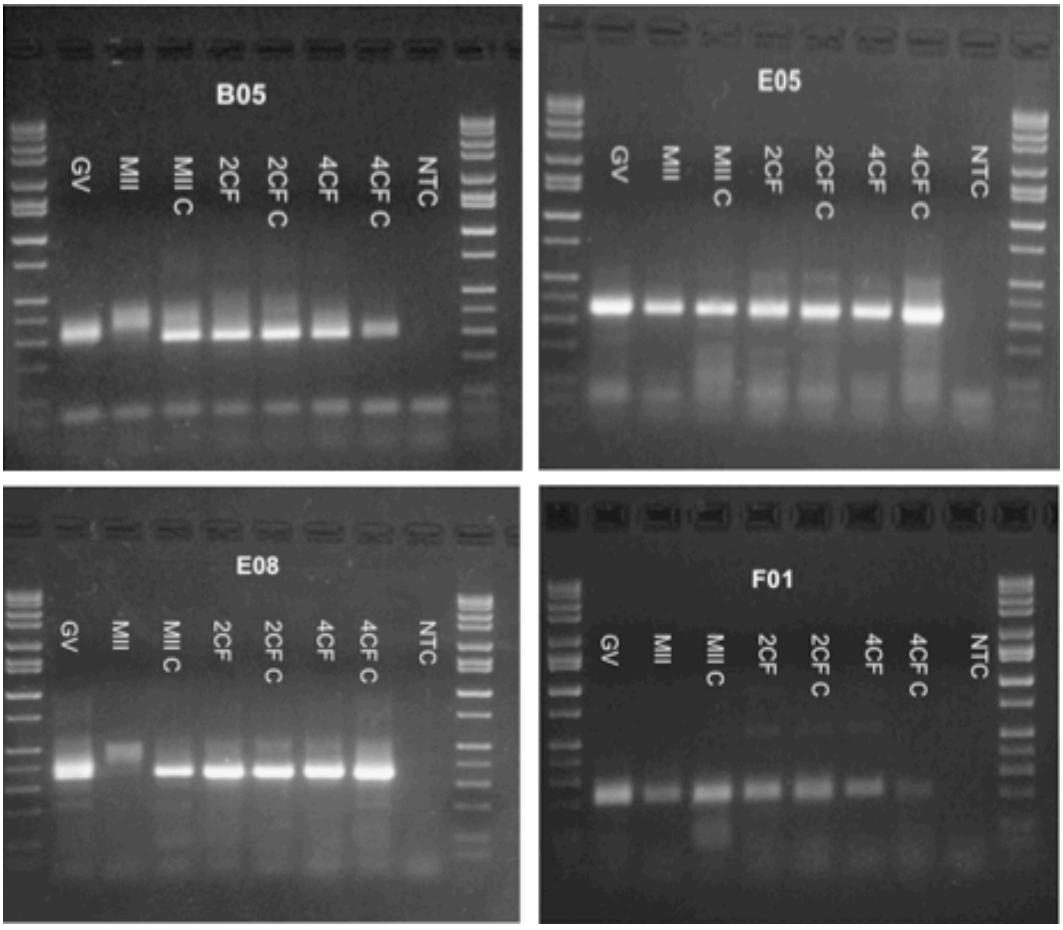


Table 1. CPE target sequence and transcript name associated with that CPE sequence.

