THESIS

REGULATORY MICRORNA DELIVERED TO STALLION SPERMATOZOA DURING EPIDIDYMAL MATURATION

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ABSTRACT

REGULATORY MICRORNA DELIVERED TO STALLION SPERMATOZOA DURING EPIDIDYMAL MATURATION

Stallion spermatozoa are produced in the seminiferous tubules of the testis. After spermatogenesis, spermatozoa migrate through the seminiferous tubules to the rete testis then efferent ducts which converge to form a single duct within the caput of the epididymis. The epididymis is a convoluted tubule with region-specific luminal profiles. The epididymis consists of three commonly descried regions; caput, corpus, and cauda. Each region performs distinct functions in epididymal maturation of spermatozoa. The caput is responsible for concentration of spermatozoa by reabsorbing excess fluid from the epididymal lumen. The corpus is where majority of maturation occurs as spermatozoa gain motility and shed their cytoplasmic droplet. The cauda primarily serves as a storage site for the spermatozoa until ejaculation or spontaneous emission. All regions of the epididymis are lined with multiple epithelial cell types, each with different functions to provide the ideal luminal environment for the maturation. These epithelial cells also create apical blebs containing small, membranous vesicles named epididymosomes. The apical blebs are released from the apical surface via apocrine secretion and will disintegrate in the lumen, releasing epididymosomes. Epididymosomes transport proteins from the epithelium to the spermatozoa in the lumen. They also contain microRNAs (miRNAs). MicroRNAs are small, non-coding post-transcriptional regulators of mRNAs and can interfere with mRNA transcription through translational repression or degradation. We hypothesize that epididymosomes also transfer miRNA from the epididymal epithelium to spermatozoa.

Quantitative real-time polymerase chain reaction was used to determine the miRNA profile of epididymal tissue from the caput and cauda, epididymal spermtozoa from the caput and cauda, and epididymosomes from the caput, proximal corpus, distal corpus, and cauda. Our focus turned to 33 newly-acquired miRNAs with expression specific to the spermatozoa located within the cauda as these are fully mature cells. Comparing the miRNAs present in each sample, 11 miRNAs had a distinct path from epididymal tissue to epididymosomes to spermatozoa, suggesting that miRNAs are transported to spermatozoa from the epididymal epithelium via epididymosomes. Pathway analysis was performed using DIANA tools on the 33 miRNA with expression on in caudal spermatozoa using an a posteriori method with predicted pathways considered significant with $P \leq 0.05$. Fifty-one predicted pathways were statistically significant. Some of those predicted pathways suggest a role in cell motility and viability, while others influenced factors in the oocyte or embryo. By developing a better understanding of the mechanisms behind the processes that regulate sperm maturation as well as the roles in embryogenesis better diagnostics for infertility in the horse may be generated.

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CHAPTER 1: LITERATURE REVIEW

Introduction

Spermatozoa are not yet fully mature following spermatogenesis in the testes. They are immotile and unable to recognize and fertilize an oocyte. It is not until the newly-developed sperm reach the epididymis that they gain these traits. During epididymal migration, spermatozoa experience different luminal environments that cause changes associated with maturation. Mechanisms behind these changes are not yet fully understood. Epididymosomes are small microvesicles that carry proteins to the sperm surface within the epididymis. They also carry microRNAs (miRNAs). It is believed that these proteins and miRNAs are responsible for sperm maturation. Developing a better understanding of the miRNA profiles in the various regions of the stallion epididymis may aid in determining the role of these miRNAs in sperm maturation.

Spermatogenesis in the Stallion Testis

Spermatogenesis is the production of spermatozoa within the seminiferous tubule through cellular division and differentiation. The process begins with spermatogonia, which line the base of the seminiferous tubule, undergoing mitosis to become primary spermatocytes. Primary spermatocytes go through meiosis resulting in haploid spermatids (Johnson et al., 2011). At this point spermatids have a condensed nucleus and have lost their transcriptional and translational ability (Johnson et al., 2011; Dacheux & Dacheux, 2014). The spherical spermatids develop into spermatozoa through morphological differentiation without any additional cellular division. The spermatozoa enter the lumen of the seminiferous tubule in a process known as spermiation. In

total, this process takes 57 days in the stallion. Next the spermatozoa migrate out of the seminiferous tubule through hydrostatic pressure and into the rete testis followed by efferent ducts until reaching the epididymis (Johnson et al., 2011).

Spermatozoa

Mature spermatozoa can differ in size and shape between species (Garner & Hafez, 1980). Bulls, boars, and rams all have spermatozoa with flattened heads (Saacke & Almquist, 1964). Stallions have spermatozoa similar in size and shape to that of humans and other primates (Johnson et al., 1980; Johnson, 1982). Spermatozoa consist of a head, primarily composed of the nucleus, that is covered by an acrosome. The acrosomal membrane covers the apical ridge, principal segment, and equatorial segment, although the apical ridge is not as notable in the stallion as in other species. The middle piece contains mitochondria that will be used for energy followed by a tail with a neck region that attaches to the head. The tail has the ability to become motile following maturation but spermatozoa remain quiescent until ejaculation. Spermatazoal movement is due to the axoneme consisting of doublets and a central pair of microtubules. The axoneme runs through the middle, principal, and end pieces of the tail and is surrounded by nine pair of dense fibers from the middle piece to the principal piece. Spermatozoa also have a cytoplasmic droplet located near the head which contains excess cytoplasm that was not removed during spermiation (Johnson et al., 2011). This cytoplasmic droplet will migrate down the tail and be removed during maturation usually in the cauda epididymis (Varner & Johnson, 2011).

The Stallion Epididymis

Spermatozoa are considered mature when they gain motility and the ability to bind the zona pellucida and fuse with the plasma membrane of an oocyte, which is not possible prior to epididymal maturation (Robaire & Hermo, 1988). The epididymis is a coiled, convoluted tubule suspended in mesorchium dorsal to stallion testis (Arrotéia et al., 2012). It begins as 13 to 15 highly-coiled efferent ducts as they enter the proximal caput and will converge in the caput (Amann et al., 1977; Hemeida et al., 1978) and approximately 80 meters long in the stallion (Sullivan & Saez, 2013). Connective tissue septa provide support for the epididymis and divide it into regions with specific gene expression in the epithelial lining allowing specific protein expressions (Kirchhoff, 1999; Cornwall, 2009). The epididymis consists of three primary regions, each with a different function; the caput, corpus, and cauda. The caput is relatively flat and closely attached to the anterior aspect of the testis. The corpus is loosely attached dorsally to the testis and cylindrical in shape. The cauda is large and also loosely attached to the posterior end of the testis (Amann et al., 2011). The epididymis is thickest at the proximal caput and thins near the cauda (Arrotéia et al., 2012). However, the luminal diameter and smooth muscle surrounding the epididymis increases from the caput to the cauda (Yanagimachi et al., 1985; Cornwall, 2009). Peristaltic contractions move the spermatozoa through the caput and corpus while the cauda remains relatively quiescent unless stimulated (Sostaric et al., 2008). Migration through the caput and corpus regions of the epididymis always occurs over a four-day time span (Swierstra et al., 1975; Amann, 1981; Gebaeur et al., 1974). However, storage times in the cauda may vary and a reduction of about 30% of the spermatozoa within this region can occur if the stallion ejaculates once every other day (Amann et al., 1979). Tight junctions form between the epithelial cells of the epididymis to create the blood-epididymal barrier which protects the

spermatozoa within the lumen by restricting ions, solutes and macromolecules from entering (Robaire & Hermo, 1998; Dacheux et al., 2005). Specific cell-to-cell interactions affect luminal environment conducive to spermatozoal maturation (Cornwall, 2009). Luminal environment must be at the proper temperature, pH and oxygen tension and amount of energy (Dacheux et al., 2005). Exposure to this quintessential environment is key to maturation (Bedford, 1967; Orgebin-Crist, 1967). Epididymal fluid changes due to various secretions and absorption, along with androgen and luminal factors from the testis (Cornwall, 2009). As spermatozoa migrate through various regions of the epididymis, changes will occur in their size and appearance as well as the acrosome and nucleus (Olson et al., 2002). For example, acrosome molecules are rearranged and compartmentalized in a stage-specific manner during epididymal transit (França et al., 2005). About 99% of fluid is absorbed in the caput of the epididymis resulting in a concentration of spermatozoa and luminal contents (Clulow et al., 1998). Maturation continues through the corpus and majority of mammalian sperm have some fertilizing potential from the middle corpus (Dacheux & Paquignon, 1980). The corpus is also the region in which the cytoplasmic droplet will migrate down the tail until lost (Varner & Johnson, 2011). Maturation is essentially complete by the time spermatozoa reach the proximal cauda (Jones & Murdoch, 1996). When spermatozoa reach the cauda of the epididymis, they are capable of motility similar to that of ejaculated sperm, and the cauda primarily serves as a storage site until ejaculation or spontaneous emission through the urethra (Johnson et al., 1980; Varner & Johnson, 2011). The caudal lumen provides an environment suitable of survival for weeks (Moore, 1995). Bulls and stallions can store enough spermatozoa for around 10 ejaculates (Sullivan et al., 2007). Approximately 90 billion sperm will reside in the excurrent duct in the stallion with nearly 60

billion in the cauda epididymis (Armann et al., 1979). Transit of stallion spermatozoa through the epididymis can take anywhere from five to fourteen days (França, 2005).

Epididymal Epithelium

The epididymis is lined by various epithelial cell types including principal cells, basal and apical cells, narrow cells, and clear cells (Robaire et al., 2006). Principal cells are most abundant and responsible for 80% of the population in the initial segment of the epididymis and lowering to 65% of the epithelial population in the cauda (Robaire & Hermo, 1988). Principal cells aid in formation of the blood-epididymal barrier and secrete majority of luminal proteins (Cornwall, 2009; Robaire et al., 2006). Basal cells are the second most common epithelial cell and make up 15 to 20% of the cellular population (Robaire et al., 2006). Although relatively abundant, their function within the epididymis is not yet well understood (Robaire et al., 2006). However, basal cells work together with principal cells to polarize the epididymis (Lasserre et al., 2001; Dacheux et al., 2005). Apical cells only constitute around 10% of the population in majority of the epididymis, but only 1% in the cauda (Adamali & Hermo, 1996). The roles of these apical cells include maintaining sperm quiescence and regulating the lumen pH through production of enzymes from the carbonic anhydrase family (Hermo et al., 2005). Narrow cells increase in numbers from three percent in the initial segment to around six percent in the corpus and provide intracellular transport between the lumen and epithelial cells (Adamali & Hermo, 1996; Robaire et al., 2006). Narrow cells degrade proteins as well as carbohydrates within the lumen via lysosomes and protect sperm from changes in the environment by harmful electrophiles (Adamali & Hermo, 1996; Robaire et al., 2006). Clear cells, along with halo cells, make up less than five percent of total epithelial population but are equally distributed

throughout the epididymis (Hermo et al., 1994; Hermo et al., 2005; Robaire et al, 2006). Clear cells are endocytic in nature and aide in the clearance of luminal proteins and take up cytoplasmic droplet contents (Hermo et al., 1994; Hermo et al., 2005; Robaire et al., 2006). These cell types all work together to provide proper luminal environment for the spermatozoa.

