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IDENTIFICATION OF MICRORNAS IN BOVINE SPERMATOZOA WITH IMPLICATIONS OF FERTILITY

By

LaShonda S. Robertson

A Thesis
Submitted to the Faculty of
Mississippi State University
In Partial Fulfillment of the Requirements
For the Degree of Master of Science
in Agriculture and Life Sciences with a concentration in Genetics
in the Department of Agriculture and Life Sciences

Mississippi State, Mississippi

August 2009

IDENTIFICATION OF MICRORNAS IN BOVINE SPERMATOZOA WITH

IMPLICATIONS OF FERTILITY

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MicroRNAs are small RNA molecules that could possibly play a major role in fertility. In the experiment, spermatozoa were extracted from bovine followed by an extraction of total RNA. Bovine spermatozoa were extracted from two bulls of different fertility, high and low fertility. An expression array was done to compare the expression levels of the microRNAs. It was shown that thousands of microRNAs are present in bovine spermatozoa but only a small amount was significantly expressed. The microRNAs from low fertility bulls were more highly expressed than those in high fertility bulls. A Bioanalyzer gel was used to confirm the results of the microarray data. The microRNAs were present in the bull's spermatozoa at 25 nucleotides. The functions of the significantly expressed microRNAs are not known but there is a great possibility that their functions affect fertility.

DEDICATION

I dedicate this thesis to my mother, Ethel L. Robertson, who has pushed me to be all that I can be and encouraged me to stick with it when I wanted to quit. I also dedicate this thesis to my sister, LaKeshia N. Robertson, brother, Le'Derrick Holden, and dad, Royal T. Holden. I could not have done any of this without you!

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LIST OF ABBREVIATIONS

Ago 3	Argonaute 3
Aub	Aubergine
cDNA	complementary DNA
C. elegans	*Caenorhabdhitis elegans*
dpp	day postpartum
dsRNA	double stranded RNA
FSH	follicle stimulating hormone
GV	germinal vesicle
IVF	in vitro fertilization
mRNA	messenger RNA
miRNA	microRNA
ncRNAs	non-coding RNAs
NR	nonreturn rate
nt	nucleotide
2'-OMe	2'O-methyl
PAZ	Piwi Argonaut and Zwille
PCR	polymerase chain reaction
piRC	piwi-interacting RNA complex

piRNA	piwi-interacting RNA
pri-miRNA	primary miRNA
pre-miRNA	miRNA precursor
RISC	RNA-induced silencing complex
RNAi	RNA interference
rRNA	ribosomal RNA
RT	reverse transcriptase
siRNA	small interfering RNA
stRNAs	small temporal RNAs
tRNA	transfer RNA
TRα1	thyroid hormone receptor α1 gene

CHAPTER 1

INTRODUCTION

Reproduction is a very important mechanism by which generations of animals continue to exist upon this earth by creating new individuals from existing ones. There are two types of reproduction: asexual reproduction and sexual reproduction. Asexual reproduction is the creation of new individuals whose genes come from one parent without the fusion of an egg and sperm. Conversely, sexual reproduction is the creation of new individuals or offspring by the fusion of gametes to form a zygote. The female gamete is the ovum (unfertilized egg) and the male gamete is the spermatozoon. Sexual reproduction increases the genetic variability among offspring by generating unique combinations of genes inherited from the parents.

For many people, animal reproduction has been a profitable investment. For example, breeding beef cattle with the bull that produces the best meat quality enables a producer to gain a great profit. Furthermore, if a farmer's cattle do not have calves they cannot yield any meat or milk. The producers would have to spend more money to keep unproductive cattle on their farm which will cost a great deal. Because of this, there is a need to improve animal genetics and reproduction efficiency. To improve animal genetics and reproduction, every component that leads to this process must be explored. This includes oogenesis, spermatogenesis, fertilization, and embryogenesis.

Fertilization, fusion of the sperm and egg, is an essential component of sexual reproduction. There are two main functions of fertilization: combining haploid sets of chromosomes from two individuals into a single diploid cell and activation of the egg. During mammalian fertilization, the sperm migrates through the coat of follicle cells and binds to receptor molecules in the zona pellucida of the egg. This binding induces the acrosomal reaction, in which the sperm releases hydrolytic enzymes into the zona pellucida. With the help of these enzymes, the sperm reaches the plasma membrane of the egg, and membrane proteins of the sperm bind to receptors on the egg membrane. The plasma membranes fuse, making it possible for the contents of the sperm cell to enter the egg. Enzymes released during the egg's cortical reaction harden the zona pellucida, which now functions as a block to polyspermy. Contact of the sperm with the egg's surface triggers the onset of embryonic development.

These small, yet intricate processes in fertilization are very important for the development of an embryo. Any variation or disturbance in the normal process of spermatogenesis or oogensis, ultimately affects embryogenesis. The process of fertilization is able to take place only if disturbances to these normal processes are held to a minimum. Embryogenesis, the process by which an embryo is formed and develops, begins when the egg is fertilized and involves multiple steps of cellular divisions and cellular differentiation. Embryogenesis occurs in plants, animals, and insects with minute distinction among the different species. Although each animal possesses its own advantages and restrictions and there is a great diversity of embryonic types and adult forms in the animal kingdom, there are strong similarities from one species to another in the major patterns of embryogenesis (Paquereau and Audigier, 1995).

In mammalian embryogenesis, cellular division and differentiation occur in the early prenatal development of the embryo. There are a number of hormones and proteins that are released and genes that are expressed that control these critical developmental stages. For instance, the Sp1 transcription factor is essential for normal mouse embryogenesis (Marin et al., 1997) and TRα1 acts as a repressor during early zebrafish development (Essner et al., 1997).

Decreasing fertility has become a major problem for both the beef and cattle industry. According to Koops et al. (1995) who studied of bull fertility, bovine reproductive efficiency is the total contribution of the male, the female, and the environment. Reproductive efficiency is usually measured by the nonreturn rate (NR). The nonreturn rate is the proportion of cows that were inseminated and did not return to service within a specific number of days (den Daas, 1998).

For many years, it has been thought that sperm contributes only half of the genetic material to the embryo. However, sperm factors are involved in syngamy, cleavage, and epigenetic regulations (Nanassy and Carrell, 2008). Nanssay and Carrell (2008) reviewed the male factors that affect fertilization events and early embryogenesis. These factors originate from epigenetic and genetic abnormalities during spermatogenesis, and include: DNA methylation patterns established during germ cell development; spermatocytogenesis giving rise to chromosome nondisjunction during meiosis I and II along with double strand breaks, abnormal histone modification, and alterations in the expression of mRNA and other non-coding RNAs; abnormal protamine replacement or centrosome formation during the final stage of spermatogenesis; and DNA fragmentation

as a result of apoptosis following double strand breaks or abnormal protamination during spermeogenesis (Nanassy and Carrell, 2008).

We know that abnormalities in spermatogenesis affect fertilization and ultimately embryogenesis. We also know that alterations in the expression of mRNA and other non-coding RNAs affect fertilization. Non-coding RNAs (ncRNAs) are RNAs that do not encode a protein, but it does not mean that there is no function or information in these RNAs (Mattick and Makunin, 2006). It was hypothesized that sperm bring tiny non-coding RNA molecules called microNRAs into the egg that may play important roles in fertilization and early embryonic development. Therefore, this study will be focused on identifying miRNAs in bovine spermatozoa from bulls with different fertilities.

CHAPTER 2

REVIEW OF RELEVANT LITERATURE

2.1 MicroRNAs

MicroRNAs (miRNA) are small RNA molecules that are about 19 to 26 nucleotides (nt) in length. MicroRNAs are endogenous small RNA species that act posttranscriptionally to regulate mRNA stability. These recently discovered small RNAs are produced by the cell using a unique process, involving RNA polymerase II, micropressor protein complex, and RNAase II/Dicer endonuclease complex in a miRNA ribonucleoprotein complex. About 2000 miRNAs have been identified since the discovery of the first miRNA, *lin-4,* in *Caenorhabdhitis elegans* (Song and Tuan, 2006; Lee et al., 1993). The *lin-4* and *let-7* antisense RNAs are temporal regulators that control the timing of developmental events in *C. elegans* by inhibiting translation of target mRNAs (Lee and Ambros, 2001). MicroRNAs are predicted in a wide array of organisms, including plants, zebrafish, *Drosphila,* mice and humans; the miRNAs regulate the expression of protein-coding genes in plants and animals (Song and Tuan, 2006; Lim et al. 2003). These miRNAs are located within the intronic regions of the host genes, presumably transcribed with the host gene from the same promoter (Song and Tuan, 2006; Rodriguez et al., 2004). This suggests that miRNAs fine tunes the translation

of mRNA. Furthermore, miRNAs are conserved among closely related species, such as, mice and humans (Song and Tuan, 2006, Lagos-Quinitana et al., 2003).