CPE Sequence	Figure Name	Distance of CPE to Poly (A) tail start (in bps)	Transcript name	GB accession number
UUUAAAA	cMOS	20	cMOS	NP_001106690
	B05	37	Karyopherin alpha 7 (importin alpha 8)	CN032506
UUAAAAA	10C06	32		GT641592
	E12	161	Similar to TRAF and TNF receptor-associated protein	GT641314
AUAAAAA	F01	40		GT6411484
	E08	72	H1FOO protein	GT640414
AUUAAAA	4C06	136		CN032732
	E09	15		CN032062
UUAAAAU	6_E07	231		CN031738
	4_E07	258		CN032312
UUUAAAA, UUAAAAU	A03	78	Presenilins associated rhomboid-like protein mitochondrial precursor	GT640950
	G06	354		GT641592
AUUAAAA, UUAAAAA	B06	1	Similar to Protein BTG4	CN032166
	G10	283		CN032256
UUUAAAA, UUAAAAA	7_E08	28	Ubiquitin carboxyl terminal hydrolase	GT641310
UUUAAAA, AUAAAAA, UUAAAAA	H02	2	DNA-Binding protein inhibitor ID-3	GT641600
UUUAAAA, UUAAAAA, AUUAAAA	B09	30	Signal peptidase complex subunit 2	CN032312
	E05	63	Elongation Factor	CN032873

Genebank accession numbers are attached. Distance of CPE to Poly (A) start was determined by the distance in bases of the closest CPE sequence to the start of the Poly (A) tail.

Table 2. **qPCR and PAT assay primers used throughout experiment.**

Primer Identification	Primer Sequence
Mos set 1 forward	5'-AAATCAGCGACTTTGGTTGCTCCG-3'
Mos set 2 reverse	5'-ATGGCAAAGGAGTAGATGTCCGCT-3'
h02 Set 2 For.	5'-TTTAGGCCACCCAAGTTCACAGTC-3'
h02 Set 2 Rev.	5'-ACTTTGTCGTGCCCACTTGACTTC-3'
b05 Set1 For.	5'-ATGGTCTTGGAGCAGGGCTGATAA-3'
b05 Set1 Rev.	5'-AGGCTTTGCAAATGCACGAGAACC-3'
e12 Set 1 Rev.	5'-TGCTGCAGGTACAGCTCAGATTCA-3'
e12 Set 1 For.	5'-GCCATTTGCCTCTGTTGGTGACAT-3'
a03 Set 1 Rev.	5'-TGGCATGAAATGAGGACTAACGGC-3'
a03 Set 1 For.	5'-TGTCTTCCAGGCAGGAGCAAAGAT-3'
g06 Set 1 Rev.	5'-TCAGTGGGTTAAGGATCCGGCATT-3'
g06 Set 1 For.	5'-CTACACCAGAACCACAGCAACACA-3'
f01 Set 1 Rev.	5'-CCGGAAACGAGAAACTGTGTCCAT-3'
f01 Set 1 For.	5'-TGAAGTCCATGTACTGGTGGCACT-3'
b09 Set 1 Rev.	5'-TGCCCGTTTCAATAGCTGTTTCCC-3'
b09 Set 1 For.	5'-CCCAAGTCAGATATATACACACCCAC-3'
g10 Set 1 Rev.	5'-CTGTATGTAGCAGGGCTTCCTTAATT-3'
g10 Set 1 For.	5'-AGCCACCATATTGAAAGCGGAGAG-3'
c10 Set 1 Rev.	5'-AATGACCAAACGACCTTCTGCCAC-3'
c10 Set 1 For.	5'-GAAGTCAAGTGGGCACGACAAAGT-3'
e08 Set 1 Rev.	5'-GGGAACAAGATCCCTAAAGAGGCT-3'
e08 Set 1 For.	5'-TTCTTACTGGCCACCTTGGAAGAG-3'
e05 Set 1 For.	5'-TGAGACCGTTCTTCCACCACTGAT-3'
e05 Set 1 Rev.	5'-CACCAAGTCTGCTCAGAAAGCTCA-3'
b06 Set 1 For.	5'-CACCCTTGTCCCTCATGTGGTTAT-3'
b06 Set 1 Rev.	5'-ACAGGTACCACTGGGTCAACACAA-3'
6e_07 Set 1 For.	5'-AATGGGAGAGGGAAAGGACACTGA-3'
6e_07 Set 1 Rev.	5'-AGAGCCCAACATGGAAACTGGCTA-3'
4e_07 Set 1 For.	5'-AGCAACATGAGACAAGTGGGTCAG-3'
4e_07 Set 1 Rev.	5'-GTGGACATGATCCATCTTCAGCCA-3'
10c06 Set 1 For.	5'-AAAGATACTCTGCTACAGCCGGGA-3'
10c06 Set 1 Rev.	5'-TGTTTCTACTCTCAACGCTGGGCT-3'
e_09 Set 1 For.	5'-ACTTTGGACCTTCCTGCTCTTCCT-3'
e_09 Set 1 Rev.	5'-ATGCTCTCCAGTACTGAGTCGTCTGT-3'
4c06 Set 1 For.	5'-TTCCACACGAATGCTTCCAGTCCT-3'
4c06 Set 1 Rev.	5'-AATTATCGAGGGTCACCTGGCAGA-3'
PAT Assay 3' Primer end	5'-GCGAGGTCCGCGGCGGCGTTTTTTTT-3'