Exosomes

Studies have shown cells can communicate through spherical membrane vesicles (Ratajczak et al., 2006). Exosomes are smaller membrane vesicles and range in size from 40 to 100nm. Invagination into the lumen of the late endosome will capture material from the cytoplasm and form exosomes containing cellular contents. Exosomes are released when multivesicular endosomes fuse with the plasma membrane of cells (Harding et al., 1984; Pan et al., 1985). Such vesicles have been reported in a multitude of bodily fluids including semen, blood, urine, and breast milk (Ronquist & Brody, 1985; Park et al., 2011; Aalberts et al., 2012; Caby et al., 2005; Pisitkun et al., 2004; Admyre et al., 2007).

Epididymosomes

Epididymosomes are extracellular membrane vesicles similar in structure to exosomes that allow intercellular communication (Sullivan et al., 2005). They range in size from 25-300 nanometers in diameter and transfer proteins and potentially other contents to sperm during maturation (Girouard et al., 2011; Sullivan et al., 2005). Epididymosomes are characterized by their high cholesterol to phospholipid ratio with the primary phospholipid being sphingomyelin (Rejraji et al., 2006). Epididymosomes also contain cytoplasmic contents like proteins, but are void of organelles such as mitochondria, ribosomes, and endoplasmic reticulum (Sullivan, 2015).

Unlike exosomes, epididymosomes are released into the lumen of the epididymis through a process called apocrine secretion (Sullivan et al., 2005). Apocrine secretion was first noted in 1922 by the German histologist P. Schiefferdecker (Sullivan & Saez, 2013). This form of secretion occurs within the prostate and seminal vesicles, vas deferens, epididymis, and uterus (Aumuller et al., 1997; Manin et al., 1995; Hermo & Jacks, 2002; Rejraji et al, 2006; Griffiths et al., 2008). During apocrine secretion, apical blebs begin to protrude from the apical side of principal cells into the lumen (Hermo & Jacks, 2002). These apical blebs will eventually detach and disintegrate in the lumen, releasing contents, including epididymosomes, from within (Nickel et al, 2003). Throughout this process, there is no use of the endoplasmic reticulum or Golgi apparatus in comparison to other forms of secretion (Sullivan et al, 2005).

Epididymosomes are present in multiple species including the hamster, rat, mouse, ram, bull, human, and horse (Legare et al., 1999; Fornes et al., 1995; Grimalt et al., 2000; Rejraji et al., 2006, Griffiths et al., 2008; Ecroyd et al., 2004; Gatti et al., 2004, Frenette & Sullivan, 2001; Thimon et al., 2008; Sostaric et al., 2008). Epididymosomes were first noted in 1985 in the Chinese hamster (Yanagimachi et al., 1985). Yanagimachi et al. (1985) described the addition of small vesicles to the spermatozoa within the lumen of the distal caput that were not within the proximal caput. In the rat epididymis, more apical blebs occur in the cauda than the caput of the epididymis and even fewer in the corpus (Hermo & Jacks, 2002). Girouard et al. (2011) noted 555 proteins within epididymosomes from the caput of bull epididymides and 438 within epididymosomes from the cauda, with only 231 in common between them indicating that epididymosomal contents change through various regions of the epididymis. In the Chinese hamster, more epididymosomes were discovered on the sperms' acrosome in the caput than in the cauda of the epididymis as well (Yanagimachi et al., 1985). Gatti et al. (2004) and Frenette et

al. (2004) noted a difference in protein composition between the epididymosomes located in luminal fluid and spermatozoa from the same regions of the epididymis in rams and bulls. This provides evidence that these proteins vary throughout different regions of the epididymis. Although the primary described function of epididymosomes has been their ability to transfer proteins to spermatozoa, epididymosomes are also known to carry small RNAs called microRNAs (Valadi et al., 2007). Different miRNA profiles exist within epididymosomes of different epididymal regions, and miRNAs within these epididymosomes are transferred to the epithelium of the epididymal lumen (Belleannée et al., 2013).

Small RNAs

Small RNAs are small (18-30 nucleotides), non-coding RNAs (Krawetz et al., 2011). This family of RNAs consists of three primary groups: short interfering RNAs (siRNAs), microRNAs (miRNAs) and piwi-interacting RNAs (piRNAs) (Carthew & Sontheimer, 2009). Short interfering RNAs aid in defending the genome from foreign or invasive nucleic acids such as viruses while miRNAs regulate endogenous genes and piRNAs function primarily in the germ line (Carthew & Sontheimer, 2009). Both siRNAs and miRNAs stem from a double-stranded precursor while piRNAs descend from single-stranded precursors (Carthew & Sontheimer, 2009). Both siRNAs and miRNAs also bind to Ago family of Argonaute proteins to support silencing whereas piRNAs bind to members of the Piwi family (Tomari et al., 2005; Carthew & Sontheimer, 2009). Similarities continue amongst siRNAs and miRNAs as both require DICER enzyme in their formation from their precursors (Meister & Tuschl, 2004). Although similar, siRNAs and miRNAs also have differences in that miRNAs are endogenous while siRNAs are exogenous and derived directly from foreign or invasive nucleic acids (Tomari et al., 2005).

MicroRNA also evolve from an incomplete stem-looped, double-stranded precursor but siRNAs are from a long and fully complemented double-stranded RNA (Tomari et al., 2005).

MicroRNAs

The first miRNA, lin-4, was discovered in 1993 by Victor Ambrose and Gary Ruvkun in the *Caenorhabditis elegans* (Lindow & Kauppinen, 2012). While studying nematodes, they noted lin-4 regulated developmental progression (Lee et al., 1993). Soon after, let-7 was discovered to have a similar role on later development (Reinhart et al., 2000). To this day, lin-4 is considered the founding member of the miRNA family (Lagos-Quintana et al., 2001; Lau et al., 2001, Lee & Ambrose, 2001).

MicroRNAs (miRNAs) are small, non-coding endogenous RNAs about 22 nucleotides in length (Bartel, 2004). They play a role in regulating gene expression with post-transcriptional interaction with the 3' untranslated region (UTR) of target mRNAs (Krawetz et al., 2011). MiRNA can either degrade or cause translational repression (Bushati & Cohen, 2007). MiRNAs are derived from long, capped polyadenlated pri-miRNAs (Davis-Dusenbery & Hata, 2010). During miRNA biogenesis, RNAse III enzymes DROSHA and DICER work together to cleave the precursor pri-miRNAs into miRNAs (Kim, 2005). First, DROSHA cleaves pri-miRNA into a hairpin-structured pre-miRNA (Davis-Dusenbery & Hata, 2010). Exportin then transfers pre-miRNA from the nucleus into the cytoplasm (Davis-Dusenbery & Hata, 2010). At this point, DICER will remove the hairpin structure to generate a miRNA about 22 nucleotides in length (Davis-Dusenbery & Hata, 2010). These newly formed miRNA now attach to mRNA inducing silencing complex (RISC), which mediates gene silencing by way of translational inhibition or degradation of target mRNAs (Davis-Dusenbery & Hata, 2010). An imperfect base-pairing is possible between miRNA and miRNA recognition site at the 3' UTR of target mRNA, meaning a

single miRNA may bind thousands of mRNAs (Selbach et al., 2008; Friedman et al., 2009; Baek et al., 2008). Interactions with mRNAs likely influence output of various protein coding genes (Bartel, 2004). MicroRNAs play a role in regulating gene expression during many processes including development, differentiation, apoptosis, metabolism and stress response (Björkgren et al., 2012; Plasterk, 2006; Schickel et al., 2008).

MicroRNAs in Reproduction

Although miRNAs were first discovered for their role in nematode development, they are also in other regions of the body, including spermatozoa and components of the reproductive tract. MicroRNA have a role in sperm function, fertility and reproduction (Das et al., 2013). They regulate gene expression allowing differentiation of epididymal epithelium that supports male fertility (Papaioannou et al., 2010; Hawkins et al., 2011; Björkgren et al., 2012). For example, mice without DICER1, which prevents the cleavage of pre-miRNA to miRNA, have no differentiation of epididymal epithelium and produce infertile spermatozoa (Björkgren et al., 2012). Some miRNAs are unique to the epididymis in the mouse, human, rat, and bull among others and have differential expression within the various regions (Belleannée et al., 2012; Landgraf et al., 2007; Zhang et al., 2010). Spermatozoal miRNAs were first identified in human followed by that of the mouse and boar (Ostermeier et al., 2005; Amanai et al., 2006; Curry et al., 2009). Over 200 miRNAs are also recognized within the human epididymis (Zhang et al., 2010). Mouse epididymal spermatozoa contain different miRNA profiles dependent upon the various regions (Nixon et al., 2015). Differential expression of miRNAs has also been identified between normal spermatozoa and those with low motility or abnormal morphology (Curry et al., 2011).

The effect of miRNAs extends beyond the spermatozoa. These miRNAs can have an effect on oocyte and developing embryo as well. For example, without miRNA-34c directly from spermatozoa, a mouse embryo will fail to begin the first cleavage division (Liu et al., 2011). Additional miRNAs are delivered to the oocyte at fertilization, along with paternal mRNA and haploid genome, and will remain until the developing embryo's genome begins transcribing (Boerke et al., 2007; Dadoune, 2009). miRNAs can alter stability and translational efficiency of maternal transcripts until activation of the zygote's genome (Liu et al., 2012; Ostermeier et al., 2004; Rodgers et al., 2013). Unfortunately, poor-quality spermatozoa may have negative effects on the embryo's miRNA expression and could affect the embryo's phenotype (McCallie et al., 2010).