The expression of miRNAs in multicellular organisms exhibit spatiotemporal, and tissue and cell specificity. This suggests miRNAs have an involvement in tissue morphogenesis and cell differentiation (Song and Tuan, 2006). Recent studies have demonstrated the importance of miRNAs in embryonic stem (ES) cell differentiation, limb development, adipogenesis, myogenesis, angiogenesis, and hematopogenesis, neurogenesis, and epithelial morphogenesis (Song and Tuan, 2006). For example, the *let-7* miRNA plays a role in controlling the timing of developmental transition, and it is detected in humans, *Drosophila,* and eleven other bilateral animals (Bartel, 2004; Pasquinelli et al., 2000). Paralogs and orthologs of the *C. elegans lin-4* and *let-7* RNAs have stage-specific expression in development as if they, too, function in small temporal RNAs (stRNAs). StRNAs function to control temporal identity during development in *C. elegans* and other organisms.

Various studies have shown that the absence of the production of miRNAs or the deletion of Dicer or Argonaute proteins, that promote the production of miRNA, results in embryonic lethality (Pasquinelli et al., 2005). For example, oocytes lacking Dicer (mutants) that were fertilized with wild type sperm fail to proceed through the first cell division. These mutants were infertile and showed reduced disorganized spindles and improperly aligned chromosomes (Tang et al. 2007). This study showed that maternal miRNAs are crucial for early mammalian development.

According to Tesfaye *et al.,* (2009) prediction of the target mRNAs of the corresponding miRNAs identified genes related to growth regulating proteins,

transcription factors, and tumor necrosis factors. Investigating the corresponding target mRNAs and protein products during oocyte maturation will provide insight into miRNAs controlled regulation of maternal gene expression.

Expression array technology has been adapted to examine miRNAs and has revealed distinct expression patterns in different developmental stages. Because of the different genes and expression patterns, it is reasonable to propose that every metazoan cell type at each development stage might have a distinct miRNA expression profile. For instance, while miR-122 is detected in liver, miR-223 is present primarily in the granulocytes and macrophages of mouse bone marrow, and miR-1 is found in mammalian heart. Furthermore, miRNAs play roles in skull development and have been implicated to have functions in craniofacial development (Bartel, 2004). Of the >250 miRNAs present in the vertebrate genome, very few have been assigned a function during development.

There are currently two approaches to elucidate the function of a specific miRNA in cells. These approaches are overexpression of miRNA or inactivation of miRNA. Overexpression is usually achieved by constructing a plasmid or viral vector that contains a miRNA precursor sequence (Zeng et al., 2002). To achieve inactivation, the cells are transfected with miRNA-specific antisense 2'O-methyl (2'-OMe) oligoribonucleotides ("antagomers") or chemically modified, cholesterol-conjugated single-stranded RNA analogs (Krutzfeldt et al., 2005). Although thousands of miRNAs have been identified from somatic tissue, little is known about their expression in germ cells (Yan et al., 2007).

2.2 Spermatogenesis

Spermatogenesis occurs in mitotic, meiotic, and post-meiotic phases (Eddy, 1998). It is a process by which mature male germ cells, spermatozoa, are formed in the seminiferous tubules of the testis of sexual mature males (Yan et al., 2007). The stem cells give rise to spermatogonia and are located at the periphery of each seminiferous tubule (Figure 2.1). The developing sperm cells move toward the central opening (lumen) of the tubule as they undergo meiosis and differentiation. The cells that result from meiosis all develop into mature sperm cells.

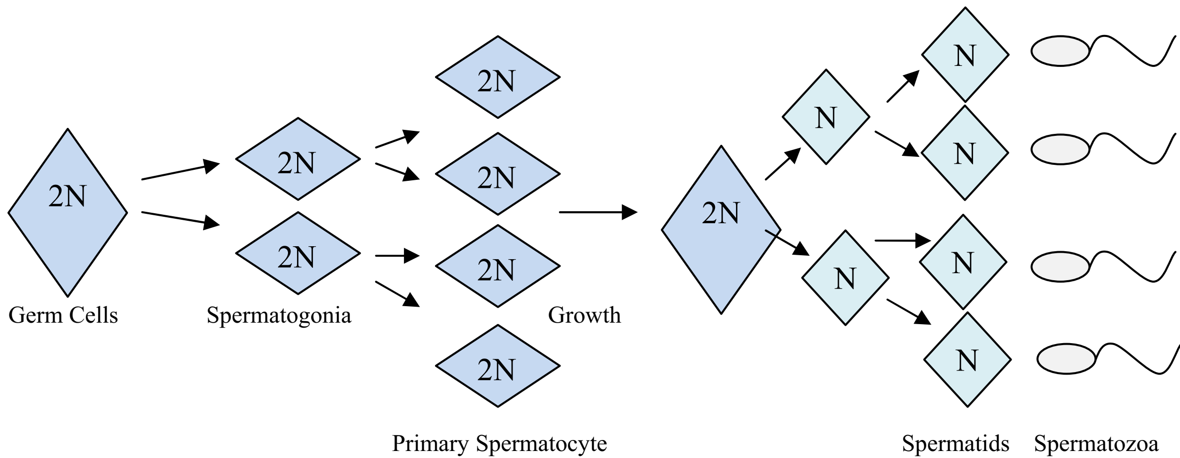

Figure 2.1 Spermatogenesis

Note: The primordial germ cells undergo differentiation giving rise to spermatogonia. The spermatogonia go through mitotic division and differentiation and at the onset of meiosis I it produce primary spermatocytes. Meiosis reduces the number from diploid (2n) to haploid (n) producing secondary spermatocytes. The secondary spermatocytes give rise to spermatids during meiosis II. The spermatids differentiate and produce spermatozoa.

Sperm structure is different among species. Mammalian sperm heads can be slender or spindle, comma or hook-shaped, oval or paddle shaped, or spherical in form (Table 2.1). However, in most species, the head, containing the haploid nucleus, is tipped with a special body-the acrosome. The acrosome contains enzymes used to penetrate the egg. Behind the head, the sperm cells contain large number of mitochondria that provide ATP for the movement of the tail, which is the flagellum.

Table 2.1 Sperm Structure in Different Species

Species	*Head Shape*
Humans	Paddle-shaped
Bulls	Paddle-shaped
Rat	Hook-shaped
Rooster	Spindle-shaped

Spermatogenesis is a continuous and prolific process in the adult male. In spermatogenesis, the male germ cells exhibit high transcriptional activity, strictly regulated spatiotemporal gene expression, and profoundly repressed translation (Ro et al., 2007). The profoundly repressed translational activity of the germ cells indicates the presence of miRNA in spermatogenesis. It is suspected that miRNAs play essential roles in spermatogenesis and any small change in their synthesis may affect male fertility.

2.2.1 Fertility

Poor semen morphology is an important indicator of decreased fertility in men, stallions, bulls, and goats (Esteso et al., 2006). In other words, normal sperm morphology is the best indicator of male fertility (Coetzee et al., 2001). In bulls there are two factors that are associated with semen quality and contribute to fertility levels. These factors are compensable and uncompensable traits. Compensable factors can be improved by increasing semen quantity; whereas, the uncopmensable traits are associated with the imcompetent sperm (Evenson, 1999). The incompetent sperm are the sperm cells that initiate fertilization, but are incapable of completing the fertilization process and sustaining early embryogenesis or fetal development (Ostermeier et al., 2001). According to Ostermeier et al., (2001) it is the noncompensable or uncompensable factors that contribute most to the fertility level of a bull.