Table 3. P-values for the different types of interactions from Figure 1 A-I Real-time data.

CPE Sequence	Transcript	Stage	Activation	Repetition	Act.* Stage	Transcript* Act	Transcript* Stage
UUAAAAA	p<0.01	p<0.01	p<0.01	p=0.13	p=0.01	p<0.01	p<0.01
AUUAAAA	p=0.73	p<0.01	p<0.01	p=0.79	p<0.01	p=0.97	p=0.68
UUAAAAU	p<0.01	p=0.72	p=0.28	p=0.81	p=0.48	p=0.13	p<0.01
UUUAAAA	p=0.06	p<0.01	p=0.68	p=0.22	p=0.59	p=0.07	p=0.11
AUAAAAA	p<0.01	p<0.01	p=0.38	p=0.93	p=0.80	p=0.30	p=0.01
UUUAAAA, UUAAAAU	p<0.01	p=0.07		p=0.42			p=0.10
UUAAAAA, AUAAAAA, UUUAAAA	p=0.66	p<0.01		p=0.85			p=0.93
AUUAAAA, UUAAAAA, UUUAAAA	p=0.13	p<0.01		p=0.54			p=0.16
AUUAAAA, UUAAAAA	p=0.11	p=0.57		p=0.47			p=0.11

Significance was considered at p<0.05

Table 4. P-values for the different types of interactions from Figure 1 J-L.

Transcript Comparison	Transcript	Stage	Treatment	Repetition	Transcript* stage	Treatment* stage
E08 and F01 Treated and Non-Treated	p<0.01	p=0.42	p=0.87	p=0.85	p<0.01	
E05 Treated and Non-Treated		p<0.01	p=0.45	p=0.51		p=0.63
B05 Treated and Non-Treated		p=0.48	p=0.95	p=0.42		p=0.36

Significance was considered at p<0.05

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APPENDIX A: 2010 IETS and 2009 University of Missouri Life Science Week ABSTRACTS

CHARACTERIZATION OF CYTOPLASMIC POLYADENYLATION SITES DURING PORCINE EMBRYOGENESIS

K. B. Dobbs, W. G. Spollen, G. Springer, R. S. Prather

University of Missouri, Columbia, MO, U.S.A.

During the development of a germinal vesicle (GV) stage porcine oocyte to the 4-cell stage embryo there is a transcriptionally silent period during which the oocyte/embryo relies completely on the maternal contribution of mRNA. Regulation of transcription and degradation of the maternal mRNA is at least partially dependent upon cytoplasmic polyadenylation elements (CPEs) within the 3′ untranslated region of the mRNA. The goal of this study was to determine how single, double and triple CPE sites affect the transcript levels of mRNA during embryogenesis. Expressed sequence tags from oocytes and 4-cell stage embryos were scanned for the presence of consensus CPEs (TTTAAAA, TTAAAAA, ATAAAAA, TTAAAAT, ATTAAAA). We choose 19 different transcripts containing one to three CPE sites, to examine their mRNA levels in GV, metaphase II (MII), 2-cell and 4-cell stage embryos via real-time PCR. Ovaries were collected from a local slaughterhouse, aspirated and those oocytes surrounded by cumulus cells were matured in vitro, fertilized and cultured to the 2- or 4-cell stage. At each stage, 30 oocytes/embryos were pooled and flash-frozen in liquid nitrogen followed by storage at -80 C. Oocyte/embryo pools then