Conclusion

Up to 40% of unknown causes for infertility in men may be due to a maturational disorder of the spermatozoa (Cornwall, 2009). A better understanding of various miRNA profiles within stallion epididymal tissue, epididymosomes, and spermatozoa may lead to determining the mechanism behind maturation and oocyte and embryo development. This could result in more efficient in vitro maturation as well as a development of diagnostics and treatment for males of various species to overcome infertility. We know miRNA in spermatozoa are involved in fertilization and embryogenesis. To date, no study has demonstrated where or how miRNAs get to spermatozoa.

CHAPTER II: EPIDIDYMOSOMES TRANSFER MICRORNAS FROM EPIDIDYMAL EPITHELIUM TO EQUINE SPERMATOZOA

Introduction

As spermatozoa leave the testis of the stallion, they are immotile and incapable of recognizing and fertilizing an oocyte. During their migration through the epididymis, spermatozoa are exposed to various environments in the lumen that have post-transcriptional effects on the spermatozoa and aid in maturation (Dacheux & Dacheux, 2014). The epididymis is comprised of three main regions, each with a different function: caput, corpus and cauda. Although epididymal functions are well known, the mechanisms behind sperm maturation are still unclear. Small microvesicles and epididymosomes are generated from epididymal epithelium via apocrine secretion (Hermo & Jacks, 2002; Sullivan et al, 2007). Epididymosomes have been noted in many species including bulls, rats, mice, hamsters, rams, humans, and horses (Sullivan & Saez, 2013; Gatti et al, 2004). These epididymosomes transport proteins from epithelium to spermatozoa and transport small, non-coding RNAs called microRNAs (Valadi et al, 2007). MiRNAs silence genes through translational inhibition or degradation of target mRNA (Davis-Dusenberry & Hata, 2010). Spermatozoa contain miRNA (Valadi et al., 2007). We hypothesize that epididymosomes transport miRNA from epididymal epithelium to the spermatozoa during their migration in the epididymis.

Materials and Methods

Horse Care

The Colorado State University Institutional Care and Use Committee approval was not required for this project due to the use of samples from elective procedures. All whole testes used in this study were obtained from the Harmony Equine Center in Franktown, Colorado, from mature stallions, ranging in age from four to eight years old, with fully-descended testes.

Epididymal Tissue and Luminal Fluid Collection

An epididymis was removed from one testis from each stallion (n=4). Fascia was carefully removed from around the epididymides. Each epididymis was divided into four sections: caput, proximal corpus, distal corpus, and cauda first by ligation with hemostats then excision. Each transected region was further divided into two sections- one for epididymosomes samples, another for spermatozoa samples. To flush the lumen, tissue samples were placed in 15 mL conical tubes with 2 mL sterile phosphate buffered saline (PBS), centrifuged for five minutes at 400 x g, punctured with a sterile needed and centrifugation repeated. Tissue was removed from each conical tube and rinsed three times in PBS then frozen until further analysis. Luminal fluid for epididymosome extraction was also frozen at -80°C until further use. Luminal fluid for spermatozoa isolation was carefully placed in 15 mL conical tubes on 3 ml of room-temperature EquiPure© (Nidacon, Sweden). Each sample was then centrifuged at 200 x g for 30 minutes. The EquiPure supernatant was removed and the remaining spermatozoa were resuspended in PBS and frozen at -80°C. Figure 1 depicts the dissection of each epididymis.

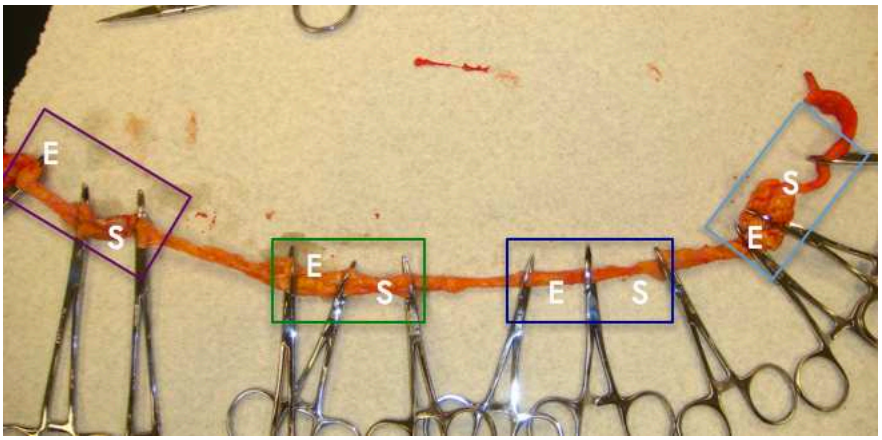

Figure 1

After removal of fascia, epididymides were ligated into four regions using hemostats then excised using the regions labeled E for epididymosome samples and those labeled S for spermatozoa samples.

Epididymosome Isolation

Epididymosomes were isolated from luminal fluid of each region via ultracentrifugation (Frenette et al., 2006). Luminal fluid was diluted with 150 mM NaCl then centrifuged at 700 x g for 10 minutes to eliminate spermatozoa and repeated. A 30-minute spin at 3,000 x g followed to remove remaining cellular debris. The supernatant was ultracentrifuged at 120,000 x g for two hours. The pellet was resuspended in 150 mM NaCl and re-ultracentrifuged. Epididymosomes were resuspended in 250 μL of 150 mM NaCl and frozen at -80°C.

RNA Isolation and Quantification

Total RNA was isolated from mechanically homogenized epididymal tissue, spermatozoa, and epididymosomes using 750 μL TRI Reagent (Molecular Research Center, Cincinnati, OH) and 8 μL Polyacryl Carrier (Molecular Research Center, Cincinnati, OH) held at room temperature for 10 minutes followed by the addition of 200 μL of chloroform. Samples were vortexed and incubated at room temperature for 10 minutes then centrifuged at 12,000 x g

for 15 minutes at 4°C. After centrifugation, the aqueous phase was transferred to a new tube for RNA precipitation with the addition of 500 μL of isopropanol followed by a 10 minute incubation at room temperature then centrifugation at 12,000 x g for 10 minutes at 4°C. Supernatant was removed and 1 mL of 75% ethanol was added and vortexed for an RNA wash. Centrifugation at 12,000 x g for 8 minutes at 4°C and the ethanol wash was repeated. Supernatant was removed and the pellet was air dried. 20 μL of nuclease-free water was added to dissolve the pellet and incubated at 55°C for 15 minutes. After incubation, 2 μL of 10x DNAse I Buffer (Ambion, Waltham, MA) and 1 μL rDNAse I (Ambion, Waltham, MA) were added to each sample and incubated for 30 minutes at 37°C. 2 μL of DNAse Inactivation Reagent (Ambion, Waltham, MA) was added and incubated at room temperature for two minutes while vortexing periodically. Centrifugation at 10,000 x g for 1.5 minutes followed and supernatant was transferred to a new tube for RNA quantification and purity assessment using the Nanodrop Spectrophotometer ND-1000 (Thermo Scientific, Wilmington, DE).

Reverse Transcription

cDNA was generated using miScript II RT kit (Qiagen, Valencia, CA) following manufacturer's protocol. 20 μL reactions were made up with 4 μL of 5x HiSpec Buffer, 2 μL of 10x Nucleics Mix, 1,000 ng of template RNA and RNAse-free water and 2 μL of Reverse Transcriptase Mix. They were incubated at 37°C for 60 minutes then at 95°C for five minutes. cDNA was immediately used in qRT-PCR reaction mix for PCR analysis.

Quantitative Real-Time PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using cDNA and miScript SYBR Green PCR kit (Qiagen, Valencia, CA). Each reaction consisted of 2.4 ng of cDNA, 3 μL of SYBR Green, 0.60 μL of 10x Universal Primer, and 1.34 uL of nuclease-free water in addition to 1.5 μL of equine miRNAs (Fischer Scientific, Waltham, MA). Epididymal spermatozoa samples were run with 346 equine miRNA primers (Table 1) loaded into 384-well LightCycler 480 Plates (Roche, Indianapolis, IN) and analyzed using the LightCycler 480 PCR System (Roche, Indianapolis, IN) or QuantStudio 5 Real-Time PCR System (Thermo Fisher, Waltham, MA). Epididymal tissue samples were run with 324 equine miRNA primers identified in epididymal spermatozoa (Table 1). Epididymosome samples were run with a subset of 92 equine miRNA primers noted only in the cauda or caput of either epididymal tissue, or spermatozoa, or miRNAs differentially expressed between the samples from those regions (Table 1). MiRNAs were considered present if the cross point cycle (Cp) value was less than 40.0 and the melt curves appeared normal. Samples from each region were run in duplicate and normalized to internal controls- miRNAs present within all samples with a standard deviation between samples less than one. Internal controls for each sample type were as follows: epididymal spermatozoa- eca-mir-129a5p, eca-mir-130b, eca-mir-1403p, eca-mir-3233p and eca-mir-5083p, epididymal tissue- eca-mir-129a5p, eca-mir-3233p, eca-mir-433, and eca-mir-5083p, epididymosomes- eca-mir-1403p, eca-mir-5083p and eca-mir-653. MiRNAs were considered present within samples if noted in three of the four epididymides for each region.

Table 1. Equine miRNA ID and Sequence

Spermatozoa samples were compared to 346 known equine miRNAs. All 346 are provided below with the corresponding sequence. Those used in **tissue** samples are bolded and those also used in ***epididymosome*** samples.