Ostermeier et al. (2001) did a study to determine if a sperm nuclear shape is related to bull fertility. The Fourier harmonic analysis was used to determine multivariate parameters that describe the shape of sperm nuclei from bulls of different fertility. They also investigated the relationship of sperm chromatin structure to nuclear shape. It was found that the sperm nuclear shape was related to bull fertility. The high fertility sperm was identified to be more elongated and tapered than the sperm of the lower fertility (Ostermeier et al., 2001). They saw that bulls of the high fertility sire embryos that are more likely to develop to the morula/blastocyst stage than the bulls of lower fertility. Furthermore, zygotes sired by high-fertility bulls started S phase (DNA synthesis) earlier, stayed in S phase longer, exited S phase at the same time, had a shorter gap-2 phase and mitotic phase than zygotes sired from lower-fertility bulls. The

differences between the different fertility bulls were speculated to be due to DNA damage and unreplicated DNA which explains the differences observed in sperm nuclear shape.

So, the small but significant differences in sperm nuclear shape between the high and low fertility bulls reflect differences in chromatin structure and its ability to resist DNA denaturation. The results suggest that the shape of the sperm nucleus not only reflects chromatin structure, but additional aspects of noncompensable fertility traits as well (Ostermeier et al., 2001).

2.3 Oogenesis

Oogeneis is the development of ova, or mature, unfertilized egg cells. Oogonia, the stem cells that give rise to ova, multiply and then begin meiosis, but the process stops in prophase I. At this stage, the cells are called primary oocytes. The primary oocytes remain inactive within small follicles until puberty when they are reactivated by hormones. Beginning at puberty, FSH (follicle stimulating hormone) periodically stimulates a follicle to grow and induces its primary oocyte to complete meiosis I and start meiosis II. Meiosis stops again, and the secondary oocyte is released during ovulation and does not continue meiosis immediately. Penetration of the egg cell by the sperm triggers the completion of meiosis and only then is oogenesis complete.

Bovine oocyte maturation involves the continuation and completion of the first meiotic division from the germinal vesicle (GV) stage to metaphase II, with corresponding cytoplasmic maturation (Tesfaye et al., 2009). Optimal storage and timely availability of mRNA is important for oocyte quality and developmental capability.

Oocyte competence is the ability of the oocyte to complete maturation, undergo successful fertilization, and reach the blastocyst stage (Assidi et al., 2008). The earliest stages of embryogenesis are regulated by maternally inherited components stored within the oocyte (Tesfaye et al., 2009).

2.4 Expression Analysis

2.4.1 Microarray

Microarray technologies evolved from Southern blotting and were made by spotting cDNAs onto filter paper with pin-spotting devices (Tofano et al., 2006). An array can contain tens of thousands of probes and a microarray experiment can accomplish many genetic tests. Because of this, arrays have dramatically accelerated many types of inventions and are used to explore the expression patterns in different organisms.

Microarray-based techniques are able to screen large numbers of miRNAs simultaneously. The miRNA microarray analysis provides a method that is parallel and highthroghput for the detection of thousands of miRNAs (Yan et al., 2007). Also, a solution-based sandwich assay format where the target miRNA simultaneously hybridizes to a pair of spectrally distinguishable fluorescent oligonucleotide probes. These probes are each complementary to one half of the target miRNA or a surface-based assay measuring the sequence specific adsorption onto different probe array elements created on a gold thin film can be used to directly detect miRNA. After microarray

identification of differential expression, these differences are confirmed by quantitative RT-PCR (Yan et al., 2007).

CHAPTER 3

REGULATORS OF DEVELOPMENTAL GENE EXPRESSION

3.1 Small RNAs

Over the course of time many studies have focused on small RNAs. One of the functions of RNA (ribonucleic acid) is to translate DNA into proteins. The three main types of RNA are: messenger RNAs (mRNAs), transfer RNAs (tRNAs), and ribosomal RNAs (rRNAs). Of these RNAs, tRNA and rRNA are non-coding RNAs that have housekeeping roles during mRNA translation (Groβhans and Filipowicz, 2008). RNA molecules have several functions such as catalytic activity and the ability to act as structural components; there are also non-coding RNAs that play essential roles in biological systems without encoding proteins (Review by Kawaji and Hayashizaki, 2008). Likewise, small RNAs are not translated into proteins.

Small RNAs are involved in important biological processes like mammalian cell and developmental biology (Mattick and Makunin, 2005). There are several classes or forms of small RNAs. The best understood classes are piwi-interacting RNA (piRNAs), small interfering RNAs (siRNAs), and microRNAs (miRNAs). These small regulatory RNAs were first discovered in the 1990s and range from 17-30 nt in length. Small RNAs regulate gene expression at the transcriptional and post transcriptional level (Kurth and Mochizuki, 2009).

The lack of small RNAs causes transposons to jump (wreaking disorder on the genome), stem cells to be lost, failure of brain and muscles to develop, plants to succumb viral infection, flowers to take on unfamiliar shapes, and failure of cells to divide because of non-functional centromeres (Zamore and Haley, 2005). On the other hand, small RNAs behave as a guide to direct mRNA degradation, translational repression, heterochromatin formation and DNA elimination (Cerutti, 2003).

Furthermore, small RNAs guide Argonaute proteins to target molecules, which leads to gene silencing. The Argonaute proteins are defined by PAZ (Piwi Argonaut and Zwille) and PIWI domains (Narry Kim, 2006). The Argonaute family is divided into the Ago subclade and the Piwi subclade. The Ago members are associated with miRNAs and siRNAs and are expressed ubiquitously; whereas, the Piwi members are restricted to germ-line cells and stem cells. In mice, there are four Ago members (Ago 1-4) and three PIWI members (MIWI, MILI/PIWIL2, and MIWI2/PIWIL4) (Narry Kim, 2006).

3.1.1 Novel Class – piRNA

The piRNAs are a distinct class of small RNAs. They are ~24 to 30 nt in length. They are produced by a Dicer independent mechanism. The piRNAs have been studied in zebrafish, mice, and flies. They have been found to maintain germline DNA integrity and silence DNA elements. It has also been found that piRNAs are derived from repeated or complex DNA sequences (Aravin et al., 2007; Brennecke et al., 2007; Houwing et al., 2007).

A study was done to report the identification and characterization of a novel class of small RNAs in mouse spermatogenic cells (Grivna et al., 2006). In this study, an

abundance of small RNA species were identified and their expressions levels were significantly reduced on 24 day postpartum (dpp). Because spermatogenesis in *miwi*-null testes is arrested uniformly at the onset of spermiogenesis at 24 dpp, the results suggested that this novel class of small RNAs are expressed in spermatocytes and spermatids and that their expression is dependent on MIWI function (Grivna et al., 2006). Since the expression of the novel RNAs required *miwi*, a piwi subfamily gene, those small RNAs were referred to as piRNAs. Furthermore, this study showed piRNAs were only found in mouse testes and not other organs. Their expression profile showed that piRNAs were predominately produced post-meiotically in early round spermatids in a MIWI-dependent method. This suggests a possible role for piRNAs in translation regulation since MIWI is involved in translational regulation (Grivna et al., 2006).

PiRNAs were studied in the fly, *Drosophila* to gain an understanding of the mechanism by which piRNAs are produced. This method of production is often referred to as the ping-pong model for piRNA production (Figure 3.1). Argonaute 3 (Ago 3) associates with mature sense-strand piRNA to form an Ago3-piRNA complex. This complex cleaves the antisense transcripts and produces a piRNA precursor. Aubergine (Aub) and piwi associate with antisense piRNA precursor to form a complex that guides the production of mature antisense-strand piRNAs. Also, Aub associates with mature antisense-strand piRNA to form an Aub-piRNA complex. This complex cleaves sense transcripts and produces sense piRNA precursors. The sense piRNA precursor associates with Ago3 and forms a complex that guides the production of mature sense-strand piRNAs. The piRNA binds to piwi proteins and forms a complex called the piwi-

interacting RNA complex (piRC) that regulates mRNA expression and gene silencing (He et al., 2009).