had their RNA extracted and reversed transcribed into cDNA using an oligo dT primer accompanied with purification using Tris. Real time PCR was performed with primers designed for the corresponding transcripts using three biological and two technical replicates for each stage. Starting quantities were calculated based on the standard curve method and were standardized to the housekeeping gene, YWHAG and then MII, 2-cell and 4-cell were compared to the GV stage. One-way ANOVA was done on the individual samples using proc GLM and proc power in Statistical Analysis System (SAS). Individual comparisons were made by a protected ($P<0.05$) LSmeans. The relative abundance of one of the two clones that contained the consensus TTAAAAA decreased after MPII, while the other did not change. One of the two clones that contained the consensus TTTAAAA decreased (c-MOS) after MPII, while the other did not change. One of the two clones with the consensus ATAAAAA decreased after the GV stage while the other did not change. Of the two clones with the consensus TTAAAAT one increased after the 2-cell stage, while the other decreased after GV. The two clones that contained the ATTAAAA consensus did not change over the stages of embryos evaluated. Two of five clones with two CPEs showed no change during these stages, while all four clones with three CPEs decreased from GV through the 4-cell stage.

Our results show that there was no significant trend when comparing two transcripts that contained the same type and number of CPE sites, and that combinations of CPE sites can change the exact nature of when the transcript is

available for translation. Thus CPEs may not be the only factors that regulate message stability.

HEAT STRESS AND HEAT SHOCK PROTEIN PRODUCTION IN EARLY PORCINE EMBRYOS *IN VITRO*

Kyle Dobbs and Randall S. Prather

Department of Animal Science, University of Missouri-Columbia

Environmental stressors can negatively impact embryo development. Generally a cell responds to such stressors by transcribing and translating chaperones. Unfortunately, in the pig, zygotic RNA synthesis does not occur from germinal vesicle breakdown until the four-cell stage, which, *in vitro*, is about 48 to 50 hours after fertilization. This presents a problem when ambient temperatures are high. Heat shock proteins are expressed in higher amounts under environmental stress during *Bos taurus* embryogenesis, while little is known about how *Sus scrofa* embryos respond to heat stress.

We proposed to investigate whether or not injection of mRNA coding for Heat Shock Protein (HSP) 70.6 or 90β will increase the survival of a heat stressed embryo to the 8-cell stage of development. Molecular vector design was used to create mRNA for the two HSP, which were used for microinjection into 1-cell porcine embryos right after in vitro fertilization. At 22 hrs post-injection the HSPs should have been translated. Then the temperature will be elevated for 3 hrs to 42.5°C. The embryos will then be returned to 39.5°C and cultured for 36-48 hours. mRNA coding for the eGFP will be used to visually verify that the injection was successful and that the translation had occurred. If injection of mRNA can rescue the heat stressed embryos a transgenic pig overexpressing

HSP in the oocyte may permit animals to initiate a pregnancy even while heat stressed.