Mature miRNA ID	Target miRNA Mature Sequence
eca-let-7a	tgaggtagtaggttgtatagtt
eca-let-7c	tgaggtagtaggttgtatggtt
eca-let-7d	agaggtagtaggttgcatagtt
eca-let-7e	tgaggtaggaggttgtatagtt
eca-let-7f	tgaggtagtagattgtatagtt
eca-let-7g	tgaggtagtagtttgtacagtt
eca-mir-1	tggaatgtaaagaagtatgtat
eca-mir-7	tggaagactagtgattttgttgt
eca-mir-9a	tctttggttatctagctgtatga
eca-mir-10a	taccctgtagatccgaatttgtg
eca-mir-10b	taccctgtagaaccgaatttgtg
eca-mir-15a	tagcagcacataatggtttgtg
eca-mir-15b	tagcagcacatcatggtttaca
eca-mir-16	tagcagcacgtaaatattggcg
eca-mir-17	caaagtgcttacagtgcaggtag
eca-mir-18a	taaggtgcatctagtgcagatag
eca-mir-18b	taaggtgcatctagtgcagttag
eca-mir-19a	tgtgcaaatctatgcaaaactga
eca-mir-19b	tgtgcaaatctatgcaaaactga
eca-mir-20a	taaagtgcttatagtgcaggtag
eca-mir-20b	caaagtgctcatagtgcaggtag
eca-mir-21	tagcttatcagactgatgttga
eca-mir-22	aagctgccagttgaagaactgt
eca-mir-23a	atcacattgccagggatttcc
eca-mir-23b	atcacattgccagggattacc
eca-mir-24	tggctcagttcagcaggaacag
eca-mir-25	cattgcacttgtctcggtctga
eca-mir-26a	ttcaagtaatccaggataggct
eca-mir-27a	ttcacagtggctaagttccgc
eca-mir-27b	ttcacagtggctaagttctgc
eca-mir-28-3p	cactagattgtgagctcctgga
eca-mir-28-5p	aaggagctcacagtctattgag
eca-mir-29a	tagcaccatctgaaatcggtta

eca-mir-29b	tagcaccatttgaaatcagtgtt
eca-mir-29c	tagcaccatttgaaatcggtta
eca-mir-30b	tgtaaacatcctacactcagct
eca-mir-30c	tgtaaacatcctacactctcagc
eca-mir-30d	tgtaaacatccccgactggaag
eca-mir-30e	tgtaaacatccttgactggaag
eca-mir-31	aggcaagatgctggcatagct
eca-mir-32	tattgcacattactaagttgca
eca-mir-33a	gtgcattgtagttgcattgca
eca-mir-33b	gtgcattgctgttgcattgc
eca-mir-34	tggcagtgtcttagctggttgt
eca-mir-92a	tattgcacttgtcccggcctgt
eca-mir-92b	tattgcactcgtcccggcctcc
eca-mir-93	caaagtgctgttcgtgcaggtag
eca-mir-95	ttcaacgggtctttattgagca
eca-mir-96	tttggcactagcacatttttgct
eca-mir-98	tgaggtagtaagttgtattgtt
eca-mir-99a	aacccgtagatccgatcttgtg
eca-mir-99b	cacccgtagaaccgaccttgcg
eca-mir-100	aacccgtagatccgaacttgtg
eca-mir-101	tacagtactgtgataactgaa
eca-mir-103	agcagcattgtacagggctatga
eca-mir-105	tcaaatgctcagactcctgtggt
eca-mir-106a	caaagtgcttacagtgcaggtag
eca-mir-106b	taaagtgctgacagtgcagat
eca-mir-107b	agcagcattgtacagggctatca
eca-mir-122	tggagtgtgacaatggtgtttg
eca-mir-124	taaggcacgcggtgaatgcc
eca-mir-125a-3p	acaggtgaggttcttgggagcc
eca-mir-125a-5p	tccctgagacccttaacctgtga
eca-mir-125b	tccctgagaccctaacttgtga
eca-mir-126-3p	tcgtaccgtgagtaataatgcg
eca-mir-127	tcggatccgtctgagcttggct
eca-mir-128	tcacagtgaaccggtctcttt
eca-mir-129a-3p	aagcccttaccccaaaaagtat
eca-mir-129a-5p	ctttttgcggtctgggcttgc
eca-mir-130a	cagtgcaatgttaaaagggcat
eca-mir-130b	cagtgcaatgatgaaagggcat
eca-mir-132	taacagtctacagccatggtcg

eca-mir-133a	tttggtccccttcaaccagctg
eca-mir-133b	tttggtccccttcaaccagcta
eca-mir-134	tgtgactggttgaccagagggg
eca-mir-135a	tatggctttttattcctatgtga
eca-mir-135b	tatggcttttcattcctatgtga
eca-mir-136	actccatttgttttgatgatgg
eca-mir-137	ttattgcttaagaatacgcgtag
eca-mir-138	agctggtgttgtgaatcaggccg
eca-mir-139-3p	ggagacgcggccctgttggagt
eca-mir-139-5p	tctacagtgcacgtgtctccag
eca-mir-140-3p	taccacagggtagaaccacgg
eca-mir-140-5p	cagtggttttaccctatggtag
eca-mir-141	taacactgtctggtaaagatgg
eca-mir-142-3p	tgtagtgtttcctactttatgga
eca-mir-142-5p	cataaagtagaaagcactact
eca-mir-143	tgagatgaagcactgtagctc
eca-mir-144	tacagtatagatgatgtact
eca-mir-145	gtccagttttcccaggaatccct
eca-mir-146a	tgagaactgaattccatgggtt
eca-mir-146b-3p	tgccctagggactcagttctgg
eca-mir-146b-5p	tgagaactgaattccataggct
eca-mir-147b	gtgtgccgaaatgcttctgcta
eca-mir-148a	tcagtgcactacagaactttgt
eca-mir-148b-3p	tcagtgcatcacagaactttgt
eca-mir-149	tctggctccgtgtcttcactccc
eca-mir-150	tctcccaacccttgtaccagtg
eca-mir-151-5p	tcgaggagctcacagtctagt
eca-mir-153	ttgcatagtcacaaaagtgatc
eca-mir-154	taggttatccgtgttgccttcg
eca-mir-155	ttaatgctaatcgtgataggggt
eca-mir-181a	aacattcaacgctgtcggtgagt
eca-mir-181b	aacattcattgctgtcggtgggt
eca-mir-182	tttggcaatggtagaactcacactg
eca-mir-183	tatggcactggtagaattcact
eca-mir-184	tggacggagaactgataagggt
eca-mir-186	caaagaattctccttttgggct
eca-mir-187	tcgtgtcttgtgttgcagccgg
eca-mir-188-3p	ctcccacatgcagggtttgca
eca-mir-188-5p	catcccttgcatggtggaggg

eca-mir-190	tgatatgtttgatatattaggt
eca-mir-190b	tgatatgtttgatattgggtt
eca-mir-191	caacggaatcccaaaagcagctg
eca-mir-192	ctgacctatgaattgacagcc
eca-mir-193a-3p	aactggcctacaaagtcccagt
eca-mir-193a-5p	tgggtctttgcgggcgagatga
eca-mir-193b	aactggcccacaaagtcccgct
eca-mir-194	tgtaacagcaactccatgtgga
eca-mir-195	tagcagcacagaaatattggc
eca-mir-196a	taggtagtttcatgttgttggg
eca-mir-196b	taggtagtttcctgttgttggg
eca-mir-197	ttcaccaccttctccacccagc
eca-mir-199a-3p	acagtagtctgcacattggtag
eca-mir-199a-5p	cccagtgttcagactacctgttc
eca-mir-199b-3p	acagtagtctgcacattggtta
eca-mir-199b-5p	cccagtgtttagactatctgttc
eca-mir-200a	taacactgtctggtaacgatgt
eca-mir-200b	taatactgcctggtaatgatga
eca-mir-200c	taatactgccgggtaatgatgga
eca-mir-204b	ttccctttgtcatcctatgcct
eca-mir-205	tccttcattccaccggagtctg
eca-mir-206	tggaatgtaaggaagtgtgtgg
eca-mir-208a	ataagacgagcaaaaagcttgt
eca-mir-208b	ataagacgaacaaaaggtttgt
eca-mir-211	ttccctttgtcatcctttgcct
eca-mir-212	taacagtctccagtcacggcc
eca-mir-214	acagcaggcacagacaggcagt
eca-mir-215	atgacctatgaattgacagac
eca-mir-216a	taatctcagctggcaactgtga
eca-mir-216b	aaatctctgcaggcaaatgtga
eca-mir-217	tactgcatcaggaactgattgga
eca-mir-218	ttgtgcttgatctaaccatgt
eca-mir-219-5p	tgattgtccaaacgcaattct
eca-mir-221	agctacattgtctgctgggtttc
eca-mir-222	agctacatctggctactgggt
eca-mir-223	tgtcagtttgtcaaatacccca
eca-mir-224	caagtcactagtggttccgtt
eca-mir-296	gagggttgggtggaggctttcc
eca-mir-299	tatgtgggatggtaaaccgctt

eca-mir-301a	cagtgcaatagtattgtcaaagc
eca-mir-301b-3p	cagtgcaatgatattgtcaaagc
eca-mir-302a	taagtgcttccatgttttagtga
eca-mir-302b	taagtgcttccatgttttagtag
eca-mir-302c	taagtgcttccatgtttcagtgg
eca-mir-302d	taagtgcttccatgttttagtgt
eca-mir-323-3p	cacattacacggtcgacctct
eca-mir-323-5p	aggtggtccgtggcgcgttcgc
eca-mir-324-3p	ccactgccccaggtgctgctgg
eca-mir-324-5p	cgcatcccctagggcattggtgt
eca-mir-326	cctctgggcccttcctccagc
eca-mir-328	ctggccctctctgcccttccgt
eca-mir-329	aacacacctagttaacctcttt
eca-mir-330	tctctgggcctgtgtcttaggc
eca-mir-331	gcccctgggcctatcctagaa
eca-mir-335	tcaagagcaataacgaaaaatgt
eca-mir-337-3p	ctcctatgagatgcctttcctc
eca-mir-337-5p	gaacggcttcatacaggagct
eca-mir-338-3p	tccagcatcagtgattttgttg
eca-mir-338-5p	aacaatatcctggtgctgagtg
eca-mir-340-5p	ttataaagcaatgagactgatt
eca-mir-342-3p	tctcacacagaaatcgcacccgt
eca-mir-342-5p	aggggtgctatctgtgattgag
eca-mir-345-5p	gctgactcctagtccagtgctc
eca-mir-346	tgtctgcccgcatgcctgcctct
eca-mir-361-3p	tcccccaggcgtgattctgattt
eca-mir-361-5p	ttatcagaatctccaggggtac
eca-mir-362-3p	aacacacctattcaaggattca
eca-mir-362-5p	aatccttggaacctaggtgtgagt
eca-mir-363	aattgcacggtatccatctgta
eca-mir-365	taatgcccctaaaaatccttat
eca-mir-367	aattgcactttagcaatggtga
eca-mir-369-3p	aataatacatggttgatcttt
eca-mir-369-5p	agatcgaccgtgtcatattcgc
eca-mir-370	gcctgctggggtggaacctggt
eca-mir-371-3p	aagtgccgccatttttgagtgt
eca-mir-371-5p	actcaaactgtgggggcact
eca-mir-374a	ttataatacaacctgataagtg
eca-mir-374b	atataatacaacctgctaagtg