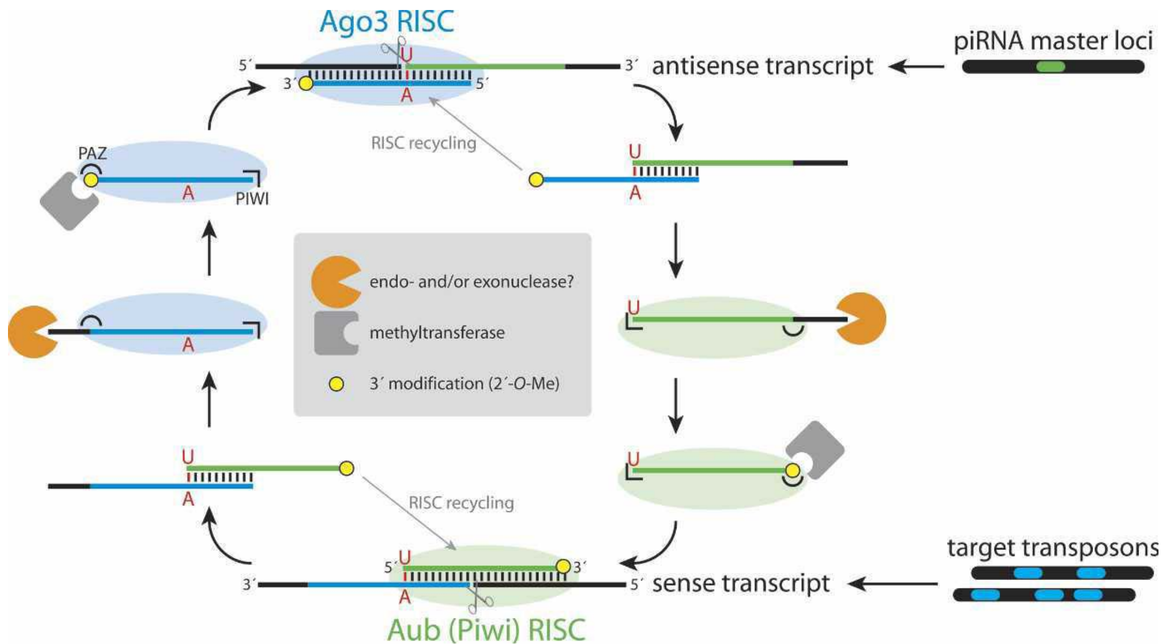

Figure 3.1 Ping-pong model for piRNA production.

Note: Sense transcripts from transposons are cleaved by Piwi or Aub RICS loaded with a piRNA guide. The cleaved transcript is not degraded but used to program Ago3 RISC. The complex cleaves the antisense transcript. The sense or antisense transcripts fuel an amplification cycle in which the 5'end of piRNAs are defined by RISC cleavage. The 3'ends are shortened by an endonuclease and/or exonuclease to the size that fits the distance between PAZ and PIWI domains (Hartig et al., 2007).

3.1.2 First Class – miRNA

MicroRNAs were first indentified in *C. elegans* as endogenous regulators of developmental timing (Bartel, 2004; Lehrbach and Miska, 2008). The first genes discovered in *C. elegans* were *lin-4* and then *let-7* (Wienholds and Plasterk, 2005). There are several steps that must take place to generate miRNAs (Figure 3.2). pri-

miRNA is transcribed from the genome by RNA polymerase II in the nucleus (Yu et al., 2006, Narray Kim, 2006). The pri-miRNA is cleaved into a stem loop intermediate called miRNA precursor (pre-miRNA) that is catalyzed by the Micropressor protein complex. This complex consists of two proteins, Drosha and DGCR8 (Tang et al., 2007). The pre-miRNA is then exported from the nucleus to the cytoplasm by Exportin 5. A 22 nt long mature miRNA complex is released by the second RNase III endonuclease complex, including Dicer (Tang et al., 2007). After being unwound, the miRNA strand is degraded. In some cases, when it exhibits a perfect complementarity to the miRNA strand it is capable of specific cleavage of the mRNA target. On the other hand, when there is a near perfect match the Argonaute protein in the RNA-induced silencing complex (RISC) cleaves the mRNA target that is destined for degradation (Song and Tuan, 2006). In humans, when there is a lesser degree of complementarities, the translational repression mechanism is used to control gene expression (Song and Tuan, 2006; Mack, 2007).

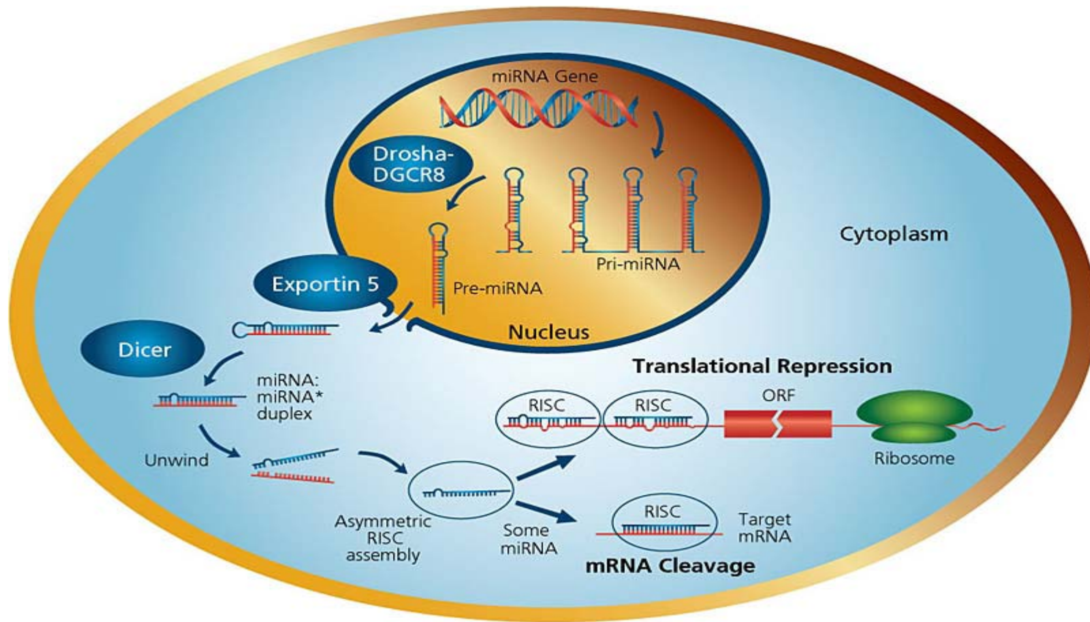

Figure 3.2 Biogenesis of MiRNA (www.sigmaaldrich.com)

Note: MiRNAs are encoded in the chromosomal DNA and transcribed as longer stem-loop precursor called primary miRNAs (pri-miRNAs) by RNA polymerase II. The pri-miRNAs are processed in to miRNA precursors (pre-miRNA) by Drosha and DGCR8. The pre-miRNA is exported from the nucleus into the cytoplasm and is cleaved by Dicer into single stranded mature miRNAs. Thus giving rise to a large class of short non-coding regions (17-25 nucleotides). The mature miRNAs repress protein production by blocking translation or causing transcript degradation.

A great deal of what is known about miRNAs has come from studies in *C. elegans*. *Lin-4* and *let-7* were the first identified and are the most understood miRNAs. The laboratory of Victor Ambros identified the first miRNA, *lin-4*, and determined that it did not encode a protein but was a small RNA that imperfectly base-paired to complementary sequences on target mRNAs to block gene expression (Lee et al., 1993). In 2000, Gary Ruvkun and his group discovered, *let-7,* the second miRNA in a similar manner as *lin-4* (reviewed in Vella and Slack, 2005). A study done by Rienhart et al. (2000) showed that *let-7* is a heterochronic switch gene. Loss of *let-7* causes

reintegration of larval cell fates during the adult stage. Increases in *let-7* gene dosage causes precocious expression of adult fate during larval stages (Reinhart et al., 2000). Because of such studies, scientists concluded that *lin-4* and *let-7*(miRNAs) have roles in regulating developmental timing.

3.1.3 Second Class – siRNA

Several years after the discovery of miRNAs, exogenous double-stranded RNA (dsRNA) that specifically silences genes through a mechanism called RNA interference (RNAi) was reported by Fire, Mello, and other colleagues (Mello and Conte, 2004). Fire, Mello, and coworkers showed that dsRNA had contaminated both sense and anti-sense RNA preparations and was responsible for the observed gene silencing (Reviewed in Zamore, 2001). SiRNAs were originally proposed to act mainly as an antiviral defense and transposon repression system via the phenomenon of RNAi, but recent findings indicate that such RNAs may play a much broader role in gene and genome regulation.