APPENDIX B: Heat Shock Protein Overexpression in Heat Stressed Porcine Embryos

Introduction

Initial research during the beginning of my Master's degree began with the thesis title "Heat shock protein (HSP) 70.6 and 90β overexpression in porcine zygotes will make them resistant to environmental stressors that normally impair development." It is known that environmental stressors can have a dramatic negative impact on embryo development (Hansen, 2007). When stress, such as that induced by free radicals or high temperature, occurs to the point of protein mis-folding, the embryo might undergo apoptosis. Generally a cell responds to such stressors by transcribing and translating chaperones (Hansen, 2007; Isom et al., 2007). Unfortunately, in the pig zygotic RNA synthesis does not occur from germinal vesicle breakdown until the four-cell stage, which, *in vitro*, is about 48 to 50 hours after fertilization (Prather, 1993). This becomes a problem for those who are breeding swine in areas of the world where temperatures are over the optimum. Research during embryogenesis in *Bos taurus* has shown that heat shock proteins are expressed in higher amounts under environmental stress, while there has been a very limited amount of research done on *Sus scrofa* embryonic heat shock proteins (Hansen, 2007).

The overall goal of this project was to determine if overexpression of heat shock proteins in porcine zygotes would result in resistance to environmental stressors. This project incorporated two different HSPs 70.6 and 90β, both of which have been studied in their interactions within the somatic cells. More specifically, this project began with in vitro work to determine if these HSPs

improve the chances of survival of the early embryo after environmental stressors. These environmental stressors are not limited to heat but also include deprivation of oxygen that could alter or stop development. If it is possible to reduce or eliminate the consequences of these environmental insults by providing either of these two HSPs during porcine embryogenesis, a window will be opened into a greater understanding of embryogenesis, and possible remedies to environmental stress. In addition, a next step may be to attach an oocyte-specific promoter to the gene(s) and create a transgenic animal that would reproduce under, for example, high ambient temperatures. A zona pellucida promoter would ensure overexpression only during oogenesis and provide the transcripts when embryonic HSP cannot be transcribed.

Discussion

RNA was generated that represented the complete coding sequence for the representative HSPs from cDNA libraries. Following mRNA production for the HSPs, eGFP mRNA was also created for use as a control injection. After the mRNA had been generated, heat shock studies were done on embryos to determine what amount of time and at what temperature the embryos would need to be incubated at to cause a statistically significant decrease in development as determined by blastocyst rate. The reason why this project stopped was due to a couple of unforeseen reasons. First, the temperature at which the embryos were shocked at (42.5ºC) was physiologically irrelevant. At such ambient temperature that would result in a uterine temperature of 42.5ºC,

most sows and gilts would have a heat load that is detrimental to their health leading to death (D'Allaire et al., 1996). The reason why a temperature was not used below 42.5ºC was due to an increase in development from zero to nine hours of heat shock at temperatures below 42.5ºC. At a heat shock of 42.5ºC, there was a decrease in development at four to five hours of treatment but at three hours of heat shock, there was no change in development rate when compared to the control. When ICC was done using antibodies for the two HSPs, there was a clearly already enough HSPs to begin with in the embryo showing that by adding more, there would probably would not be an alteration in the rate of development. These problems along with the actual injection process being extremely difficult, led to the change in projects.

Vita

Kyle Bradley Dobbs was born in St. Louis, Missouri on January 18, 1986. He grew up in a suburb of St. Louis, Webster Groves, Missouri and went to high school at Lutheran South in Afton, Missouri. His love for animals started with his first dog, Millie, a Norwegian elkhound, which led him to follow the path to pursue a DVM. Following graduation from high school in 2004 he then earned his B.S. in Biological Sciences and minor in Chemistry in 2008 from the University of Missouri, Columbia. During this time, he worked in two reproductive science laboratories, which sparked his interest for graduate school in Animal Science. Directly after receiving his undergraduate degree, he entered into the Animal Science graduate program at the University of Missouri, Columbia and earned his Master of Science degree in May 2010 under the supervision of Dr. Randall Prather. Kyle got engaged to his High School sweetheart, Lauren Kienstra, during his graduate studies and will marry June 2010. He will purse a doctorate of philosophy degree in Animal Molecular and Cellular Biology at the University of Florida, Gainesville under the supervision of Dr. Peter Hansen.