eca-mir-376a	atcatagaggaaaatccacgt
eca-mir-376b	atcatagaggaaaatccatgt
eca-mir-376c	aacatagaggaaattccacgt
eca-mir-377	atcacacaaaggcaacttttgt
eca-mir-378	actggacttggagtcagaagg
eca-mir-379	tggtagactatggaacgtagg
eca-mir-380	tatgtaatatggtccacgtctt
eca-mir-381	tatacaagggcaagctctctgt
eca-mir-382	gaagttgttcgtggtggattcg
eca-mir-383	agatcagaaggtgattgtggct
eca-mir-384	attcctagaaattgttcaca
eca-mir-409-3p	gaatgttgctcggtgaacccct
eca-mir-409-5p	aggttacccgagcaactttgcat
eca-mir-410	aatataacacagatggcctgt
eca-mir-411	tagtagaccgtatagcgtacg
eca-mir-412	ttcacctggtccactagccg
eca-mir-421	ggcctcattaaatgtttgttg
eca-mir-423-3p	agctcggtctgaggcccctcagt
eca-mir-423-5p	tgaggggcagagagcgagacttt
eca-mir-424	cagcagcaattcatgttttgaa
eca-mir-429	taatactgtctggtaatgccg
eca-mir-431	tgtcttgcaggccgtcatgcagg
eca-mir-432	tcttggagtaggtcattgggtgg
eca-mir-433	atcatgatgggctcctcggtgt
eca-mir-448	ttgcatatgtaggatgtcccat
eca-mir-449a	tggcagtgtattgttagctggt
eca-mir-450a	ttttgcgatgtgttcctaatat
eca-mir-450b-3p	ttgggaacattttgcatccata
eca-mir-450b-5p	ttttgcaatatgttcctgaata
eca-mir-451	aaaccgttaccattactgtgtt
eca-mir-454	tagtgcaatattgcttatagggt
eca-mir-485-3p	gtcatacacggctctcctctct
eca-mir-485-5p	agaggctggccgtgatgaattc
eca-mir-486-3p	cggggcagctcagtacaggat
eca-mir-486-5p	tcctgtactgagctgccccgag
eca-mir-487a	aatcatacagggacatccagtt
eca-mir-487b	aatcgtacagggtcatccactt
eca-mir-488	ttgaaaggctatttcttggtc
eca-mir-489	gtgacatcacatatacggcggc

eca-mir-490-3p	caacctggaggactccatgctg
eca-mir-490-5p	ccatggatctccaggtgggt
eca-mir-491-3p	cttatgcaagattcccttctac
eca-mir-491-5p	agtggggaacccttccatgagg
eca-mir-492	aggagctgcgggacaagattctt
eca-mir-493b	tgaaggtcttccgtgtgccagg
eca-mir-494	tgaaacatacacgggaaacctc
eca-mir-495	aaacaaacatggtgcacttctt
eca-mir-496	tgagtattacatggccaatctc
eca-mir-497	cagcagcacactgtggtttgt
eca-mir-499-3p	aacatcacagcaagtctgtgct
eca-mir-499-5p	ttaagacttgcagtgatgttt
eca-mir-500	taatccttgctacctgggtgaga
eca-mir-501	atccttcgtccctgggtgaga
eca-mir-502-3p	aatgcacctgggcaaggattca
eca-mir-502-5p	atccttgctatctgggtgcta
eca-mir-503	tagcagcgggaacagtactgcag
eca-mir-504	agaccctggtctgcactctatc
eca-mir-505	cgtcaacacttgctggtttcct
eca-mir-507	attggcacctcttagagtgaa
eca-mir-508-3p	tgattgtcacctttggagtaga
eca-mir-508-5p	tactccagagggtgtcattcaca
eca-mir-509-5p	tactgcagacagtggcaatca
eca-mir-514	attgacacctctgtgagtgga
eca-mir-532-3p	cctcccacacccaaggcttgca
eca-mir-532-5p	catgccttgagtgtaggaccgt
eca-mir-539	ggagaaattatccttgctgtgt
eca-mir-541	tggtgggcacagaatccagtct
eca-mir-542-3p	tgtgacagattgataactgaaa
eca-mir-542-5p	ctcggggatcatcatgtcacga
eca-mir-543	aaacattcgcggtgcacttctt
eca-mir-544b	attctgcatttttaacaagttc
eca-mir-545	tcaacaaacatttattgtgtgc
eca-mir-551a	gcgacccactcttggtttcca
eca-mir-551b	gcgacccatacttggtttcag
eca-mir-568	atgtataaatgtatacacac
eca-mir-582-5p	ttacagttgttcaaccagttact
eca-mir-582-3p	taaccggttgaacaactgaacc
eca-mir-590-5p	gagcttattcataaaagtacag

eca-mir-590-3p	taattttatgtataagctagt
eca-mir-592	ttgtgtcaatatgcgatgatgt
eca-mir-598	tacgtcatcgttgtcatcgtca
eca-mir-615-5p	gggggtccccggtgctcggatc
eca-mir-615-3p	tccgagcctgggtctccctctc
eca-mir-628a	atgctgacatatttactagagg
eca-mir-632	gtgcctgtttcctgtggga
eca-mir-652	aatggcgccactagggttgtg
eca-mir-653	gtgttgaaacaatctctgctg
eca-mir-655	ataatacatggttaacctcttt
eca-mir-656	aatattatacagtcaacctct
eca-mir-660	tacccattgcatatcggagttg
eca-mir-664	tattcatttatctcctagcctaca
eca-mir-670	gtccctgagtgtatgtggtgaa
eca-mir-671-5p	aggaagccctggaggggctggag
eca-mir-671-3p	tccggttctcagggctccacc
eca-mir-672	tgaggttggtgtactgtgtgtga
eca-mir-674-5p	ggtgctcacttgtcctcct
eca-mir-674-3p	aggaggccatagtggcaactgt
eca-mir-675	tggcgcggagagggcccacagtg
eca-mir-684	agttttcccttcaattcag
eca-mir-703	aaaaccttcagaaggaaagga
eca-mir-708	aaggagcttacaatctagctggg
eca-mir-711	gggacccagggagagacgtaag
eca-mir-758	tttgtgacctggtccactaacc
eca-mir-761	gcagcagggtgaaactgacaca
eca-mir-763	ccagctgggaggaaccagtggc
eca-mir-767-5p	tgcaccatggttgtctgagcatg
eca-mir-767-3p	tctgctcatactccatggttcct
eca-mir-769-5p	ggagacctctgggttctgagct
eca-mir-769-3p	ctgggatctcgggggtcttggtt
eca-mir-769b	ggaaacctctgggttctgagct
eca-mir-770	agcaccacgtgtctgggccatg
eca-mir-802	cagtaacaaagattcatccttgt
eca-mir-872	aaggttacttgttagttcagg
eca-mir-873	gcaggaacttgtgagtctcct
eca-mir-874	ctgccctggcccgagggaccga
eca-mir-876-5p	tggatttctttgtgaatcacca
eca-mir-876-3p	tggtggtggtttacaaagtaattca

eca-mir-885-5p	tccattacactaccctgcctct
eca-mir-885-3p	aggcagcggggtgtagtggata
eca-mir-889	ttaatatcggacaaccattgt
eca-mir-1179	aagcattctttcattggttgg
eca-mir-1180	tttccggctcgagtgggtgtgt
eca-mir-1185	agaggataccctttgtatgtt
eca-mir-1193	taggtcacccgtttgactatc
eca-mir-1197	taggacacatggtctacttct
eca-mir-1204	tcgtggcctggtccccactat
eca-mir-1244	gagtggttggtttgtatgagatggtt
eca-mir-1248	tccttcttgtataagcactgtgctaaa
eca-mir-1255b	cggataagcaaagaaagtggtt
eca-mir-1261	gtggattaggctttggctt
eca-mir-1264	caagtcttatttgagcacctgtt
eca-mir-1271	cttggcacctcgtaagcactca
eca-mir-1282	agtggttggtttgtatgagatggtt
eca-mir-1289	tggagtccaggaatctgcatttt
eca-mir-1291a	tggccctgactgaagaccagcagt
eca-mir-1291b	aggccctgaatcaagaccagcagt
eca-mir-1296	ttagggccctggctccatctcc
eca-mir-1298	ttcattcggctgtccagatgta
eca-mir-1301	ttgcagctgcctgggagtgatttc
eca-mir-13021	ttgggacatacttatactaaa
eca-mir-1302b2	ttgggacatacttatactaga
eca-mir-1302d4	ttgggacatacttatgctaaa
eca-mir-1302e6	ttgggatatacttatactaaa
eca-mir-1302e7	ttgggatatacttatactaaa
eca-mir-1302c5	ttgcgacatacttatactaaa
eca-mir-1461	atctctacgggtaagtgtgtga
eca-mir-1468	ctccgtttgcctgttttgctg
eca-mir-1597	tgaggagctctgcgagcatgta
eca-mir-1839	aaggtagatagaacaggtcttg
eca-mir-1842	tggctctgtgaggtcggctca
eca-mir-1892	atttggggtgggggatgggga
eca-mir-1898	aggtcaaggttcacaggggatc
eca-mir-1902	agaggtgcagtaggcatgactt
eca-mir-1905a	caccacgagccctaccacgcggtag
eca-mir-1905b	caccagccccactacgcggtag
eca-mir-1905c	caccaccagccccaccacgcggtag

eca-mir-1912	tacccagagcgtgcagtgtgaa

Pathway Analysis

An a posteriori pathway analysis was performed with DIANA TOOLS miRPath v.3 (Vlachos et al., 2015) using 33 miRNA only expressed in spermatozoa from the cauda epididymis as these spermatozoa are mature and completed their transition through the epididymis. Predicted targets were recorded using DIANA-microT-CDS algorithm and pathways were considered significant with $P \leq 0.05$ (Reczko et al., 2012).