In this mechanism, the double stranded RNA (dsRNA) recognizes and degrades homologous mRNA sequences (Jiang and Milner, 2002). The siRNAs are produced by cleavage from the RNase III endonuclease Dicer giving rise to ~21 to 22 nt long double stranded RNA molecules. siRNA remains stable, in an RNA-induced silencing complex (RISC), with endonuclease, exonuclease, helicase, and RecA plus related proteins and begins degradation of additional homologous mRNA (Jiang and Milner, 2002; Bagasra and Prilliman, 2004). The RISC guides the RNA degradation machinery or siRNA duplexes and serves as primers to transform the target mRNA and cleaves the cognate RNA in a sequence-specific, siRNA-dependent manner (Bagasra and Prilliman, 2004).

SiRNAs and miRNAs are very similar. Both miRNAs and siRNAs are found in plants and animals. siRNAs and miRNAs are generated from double stranded RNA precursors by Dicer endonucleases, and function with Argonaute-family proteins to target transcript destruction or to silence translation (reviewed in Klattenhoff and Theurkauf, 2008) as shown in Table 1. However, the specific characteristics of the ends of miRNAs and most siRNA is a key difference between these small RNAs; most species of miRNAs have highly exact ends whereas siRNAs tend to have a much more heterogeneous end composition (reviewed in Carthew and Sontheimer, 2009).

Table 3.1 Comparison of Small RNA Classes

Small RNA	**MiRNA**	**SiRNA**	**PiRNA**
Length	~17-25 nt	~21-22 nt	~24-30 nt
Origin	Endogenous	Exogenous	Endogenous
Occurrence	Plants and Animals	Plants and Lower animals	Plants, Animals, Fungi, Flies
Function	mRNA instability and translation inhibition	mRNA degradation or posttranscriptional silencing	Repression of retrotransposon & posttranscriptional regulation
Target	mRNA	mRNA	

3.4 The World of miRNAs

Since the discovery of miRNAs, more than 5,000 miRNAs have been identified. The human genome encodes 100s to 1000s miRNAs that regulate at least a third of protein-encoding genes. According to PharmaReports, a typical human cell harbors 1,000 to 200,000 miRNAs, in patterns unique to particular cell types.

Several studies have shown that miRNAs play important roles in development. For example, a study done by Hornstein et al., (2005) showed that miRNAs were important for vertebrate limb development. In this study, it was shown that miR-196 was important for hindlimb development in the hedgehog. In this mechanism, miRNA blocks the presence of Hoxb-8 in the hindlimb by shutting off transcription. Transcription inhibition of Hoxb-8 is very important in the hedgehog for limb development.

3.4.1 Roles of miRNAs in Animal Development

Many studies have shown that miRNAs have a critical role in development. For example, there has been a study that describes the first comprehensive set of miRNA expression patterns in animal development (Wienholds et al., 2005). In this study, the temporal expression of miRNAs during embryonic development of zebrafish was determined by microarray analysis. Wienholds and colleagues saw that most miRNAs could not be detected or were not visible until 1 to 2 days after fertilization. These miRNAs showed strong expression when organogenesis was almost completed and most of the miRNAs remained expressed during adulthood. They went on to show that a high degree of the miRNAs were tissue specific by dissecting the organs of adult fish. In this study, it was found that six miRNAs were expressed in different organ systems: nervous,

digestive, muscle, circulatory, excretory system, and sensory organs. Not only was the miRNA tissue specific but there was some specificity within the organ. MiR-140 was expressed and restricted to regions of the jaw, head, and fin whereas, miR-217 was only expressed in the exocrine pancreas, and miR-7 in the endocrine pancreas only (Wienholds et al., 2005). The observations showed that miRNAs were indeed expressed during development, although, there was no expression during early development. Because of this, authors believe that most miRNAs may not be essential for tissue fate establishment but rather play crucial roles in differentiation or the maintenance of tissue identity (Wienholds et al., 2005).

CHAPTER 4

MICRORNA DETECTION IN SPERMATOZOA OF DIFFERENT FERTILITY BULLS AND IMPLICATIONS FOR FERTILITY

4.1 ABSTRACT

MicroRNAs are small non-coding RNAs that regulate gene expression of messenger RNA. MicroRNAs are found in various plants and animals. They are generated from double stranded RNA precursors by Dicer endonucleases, and function with Argonaute-family proteins to target transcript destruction or to silence translation. However, the molecular mechanisms by which microRNAs regulate gene expression during gamete and embryo development are not well understood. In this study, microRNAs were isolated from high fertility and low fertility bull spermatozoa. Here we show that an abundance (thousands) of microRNAs were present in bovine spermatozoa, and only seven were significantly expressed; furthermore, the seven significantly expressed microRNAs were more highly expressed in the low fertility bulls. The results demonstrate that microRNAs are abundantly present in bull spermatozoa and that they may have important functions in regulating bovine spermatozoa function. Identifying specific microRNAs expressed in spermatozoa from bulls of different fertility bulls will help improve understanding of mammalian gametogenesis and early development.

4.2 INTRODUCTION

Early embryogenesis is a very important mechanism for the development of new animals and is critical for later development. In mammals, the union of sperm and egg give rise to a zygote, which then goes through the process of embryogenesis forming the morulae and blastocyst, leading to fetal development. It is known that developmentally regulated molecular events following fertilization include changes in the length of the cell cycle, chromatin structure and DNA methylation, synthesis of zygotic/embryonic transcripts and proteins, and degradation of maternal transcripts (Memili and First, 1999). However, molecular mechanisms including identities of transcripts, proteins, and molecular networks regulating early embryogenesis are not known in detail (Misirlioglu et al., 2006). Yet, various studies have shown that microRNAs (miRNAs) regulate gene expression and also play a major role in embryo development. These RNAs have been recognized in a wide range of animals, including bovine, and plants and are conserved through species such as: *C. elegans* through *D. melanogaster* to *Homo sapiens* (Baskerville and Bartel, 2005). The degree to which spermatozoa miRNAs regulate fertilization and early embryonic development in bovine is yet to be discovered.

miRNAs were first indentified in *C. elegans* as regulators of developmental timing by the laboratory of Victor Ambros (Lee et al., 1993; Bartel, 2004; Yu et al., 2007). About 2000 miRNAs have been identified since the discovery of the first miRNA, *lin-4,* in *C. elegans* (Lee et al., 1993; Song and Tuan, 2006). MiRNAs are small non-coding RNAs, 19 to 22 nucleotides (nt) in length, which regulate gene expression of messenger RNA (mRNA). In addition to regulating development, miRNAs regulate and are involved in other physiological processes such as birth defects and cancer (Stefani

and Slack, 2008). During the synthesis of miRNAs, a primary miRNA (pri-miRNA) transcript encoded in DNA is transcribed in the nucleus. The pri-miRNAs are processed by the enzyme Dosha and exported into the cytoplasm, where they are further processed by Dicer giving rise to single stranded mature miRNAs. The mature miRNAs repress protein production by blocking translation or causing transcript degradation (Mack, 2007).

It was shown that maternally inherited miRNAs found in the zygote are critical for early embryo development in the mouse (Tang et al., 2007). Tang et al., (2007) discovered that oocytes lacking Dicer (mutants) that were fertilized with wild type sperm fail to proceed through the first cell division. These mutants were infertile and showed reduced disorganized spindles and improperly aligned chromosomes. According to Murchinson et al. (2007) oocytes lacking Dicer, which produces miRNAs, are unable to complete meiosis because of defects in gene expression that affect spindle organization.