Statistical Analysis

Raw Cp values were normalized to internal controls for each sample type: tissue, epididymosomes, and spermatozoa. Samples from the caput and cauda of epididymal tissue and spermatozoa were compared using the proc mixed procedure. Mean separatation was performed with lsmeans with Tukey-Kramer adjustment in SAS v9.4 (SAS Institute, Inc., Cary, NC). Samples from the caput, proximal corpus, distal corpus and cauda of epididymosomes were also compared using the same method. Significant differences were reported as $P \leq 0.05$.

Results

Thirty-three miRNAs were identified within caudal spermatozoa that were not present within spermatozoa from the caput, indicating these miRNAs are acquired during migration through the epididymis. Of those 33 miRNAs, 11 miRNAs were specifically followed from the epididymal epithelium to the spermatozoa via epididymosomes. Although these newly-acquired miRNAs restricted to the caudal spermatozoa are our main interest, there were many other

miRNAs within the tissue, epididymosomes, and spermatozoa. Of the 346 miRNA evaluated in this study, 324 were identified in spermatozoa from either caput or cauda or both (Table 2). Twenty-one miRNAs were greater in quantity ($P \leq 0.05$) in the spermatozoa from the cauda while only two were in greater quantity within spermtozoa from the caput. Of the 324 spermatozoal miRNAs evaluated in epididymal tissue for caput and cauda regions, 206 miRNAs were noted in epididymal tissue from caput, cauda, or both (Table 2). Eleven miRNAs were expressed in in the caput tissue while restricted from cauda, and nine miRNAs were expressed in caudal tissue but not the caput. Seven miRNAs were also in greater quantity in the caput than the cauda of epididymal tissue. Epididymosomes from the lumen of all four epididymal regions were compared to 92 miRNAs discovered in either sperm or tissue restricted to a single region of the epididymis, or those that were in greater quantities between sections. Of which, 69 miRNAs were present in epididymosomes from at least one of the four regions.

Table 2. MicroRNA expressed in tissue, epididymosomes, and epididymal spermatozoa. MiRNA listed below include miRNAs expressed in the tissue (caput, cauda or both regions), epididymosomes (from any of the four regions or all) and epididymal spermatozoa (caput, cauda, or both regions).

miRNA in Epididymal Tissue	miRNA in Epididymosomes	miRNA in Epididymal Sperm
eca-let-7a	eca-let-7a	eca-let-7a
eca-let-7e	eca-let-7d	eca-let-7c
eca-let-7f	eca-mir-106b	eca-let-7d
eca-mir-15a	eca-mir-1193	eca-let-7e
eca-mir-17	eca-mir-122	eca-let-7f
eca-mir-19b	eca-mir-1248	eca-let-7g
eca-mir-20a	eca-mir-125a3p	eca-mir-7
eca-mir-20b	eca-mir-127	eca-mir-9a
eca-mir-21	eca-mir-128	eca-mir-10a
eca-mir-22	eca-mir-129a5p	eca-mir-10b

eca-mir-23a	eca-mir-1301	eca-mir-15a
eca-mir-23b	eca-mir-134	eca-mir-15b
eca-mir-24	eca-mir-136	eca-mir-16
eca-mir-25	eca-mir-1393p	eca-mir-17
eca-mir-26a	eca-mir-1403p	eca-mir-18a
eca-mir-27a	eca-mir-1405p	eca-mir-18b
eca-mir-27b	eca-mir-143	eca-mir-19a
eca-mir-283p	eca-mir-1468	eca-mir-19b
eca-mir-285p	eca-mir-146b3p	eca-mir-20a
eca-mir-29a	eca-mir-1515p	eca-mir-20b
eca-mir-29b	eca-mir-15a	eca-mir-21
eca-mir-29c	eca-mir-15b	eca-mir-22
eca-mir-30b	eca-mir-16	eca-mir-23a
eca-mir-30c	eca-mir-181a	eca-mir-23b
eca-mir-30d	eca-mir-1842	eca-mir-24
eca-mir-30e	eca-mir-186	eca-mir-25
eca-mir-31	eca-mir-1885p	eca-mir-26a
eca-mir-32	eca-mir-21	eca-mir-27a
eca-mir-33a	eca-mir-212	eca-mir-27b
eca-mir-34	eca-mir-215	eca-mir-283p
eca-mir-92a	eca-mir-285p	eca-mir-285p
eca-mir-92b	eca-mir-29a	eca-mir-29a
eca-mir-93	eca-mir-29b	eca-mir-29b
eca-mir-95	eca-mir-30b	eca-mir-29c
eca-mir-96	eca-mir-30c	eca-mir-30b
eca-mir-98	eca-mir-30d	eca-mir-30c
eca-mir-99a	eca-mir-30e	eca-mir-30d
eca-mir-99b	eca-mir-326	eca-mir-30e
eca-mir-100	eca-mir-3373p	eca-mir-31
eca-mir-101	eca-mir-3385p	eca-mir-32
eca-mir-103	eca-mir-34	eca-mir-33a
eca-mir-105	eca-mir-3455p	eca-mir-33b
eca-mir-106a	eca-mir-3615p	eca-mir-34
eca-mir-106b	eca-mir-3695p	eca-mir-92a
eca-mir-107b	eca-mir-376a	eca-mir-92b
eca-mir-122	eca-mir-378	eca-mir-93
eca-mir-125a5p	eca-mir-382	eca-mir-95
eca-mir-125b	eca-mir-4093p	eca-mir-96
eca-mir-1263p	eca-mir-4235p	eca-mir-98

eca-mir-127	eca-mir-449a	eca-mir-99a
eca-mir-128	eca-mir-4863p	eca-mir-99b
eca-mir-129a3p	eca-mir-489	eca-mir-100
eca-mir-129a5p	eca-mir-4905p	eca-mir-101
eca-mir-130a	eca-mir-494	eca-mir-103
eca-mir-130b	eca-mir-5023p	eca-mir-105
eca-mir-133b	eca-mir-5025p	eca-mir-106a
eca-mir-134	eca-mir-5083p	eca-mir-106b
eca-mir-135a	eca-mir-5323p	eca-mir-107b
eca-mir-135b	eca-mir-598	eca-mir-122
eca-mir-138	eca-mir-628a	eca-mir-124
eca-mir-1395p	eca-mir-632	eca-mir-125a3p
eca-mir-1403p	eca-mir-652	eca-mir-125a5p
eca-mir-1405p	eca-mir-653	eca-mir-125b
eca-mir-141	eca-mir-670	eca-mir-1263p
eca-mir-1423p	eca-mir-6715p	eca-mir-127
eca-mir-1425p	eca-mir-763	eca-mir-128
eca-mir-143	eca-mir-7695p	eca-mir-129a3p
eca-mir-144	eca-mir-92b	eca-mir-129a5p
eca-mir-145	eca-mir-96	eca-mir-130a
eca-mir-146b3p		eca-mir-130b
eca-mir-147b		eca-mir-132
eca-mir-149		eca-mir-133b
eca-mir-150		eca-mir-134
eca-mir-1515p		eca-mir-135a
eca-mir-153		eca-mir-135b
eca-mir-154		eca-mir-136
eca-mir-155		eca-mir-138
eca-mir-181a		eca-mir-1393p
eca-mir-181b		eca-mir-1395p
eca-mir-182		eca-mir-1403p
eca-mir-184		eca-mir-1405p
eca-mir-186		eca-mir-141
eca-mir-1883p		eca-mir-1423p
eca-mir-1885p		eca-mir-1425p
eca-mir-190b		eca-mir-143
eca-mir-193a3p		eca-mir-144
eca-mir-193a5p		eca-mir-145
eca-mir-194		eca-mir-146a

eca-mir-195
eca-mir-196a
eca-mir-197
eca-mir-199a3p
eca-mir-199a5p
eca-mir-199b3p
eca-mir-199b5p
eca-mir-200a
eca-mir-200b
eca-mir-200c
eca-mir-204b
eca-mir-208b
eca-mir-217
eca-mir-218
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eca-mir-299
eca-mir-301a
eca-mir-302d
eca-mir-3233p
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eca-mir-329
eca-mir-331
eca-mir-3373p
eca-mir-3375p
eca-mir-3383p
eca-mir-3385p
eca-mir-3423p
eca-mir-3425p
eca-mir-3455p
eca-mir-3613p
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eca-mir-3625p
eca-mir-365
eca-mir-3713p
eca-mir-3715p
eca-mir-374b
eca-mir-376a

eca-mir-146b3p
eca-mir-146b5p
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eca-mir-148a
eca-mir-148b3p
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eca-mir-150
eca-mir-1515p
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eca-mir-154
eca-mir-155
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eca-mir-199b5p
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eca-mir-200b
eca-mir-200c
eca-mir-204b
eca-mir-205

eca-mir-376c	eca-mir-206
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eca-mir-382	eca-mir-211
eca-mir-383	eca-mir-212
eca-mir-384	eca-mir-214
eca-mir-4095p	eca-mir-215
eca-mir-411	eca-mir-216a
eca-mir-412	eca-mir-216b
eca-mir-4233p	eca-mir-217
eca-mir-4235p	eca-mir-218
eca-mir-432	eca-mir-2195p
eca-mir-448	eca-mir-221
eca-mir-449a	eca-mir-222
eca-mir-450b3p	eca-mir-223
eca-mir-4855p	eca-mir-224
eca-mir-4863p	eca-mir-296
eca-mir-4865p	eca-mir-299
eca-mir-487a	eca-mir-301a
eca-mir-488	eca-mir-301b3p
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eca-mir-497	eca-mir-3245p
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eca-mir-5903p	eca-mir-3625p
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eca-mir-632	eca-mir-365
eca-mir-652	eca-mir-367
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eca-mir-660	eca-mir-370
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eca-mir-1197	eca-mir-411
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eca-mir-1302c5	eca-mir-432
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eca-mir-1468	eca-mir-448
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eca-mir-1842	eca-mir-450b3p

eca-mir-1902

eca-mir-451
eca-mir-454
eca-mir-4853p
eca-mir-4855p
eca-mir-4863p
eca-mir-4865p
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eca-mir-487b
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eca-mir-761
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eca-mir-7693p
eca-mir-769b

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eca-mir-1248
eca-mir-1255b
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eca-mir-1264
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eca-mir-1291b
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eca-mir-1298
eca-mir-1301
eca-mir-13021
eca-mir-1302b2
eca-mir-1302d4
eca-mir-1302e6
eca-mir-1302c5
eca-mir-1461
eca-mir-1468
eca-mir-1597
eca-mir-1839
eca-mir-1842
eca-mir-1898
eca-mir-1902

eca-mir-1905c
eca-mir-1912

Discussion

This is the first time miRNA profiles have been shown for the various regions of the epididymis in tissue as well as the epididymosomes and mature spermatozoa within the cauda epididymis. We found 11 different miRNAs located within epididymal tissue of either the caput or cauda that were also noted in epididymosomes of the same region or a subsequent region and then transferred to the spermatozoa by the time they are transported to the cauda. For example, miRNA-494 is present within epididymal tissue of the caput as well as epididymosomes from all four regions and caudal spermatozoa. Interestingly, this miRNA is not in spermatozoa of the caput nor caudal epididymal tissue providing indirect evidence that the spermatozoa obtained this miRNA from tissue in the caput epididymis via epididymosomes. MiRNA-670 is also restricted to spermatozoa from the cauda but is also noted in the tissue as well as the epididymosomes in this region perhaps indicating that this miRNA is acquired during migration through the cauda epididymis. Multiple miRNAs continue similar patterns as these and can be found in Figure 2.

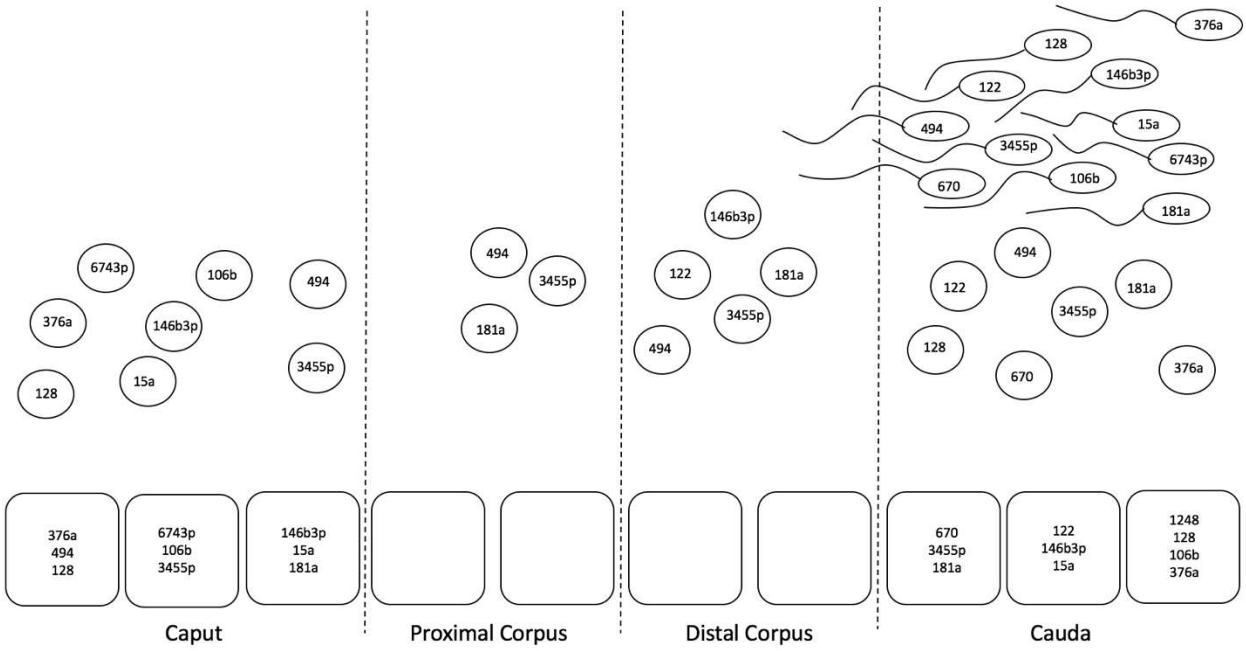

Figure 2

Of spermatozoal miRNAs with expression restricted to the cauda, 11 are present in either tissue of that region or the caput as well as epididymosomes from that region or throughout the epididymis.

Spermatozoa in the cauda should be mature and awaiting ejaculation or expulsion through the urethra. Because of this, we would expect to identify all miRNAs that may target pathways necessary for fertilization of the oocyte or embryogenesis. MiRNAs that are of interest to our laboratory are those only noted in spermatozoa of the cauda epididymis as these are added to the spermatozoa during their migration through the epididymis. To get a better understanding of the potential roles those miRNAs may play in mRNA translation, pathway analysis was performed on those miRNAs only noted in epididymal spermatozoa of the cauda. Fifty-one potential pathways were identified in our pathway analysis (Table 3). Notable pathways included those with potential roles on the oocyte as well as embryogenesis including oocyte meiosis, protein processing in endoplasmic reticulum, and mTOR signaling pathway.

Table 3. Predicted pathways of miRNAs with expression restricted to caudal spermatozoa. All 33 miRNAs expressed in the spermatozoa from the caudal epididymis but not the caput were used in the pathway analysis program DIANA tools. Below is the list of potentially targeted pathways and their corresponding p-value.

Predicted KEGG Pathway	P-value
Circadian entrainment	0.000103
Signaling pathways regulating pluripotency of stem cells	0.000103
Pathways in cancer	0.000101
Proteoglycans in cancer	0.000119
Glycosaminoglycan biosynthesis- heparan sulfate/heparin	0.000213
Glioma	0.0003
Protein processing in endoplasmic reticulum	0.000355
Renal cell carcinoma	0.00041
Axon guidance	0.00041
Retrograde endocannabinoid signaling	0.000435
GABAergic synapse	0.000478
Long-term depression	0.000593
Prostate cancer	0.000593
Rap1 signaling pathway	0.000593
PI3K-Akt signaling pathway	0.000593
Amphetamine addiction	0.000896
Adrenergic signaling in cardiomyocytes	0.000935
Wnt signaling pathway	0.000992
ErbB signaling pathway	0.001053
Nicotine addiction	0.001216
MAPK signaling pathway	0.001247
Dopaminergic synapse	0.00156
mTOR signaling pathway	0.001229
Ubiquitin-mediated proteolysis	0.002087

N-Glycan biosynthesis	0.003373
Choline metabolism in cancer	0.004384
Non-small cell lung cancer	0.005194
Cocaine addiction	0.00837
Thyroid hormone signaling pathway	0.00837
Other types of O-glycan biosynthesis	0.009043
Glutamatergic synapse	0.009327
Chronic myeloid leukemia	0.009327
Cholinergic synapse	0.009327
Pancreatic cancer	0.009327
Ras signaling pathway	0.009327
Neurotrophin signaling pathway	0.010566
Circadian rhythm	0.026467
Endocytosis	0.028116
Colorectal cancer	0.028116
Estrogen signaling pathway	0.028116
Melanoma	0.028395
Dorso-ventral axis formation	0.03276
Insulin signaling pathway	0.034726
Vasopressin-regulated water reabsorption	0.034823
p53 signaling pathway	0.035113
Oocyte meiosis	0.037214
Cell cycle	0.037214
cGMP-PKG signaling pathway	0.037214
Oxytocin signaling pathway	0.037214
Sphingolipid signaling pathway	0.047754
Prolactin signaling pathway	0.048931

Oocytes are arrested at the meiosis II phase until fertilization. The potential target of oocyte meiosis in spermatozoa is yet another indication that spermatozoa play a role in oocyte maturation and embryogenesis. MiRNA 34c is a sperm-borne miRNA required for the first cleavage division in mouse embryos (Liu et al., 2012). Other miRNAs are also delivered to the oocyte during fertilization and remain until the developing embryo's genome begins its own transcription (Boerke et al., 2007; Dadoune, 2009). MiRNAs potentially targeting oocyte meiosis may be required to allow the oocyte to leave its arrested state and continue to develop following interaction with spermatozoa.

The endoplasmic reticulum consists of tubules and cisternae extending from the nuclear membrane into the cytoplasm, and its primary function is to generate proteins for secretion (Cooper, 2000). Proteins play a vital role in the luminal environment necessary for spermatozoal maturation in the epididymis (Cornwall, 2009). Proteins are secreted from the epididymal epithelium into the lumen and directly or indirectly affect the sperm surface (Gatti et al., 2004). The miRNAs potentially targeting this pathway may promote the proper processing of proteins through the endoplasmic reticulum required for the ideal luminal environment.

Mechanistic target of rapamycin (mTOR) is a protein kinase that regulates cell growth, proliferation, motility, survival, protein synthesis, autophagy and transcription (Kanehisa et al., 2016). mTOR balances the nutrient and energy of cells during the first stages of embryonic cleavage (Land et al., 2014). The predicted role of caudal spermatozoa miRNAs on this pathway necessary for embryonic survival is yet another indication that spermatozoa may play a role in embryogenesis.

In conclusion, the miRNAs in spermatozoa from the cauda epididymis differ from those in the caput of the epididymis. These changes may play a role in maturation as well as

embryogenesis. Developing a better understanding of these mechanisms can lead to potential cures for infertility in a multitude of species. Our laboratory was able to follow miRNAs from the epididymal tissue to the spermatozoa from the cauda of the epididymis via epididymosomes. This suggests that not only are proteins necessary within the luminal environment of the epididymis for maturation, but also miRNAs. Further research will need to be conducted to determine what other miRNAs may be transported from the tissue to the spermatozoa through epididymosomes to provide a complete miRNA profile.

CHAPTER III: DISCUSSION

MicroRNA Pathways

This is the first time miRNAs have been traced from epididymal tissue to spermatozoa via epididymosomes in the stallion. Our laboratory noted 11 different miRNAs restricted to caudal spermatozoa that could be traced through the epididymal tissue and epididymosomes from one or more region. Because we limited the number of miRNAs used in our qRT-PCR analyses, there may be several other miRNAs that could be traced through the epididymis as well. Many miRNAs were located in both the tissue and spermatozoa, but have not yet been run against miRNA primers for epididymosome samples from any region. Several miRNAs were also identified within epididymosomes from one region and tissue from a subsequent region. This may be due to the endocytic characteristic of clear cells as they consume luminal proteins as well as cytoplasmic droplets and perhaps epididymosomes (Hermo et al., 1994; Hermo et al., 2005; Robaire et al., 2006). Other miRNAs were within epididymal tissue and epididymosomes from the same or ensuing regions but did not present themselves in spermatozoal samples. These miRNAs may function in ways other than on spermatozoa or they simply had not reached the spermatozoa within the cauda epididymis at the time of sample collection.