It is now evident that sperm deliver not only paternal DNA but other components such as RNA, mRNA, and miRNA to the oocyte; and the sperm RNA remains stable until the expression of the embryonic genome (Boerke et al., 2007). However, according to Amanai et al., (2006) sperm-borne miRNAs were shown to play a limited role in mammalian fertilization and pre-implantation development. Although these studies suggest that paternal miRNAs play a limited role in fertilization and development, it is recognized that miRNAs play essential roles in development and disease. It is also known that the lack of small RNAs permits transposons to jump (wreaking disorder on the genome), stem cells are lost, brain and muscle fail to develop, plants succumb to viral infection, flowers take on unfamiliar shapes, and cells fail to divide because of non-

functional centromeres (Zamore and Haley, 2005). Because of this, it is believed that miRNAs in bovine spermatozoa might have specific functions that regulate fertility and possibly embryo development which can be determined by evaluating expressions of miRNA transcripts.

The purpose of this study was to identify miRNAs in bovine spermatozoa from high and low fertility bulls and to determine the mechanisms by which miRNAs regulate fertilization and early bovine embryonic development. High fertility bulls are referred to as males with compensable seminal deficiencies, which means more sperm is required to reach maximum fertilization rate. Low fertility bulls are males that have uncompensable seminal deficiencies, producing fertilizing sperm in abnormal ejaculates (Saacke, 2008). Studies have shown that sperm RNA remained stable until the onset of major embryonic genomic activation, which is at the 8-cell stage in cattle (Boerke et al., 2007). It is expected that miRNAs in sperm samples of two bulls from different fertility will demonstrate specific roles of miRNAs in fertilization and early embryonic development. Nevertheless, the primary focus of this study is to identify miRNAs expressed in bovine spermatozoa. The results from this study will demonstrate that miRNAs are present in bovine spermatozoa, and that the levels of expression in miRNAs of high and low fertility bulls can be a major factor controlling animal reproduction.

4.3 MATERIALS AND METHODS

In this experiment, the expression of miRNAs from bovine spermatozoa in high and low fertility bulls was analyzed. This was done by the isolation total RNA from different bulls, the enrichment of miRNAs, and the performance of microarray analysis.

The microarray results were confirmed by isolating sperm RNA, running a gel using the Bioanalyzer, and performing TaqMan MicroRNA Assays.

4.3.1 Determination of Bull Fertility

Frozen semen samples and bull fertility data from two mature and progeny-tested Holstein bulls with satisfactory semen quality were provided by Alta Genetics, Inc. (Watertown, WI).

4.3.1.1 Sample and Data Sources

Fertility data that have been established for progeny testing programs is named AltaAdvantage®. This program is the industry's most reliable source of fertility information and consists of insemination records collected from 180 well managed partner dairy farms located in different geographical regions across the United States. This breeding program is advantageous because it provides DNA verification of the paternity of the offspring and pregnancies diagnosed by veterinary palpation, instead of just relying on non-return rates following 60-90 days after breeding.

4.3.1.2 Bull Fertility Prediction

To predict the fertility of the bulls from the given source, a sub-set of data was generated consisting of 962,135 insemination records from 934 bulls with an average of 1,030 breedings ranging from 300 to 15,194. The environmental and herd management factors that influence the fertility performance of the sires were adjusted using sophisticated statistical methods. Therefore, for the definition of fertility, instead of

relying only on the number pregnant cows (those verified by veterinary palpation or ultrasound examination) divided by the total numbers of cows examined for pregnancy, the effects of environmental factors were eliminated such as herd, year, season, month, cow and age of the cow, days in milk, parity, technician, synchronization method and age of the sire, in order to rank the bulls based on their breeding values for fertility. Further, the fertility index of each bull was calculated and expressed as the percent deviation of its conception from the average conception of all bulls having over 300 breedings in the data set. The average conception rate was considered as '0'.

4.3.1.3 Selection of high and low fertility bulls

For this study, an arbitrary threshold for classifying high and low fertility bulls was used (Peddinti et al., 2008; Feugang, et al., 2008). However, the bulls scoring highest and lowest fertility levels with highest reliability (>1,000 breeding/bull) were selected for this study.

The fertility difference between high and low fertility bulls was 16.6%, which was obtained from bulls having adequate records for higher reliability. While the high fertility bull scored 9.2% above the average, the low fertile bull scored 7.4% below the average.

4.3.2 Isolation of Spermatozoa

Straws containing cryopreserved sperm samples were shipped in liquid nitrogen from Wisconsin to our laboratory in Mississippi. Motile sperm cells were isolated using percoll gradient as described previously (Sagirkaya et al., 2006). A total of 12 straws were collected (6 from high fertility bull and 6 from low fertility bull). The straws were

thawed together in a water bath set at 36°C for 30 seconds. The sperm ($2x10^6$ cells) were poured (2 straws/tube) into the percoll gradient and spun at 1,509 x *g* for 15 minutes at 4°C. The supernatant, containing egg yolk, was carefully removed using a micropipette. The pellet was resuspended in 1 ml PBS and centrifuged again. The supernatant was removed and the pellet was resuspended in 1 ml of PBS and centrifuged at 9,447 x *g* for 1 minute at 4°C and then the supernatant was removed.

4.3.3 Sperm RNA Extraction

RNA was isolated using Trizol (Invitrogen, Carlsbad, CA) according to Feugang et al. (2008). Briefly, five hundred microliters of Trizol were added into the sperm cell pellet which was then homogenized at high speed for 30 seconds using the Pro 200 homogenizer (Pro Scientific Inc., Oxford CT). Glycogen (3 µl of 20 mg/ml) was added to the tubes and another 500 µl of Trizol was added followed by mixing with several pipettings. Then, the mixture was incubated for 15 minutes at 65°C. Five hundred microliters of chloroform were added, mixing was accomplished by several pipettings, and the mixture was vortexed for 40 seconds.

The samples were incubated at room temperature for 10 minutes followed by centrifugation for 15 minutes at 4°C. The upper colorless phase was collected into a new sterile eppendorf tube in which five hundred microliters of 100% isopropanol were added and then mixed by several pipettings.

Following incubation at -20°C for an hour, the samples were vortexed for 10 seconds and incubated at room temperature for 10 minutes. The RNA was precipitated by centrifugation for 10 minutes at 4°C. Following the removal of the supernatant, the

pellet was air-dried for 5 minutes and then it was rehydrated in 20 µl of deionized, diethylpyrocarbonate (DEPC) treated water. The concentration of the RNA was determined using 2 µl of the samples in Nanodrop ® ND 1000 (NanoDrop Technologies, Wilmington, DE). The remaining 18 µl of the samples were stored at -80°C and then shipped to Asuragen Inc. on dry ice.

4.3.4 Enrichment of Spermatozoal microRNAs and MicroRNA MicroArray Expression Array Experiments

Samples for miRNA profiling studies were processed by Asuragen Services (www.asuragen.com, Austin, TX), according to the company's standard operating procedures. Total RNA was dephosphorylated with calf intestinal phosphatase and the pCp-Biotin labeling molecule was ligated to the 3' ends of the RNA molecules. Labeled RNA was purified using BioSpin6 (Bio-Rad, Hercules CA). Hybridization, washing, staining, imaging, and signal extraction were performed according to Affymetrix-recommended procedures, except that the 20X GeneChip Eukaryotic Hybridization control cocktail was omitted from the hybridization.

There were six samples processed for microarray experiments using Ambion/Affymetrix DiscovArray. The signal processing implemented for the Ambion miRCHIP is a multi-step process involving probe specific signal detection calls, background estimate and correction, constant variance stabilization (Huber et al., 2002) and either array scaling or global normalization. For each probe, an estimated background value is subtracted that is derived from the median signal of a set of G-C-matched anti-genomic controls. Arrays within a specific analysis experiment were normalized together

according to the variance stabilization method described by Huber et al. (2002).

Detection calls were based on a Wilcoxon rank-sum test of the miRNA probe signal compared to the distribution of signals from GC-content matched anti-genomic probes.

4.3.5 Statistical Analysis

For statistical hypothesis testing, a two-sample t-Test with an assumption of equal variance was applied. One-way ANOVA was used for experimental designs with more than two experimental groupings or levels of the same factor. These tests define which probes are considered to be significantly differentially expressed, or "significant", based on a default p-value of 0.001 and $\log_2$ difference >1.

4.3.6 Confirming MicroRNAs using Bioanalyzer

Using the same two bulls, spermatozoa isolation and RNA extraction were performed. The bioanalyzer was used to confirm that miRNA was present (Figure 4.1). The RNA samples were stored at -80°C until TaqMan assay was performed.