Potential Targets of miRNAs in Cauda Epididymal Spermatozoa Possibly Affecting Spermatozoa Rap1 Signaling Pathway

Rap1 is a small GTPase that controls cell adhesion, cell-to-cell junction formation, and cell polarity (Kanehisa et al., 2016). Rap1 is also required prior to the release of calcium during the acrosome reaction, a process necessary for fertilization (Branham et al., 2009). The miRNA

of these selected spermatozoa are predicted to have an effect on the Rap1 signaling pathway in some manner. They may post-transcriptionally activate the pathway that signals Rap1 leading to a release of calcium from the acrosome of spermatozoa during the acrosome reaction. Without the acrosome reaction, spermatozoa cannot properly interact with the oocyte, preventing fertilization.

PI3K-AKt Signaling Pathway

Phosphatidylinositol 3'-kinase-Akt (PI3K-AKt) is activated by cellular stimuli or toxins (Kanehisa et al., 2016). PI3K-AKt regulates cellular functions including transcription, translation, proliferation, growth and survival (Kanehisa et al., 2016). Akt phosphorylates substrates involved in apoptosis, protein synthesis, metabolism, and the cell cycle (Kanehisa et al., 2016). PI3K can also be activated by most ErbBs (Kanehisa et al., 2016). Activation of the PI3K-AKt pathway in the Atlantic croaker is required for hyperactivation of spermatozoa associated with capacitation, a process that requires CatSper in many vertebrates (Tan & Thomas, 2014). Stallion spermatozoa tails contain the calcium channel CatSper, known for its role in hyperactivation in mice and human spermatozoa, but the PI3K-Akt pathway may also lead to hyperactivation because the mechanisms behind CatSper are not well understood (Loux et al., 2013). MicroRNAs of caudal spermatozoa are predicted to target the PI3K-Akt signaling pathway and perhaps this is an additional mechanism to ensure hyperactivation necessary for capacitation of spermatozoa prior to fertilizing an oocyte.

ErbB Signaling Pathway

ErbB protein family is responsible for proliferation, differentiation, motility and survival of the cell (Kanehisa et al., 2016). The ErbB protein family does not appear to have a direct role

on spermatozoa or fertilization (Naz & Ahmad, 1992). The protein family's role in other functions, may be beneficial to sperm survival while stored in the epididymis. ErbB is also a stimulator for the PI3K-Akt family (Kanehisa et al., 2016). The potential regulation of the mRNA involved in the ErbB signaling pathway via miRNAs could encourage the motility of spermatozoa during their epididymal migration as well as their overall survival prior to ejaculation.

MAPK Signaling Pathway

Mitogen-activated protein kinases (MAPK) regulate cell proliferation, differentiation, and migration (Kanehisa et al., 2016). MAPKs are also responsible for motility, hyperactivation, and the acrosome reaction of mature sperm (Almog & Naor, 2010). MicroRNA action on these pathways would prove trivial for the spermatozoa to reach the oocyte within the uterus, hyperactivate and undergo capacitation and the acrosome reaction to fertilize the oocyte.

Dopaminergic Synapse

Dopamine is a slow neurotransmitter that controls endocrine function (Kanehisa et al., 2016). Functional dopamine receptors reside in stallion spermatozoa (Urra et al., 2014). Dopamine regulates sperm viability, capacitation, and motility (Urra et al., 2014). MiRNA interaction with this predicted pathway would be important in allowing sperm survival during storage in the cauda of the epididymis as well as being capable of fertilization through proper motility and capacitation within a mare's reproductive tract.

Ubiquitin Mediated Proteolysis

Ubiquitin is a small protein required to maintain the luminal environment because of its role in maintaining intracellular activities (Rodriguez & Stewart, 2007). In mice, without ubiquitin ligase HERC4, spermatozoa motility was greatly affected due to abnormalities in the sperms' tail (Rodriguez & Stewart, 2007). MiRNAs of the caudal spermatozoa potentially target the ubiquitin-mediated proteolysis pathway. Ubiquitin-mediated proteolysis is required for many cellular processes including regulating the cell cycle, immune and inflammatory responses, development and differentiation (Chiechanover et al., 2000). By interfering with these processes, spermatozoa can maintain their current state until ejaculation followed by a potential interaction with the oocyte at fertilization.

Estrogen Signaling Pathway

The estrogen receptor varies throughout epididymal tissue of the stallion by region (Parlevliet et al., 2006). Estrogen regulates fluid reabsorption within the lumen, particularly in the caput of the epididymis where concentration occurs (França et al., 2005; Hess et al., 2011). Estrogen also targets the epididymal epithelial cells to maintain different epithelial morphologies necessary for male fertility (Hess et al., 2011; Kobayashi & Behringer, 2003). Perhaps the miRNAs located on the spermatozoa in the cauda target the estrogen signaling pathway to maintain the epididymal epithelium required during the storage of spermatozoa prior to ejaculation.

Oxytocin Signaling Pathway

Oxytocin receptors are throughout the epididymis and are stimulated to contract the smooth muscle around the epididymis during ejaculation (Yanagimoto et al., 1996). As a

potential targeted pathway of miRNAs in spermatozoa from the cauda of the epididymis, perhaps translation of mRNA promoting oxytocin is repressed to maintain quiescence in the cauda until a neural override is caused by stimulation of the penis during copulation. This would allow storage of the spermatozoa within the cauda, which is its primary function.

Prolactin Signaling Pathway

Hyperprolactinemia is a common cause for both male and female infertility (Buvat, 2003). Increased prolactin prevents proper waves of secretion of gonadotropin releasing hormone (GnRH), luteining hormone (LH), follicle stimulating hormone (FSH), and testosterone, all of which are hormones that work together to promote spermatogenesis, causing a decrease in spermatogenesis, lower motility and sperm abnormalities (Masud et al., 2007). MiRNAs within caudal spermatozoa are predicted to play a role in the prolactin signaling pathway. By interfering with the prolactin signaling pathway through translational repression or degradation, the ill effects of too much prolactin can be prevented.

Potential Targets of miRNAs in Cauda Epididymal Spermatozoa Possibly Affecting the Oocyte

Protein Processing in the Endoplasmic Reticulum

The endoplasmic reticulum consists of tubules and cisternae extending from the nuclear membrane into the cytoplasm, and its primary function is to generate proteins for secretion (Cooper, 2000). Proteins play a vital role in the luminal environment necessary for spermatozoal maturation in the epididymis (Cornwall, 2009). Proteins are secreted from the epididymal epithelium into the lumen and directly or indirectly affect the sperm surface (Gatti et al., 2004).

The miRNAs potentially targeting this pathway may promote the proper processing of proteins through the endoplasmic reticulum required for the ideal luminal environment.

Oocyte Meiosis

Oocytes are arrested at meiosis II until fertilization. The potential target of oocyte meiosis in spermatozoa is yet another indication that spermatozoa play a role in more than just fertilization. MiRNA-34c is a sperm-borne miRNA required for the first cleavage division in mouse embryos (Liu et al., 2011). Other miRNAs are also delivered to the oocyte during fertilization and remain until the developing embryo's genome begins its own transcription (Boerke et al., 2007; Dadoune, 2009). MiRNAs potentially targeting oocyte meiosis may be required to allow the oocyte to leave its arrested state and continue to develop following interaction with spermatozoa.

Potential Targets of miRNAs in Cauda Epididymal Spermatozoa Possibly Affecting Embryogenesis

mTOR Signaling Pathway

Mechanistic target of rapamycin (mTOR) is a protein kinase that regulates cell growth, proliferation, motility, survival, protein synthesis, autophagy and transcription (Kanehisa et al., 2016). mTOR balances the nutrient and energy of cells during the first stages of embryonic cleavage (Land et al., 2014). The predicted role of caudal spermatozoa miRNAs on this pathway necessary for embryonic survival is yet another indication of their role in embryogenesis.

Industry Impacts

The equine industry typically selects for pedigree and potential skill in various disciplines rather than fertility as with other industries in agriculture. If a desired stallion has fertility issues, it can become very costly to use the stud for breeding. Problems during maturation in the stallion epididymis are the cause of some fertility issues stallions may have. MiRNA profiles of spermatozoa from fertile stallions compared to those of infertile stallions can be used as biomarkers for infertility and aid in the generation of diagnostic tools.

By gathering a better understanding of the mechanisms behind maturation, diagnosis for infertility will be possible, perhaps leading to treatments at a later date. Many equine miRNAs correspond to human miRNAs. If a diagnostic method is developed to determine the cause of infertility, it may be useful in human medicine as well. For men experiencing infertility, up to 40% may be caused by maturational issues (Cornwall, 2009). Determining such causes can eventually lead to the development of treatments. For example, miRNA-210 delivered by minicircle expression cassette to adult mice improved left ventricular fractional shortening compared to controls treated with a sham (Hu et al., 2010). The minicircle expression cassette is a non-viral vector that allows greater transfection efficiency due to its small size (Hu et al., 2010). Because the minicircle contains no bacterial backbone, there is minimal immunogenicity and longer transgene expression as well (Hu et al., 2010). If certain miRNAs are identified as necessary for proper epididymal maturation, those too can be placed into a similar vector and transplanted into the effected stallion in hopes of stimulating fertility.

Recent studies indicate spermatozoal abnormalities may lead to recurrent spontaneous abortion or recurrent early pregnancy loss and more than half of these cases remain unexplained. The suspected cause of the unexplained spontaneous abortions or pregnancy loss is believed to

be “paternal effect” (Talebi et al., 2016). However, the exact mechanism of the paternal effect in this instance is unknown. Again by developing a better understanding of the mechanisms behind sperm maturation, oocyte interactions, and embryogenesis will aid in diagnostics and later treatments of various forms of infertility and pregnancy loss.

Summary

MicroRNAs may be the solution to fertility problems in many species. By developing miRNA profiles of various regions of epididymal tissue, epididymosomes, and epididymal spermatozoa, differences between fertile and infertile stallions can indicate miRNAs trivial for epididymal maturation. There are a multitude of proteins and other luminal factors required for the proper environment for maturation and miRNAs are responsible for some of these post-transcriptional regulations. Diagnostics of abnormal miRNA profiles in infertile spermatozoa will play a major role in infertility diagnosis and potential treatments in the future.

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