4.4. RESULTS

4.4.1 Dynamics of MicroRNAs in Bull Spermatozoa

The miRNA samples were analyzed using a miRNA microarray (Ambion/Affymetrix DiscovArray) containing several types of probe sets derived from organisms such as plants (i.e. rock cress and soybean) and animals (i.e., zebra fish,

chicken, mouse, and human). The bulls were categorized as having a high (D) or a low (R) fertility. Each sample was represented by a vector containing all detected probes (29, 722). The samples showed that there were general differences between the high and low fertility bulls (Figure 4.1). It also showed that there were differences among the same sample.

Approximately 14,215 probe sets were successfully analyzed above background (Table 4.1).

4.4.1.1 MicroRNAs of Bull spermatozoa

In Figure 4.1, it was made known that miRNAs were abundant in bull spermatozoa. To confirm those results, the Bioanalyzer was used to determine the purity of RNA and ensure that miRNAs were indeed present in the mature sperm. For each bull there were 3 sample repeats and the bioanalyzer showed that small RNAs were present in each bull and their repeats (Figure 4.4). MicroRNAs were visible at 25 nt on the gel showing plenty of miRNAs based on the size of the band.

4.4.2 Statistical Analysis of Differentially Expressed MicroRNAs

In Figure 4.2, the single horizontal red line corresponds to an unadjusted p-value of 0.05. Genes above this line were considered as statistically significant at a p-value of 0.05. A majority of the spots were located in box B and D, indicating unchanged genes between high and low fertile bulls. High fertile bull (D) showed some genes that were of low expression in box A and D or that were highly expressed in box C and D, when compared to low fertility bull R. The highly differentially expressed genes are indicated

by the spots colored red (Box C). There were seven of these genes and were designated as significant based on an unadjusted p-value of 0.001 and a 2-fold change (Figure 4.2).

Each sample was represented by a vector containing only significant probes. This reduction eliminated the influence of non-significant probes in the cluster analysis (Figure 4.4). Each probe was represented in each line, with the color code confirming their differential expression levels between low and high (D) fertile bulls, despite the slight variation between replicates in each bull.

The characterization of the seven probe sets are summarized in Table 1. All the probes are from humans and their annotations are still not yet complete.

4.5 DISCUSSION

MiRNAs are found in a number of species, such as *C. elegans, D. melanogaster, Bos taurus,* and *Homo sapiens.* In this study, we accomplished our objective by identifying miRNAs in bovine spermatozoa. MiRNAs play an essential part of animal gene regulatory networks and a given genome could encode nearly 1000 miRNAs (Bartel, 2004; Bentwich et al., 2005). MiRNAs provide functions in animals that are essential for normal development and cellular homeostasis (Hwang, 2009).

While examining the two bulls of different fertility, it was found that there was an abundance (thousands) of miRNAs in bovine spermatozoa. However, only a few (seven) miRNAs were significantly differentially expressed. These seven had a probe set which was from *Homo sapiens*. This could possibly mean that the sequences are conserved through species of *Bos tarus* and *Homo sapiens* although some miRNAs appear to be species specific (Bentwich et al., 2005) Furthermore, the seven significantly expressed

miRNAs were identified but there were no annotations; therefore, at the present time, their functions are unknown. Nevertheless, some studies have described possible functions of miRNAs through overexpression, misexpression, and *in vitro* knockdown (Harfe, 2005).

The miRNAs detected in the low fertility bull were expressed at higher levels than those in the high fertility bull. The higher expression levels of the miRNA in the low fertility bulls could mean that miRNAs might be down regulating expression of genes, whose products play important roles in fertilization and early embryonic development. For example, highly expressed miRNAs could be inhibiting expression of proteins involved in chromatin structure of the sperm. According to Saacke (2008), the sperm from low fertility bulls are morphologically normal but contain defected chromatin, which hinders the sperm from going through the process of decondensation to complete fertilization or sustain embryogenesis once fertilization is initiated. There are two main differentiating characteristics of the high and low fertility bulls; the competence of the spermatozoa to reach the site of fertilization and actively penetrate the zona pellucid, and the ability of spermatozoa to initiate and sustain zygotic, embryonic, and fetal development (Ostermeir et al., 2005), which we referred to as compensable and uncompensable traits.

In this study, there were some variations of miRNA expression within the same groups of bulls. This could possibly be due to the time of collection. Semen was collected within a 3 month period and it was subsequently shipped to our laboratory. It is possible that gene-environment interactions among other factors might have contributed to differences in gene expression, including the miRNAs in different ejaculates. Another

limitation was that the miRNA microarray contained probes for known miRNAs for humans, mice and rats. Thus, it is expected that additional and bovine specific miRNAs would have been detected using bovine specific miRNA microarrays if they had been available. In spermatogenesis, there are many miRNAs that are waiting to be detected but little is known about their expression level or patterns (Yan et al., 2007).

It is clear that miRNAs are abundant in bovine spermatozoa. Determining the function(s) of the significantly differentially expressed miRNAs will help to understand functional genomics of bovine spermatozoa. Distinct bovine miRNAs have been identified in bovine adipose tissue that should be useful for studying the role of miRNAs in cattle and for comparative genomic analysis of miRNA function and regulations (Gu, et al., 2007). Identifying specific miRNAs expressed in spermatozoa of different fertility, will provide a better understanding of mammalian gametogenesis. From this, we will be able to develop a model that will aid in understanding the roles of miRNAs during early development in animals as well as other mammals including humans. It would be interesting to identify target mRNAs of these miRNAs detected in bull spermatozoa.

4.6 FIGURE LEGENDS

Figure 4.1. Heat map and the result of clustering to all MicroRNA probes detected. There are two specific groups. Column 1-3 represents bull R. Columns 4-6 represents bull D. There were differences between the two groups and some differences within the same groups.

Figure 4.2. Volcano plots display the relationship between fold-change and significance between two experimental treatment groups. Slot A represents significant probes for bull D but they are not significant for the fold change.

Figure 4.3. Heat map and the result of clustering applied to the significant (as determined by statistical testing) probes only. Group R represents the low fertility bull with the seven significant probes more highly expressed than Group D that represent the high fertility bull.

Figure 4.4. Bioanalyzer gel used to describe purity of RNA and if miRNAs are present. According to the gel image there is no contamination in the RNA and the miRNA will be at or below the green line on the gel, given that miRNAs range from 17-25 nt in length.

TABLES AND FIGURES

Table 4.1 Characterization of Seven Probe Sets

IDs	Chromosome Location	Sequences	P-values	Log Ratio	Average: D vs. R
hsa-aga-3155	22	AAGCUUAUGGAGCAGAGGAUU	0.00001	3.86	4.65
hsa-aga-8197	7	UGAGUGAUAAUAGGGUCGUGAC	0.0004	1.22	5.6
hsa-aga-6727	18	UUCUGUGGCAGAUUGGGAUGGA	0.00051	1.83	4.27
hsa-aga-11796	22	GAGGCAGAGAAGGGACAGGAAA	0.00073	1.72	7.35
hsa-aga-14189	19	CAUAGCGAGACCCCGUCUG	0.00077	1.21	14.34
hsa-aga-6125	9	UUGGAUAUUGCUUGGAGGCUCU	0.0009	1.25	10.32
hsa-aga-13659	9	GACUGGAGGAGGCAUGGAGGGU	0.0009	1.05	12.93

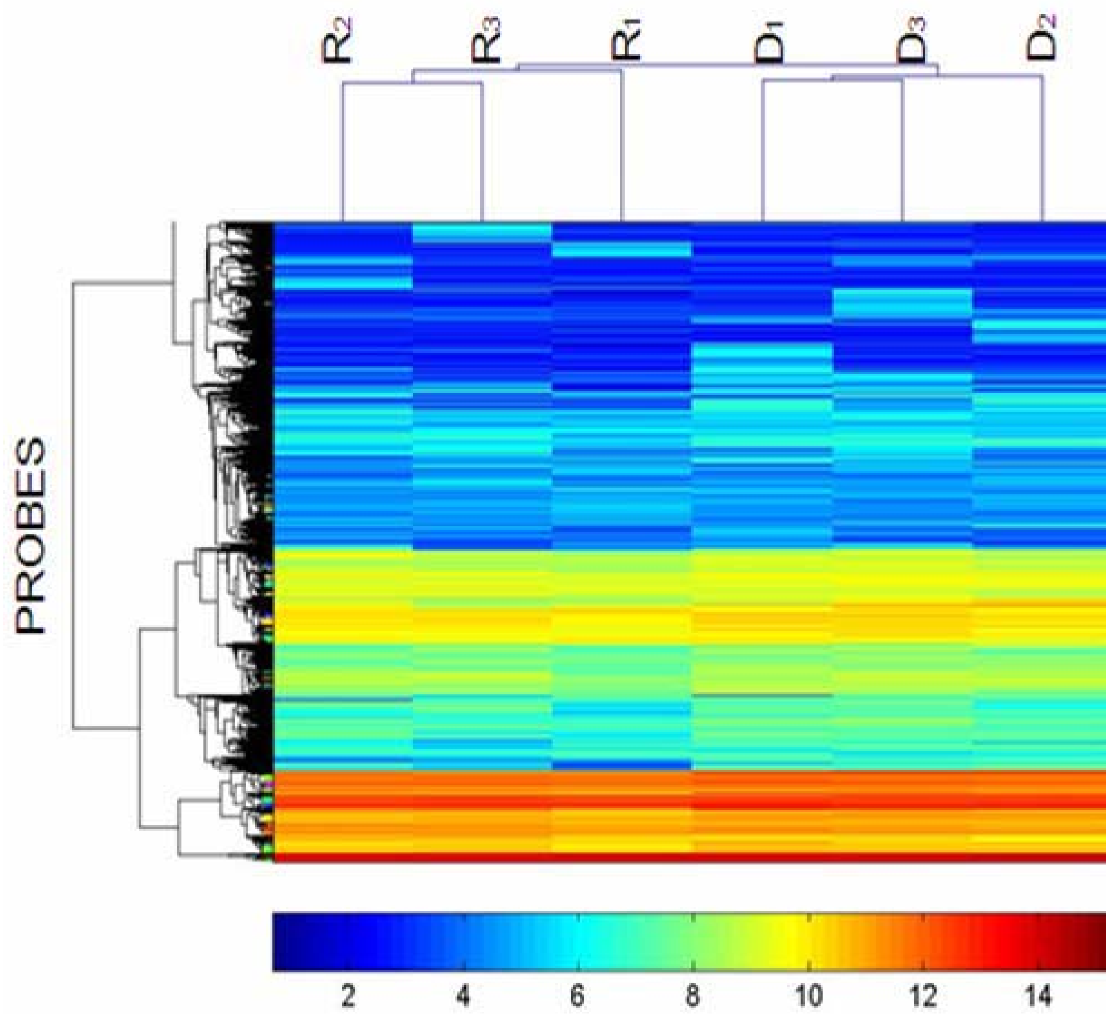

Figure 4.1 Heat Map of Detected Probes

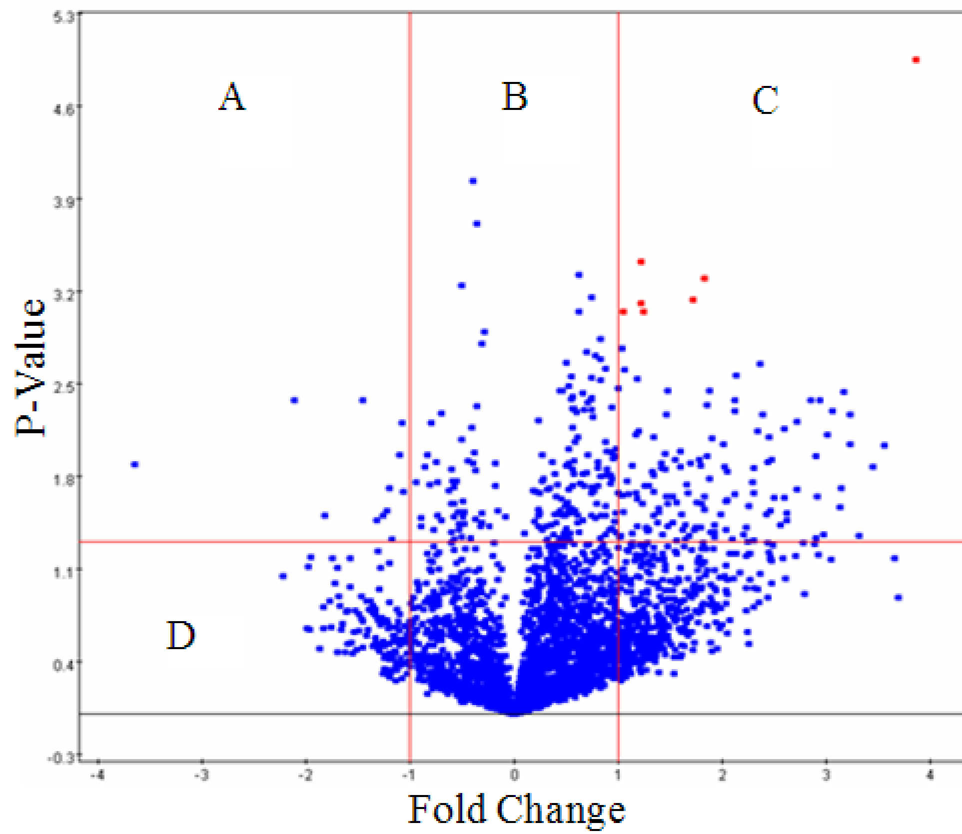

Figure 4.2 Volcano plot of D vs R

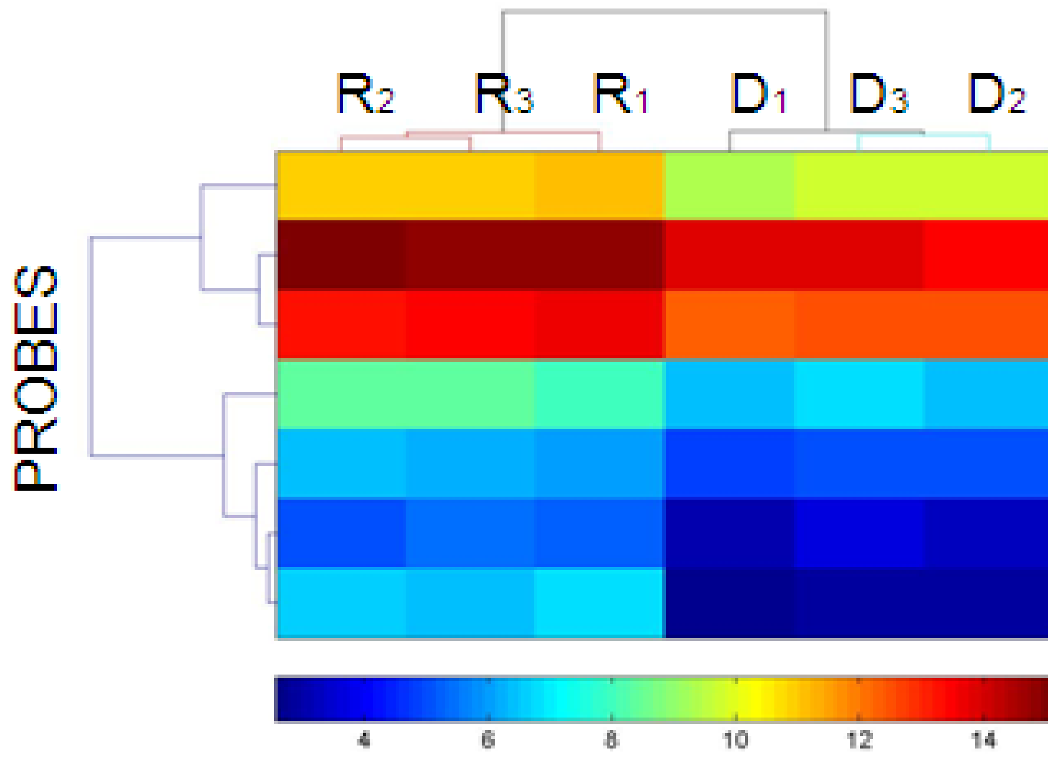

Figure 4.3 Heat Maps of Seven Significant Probes

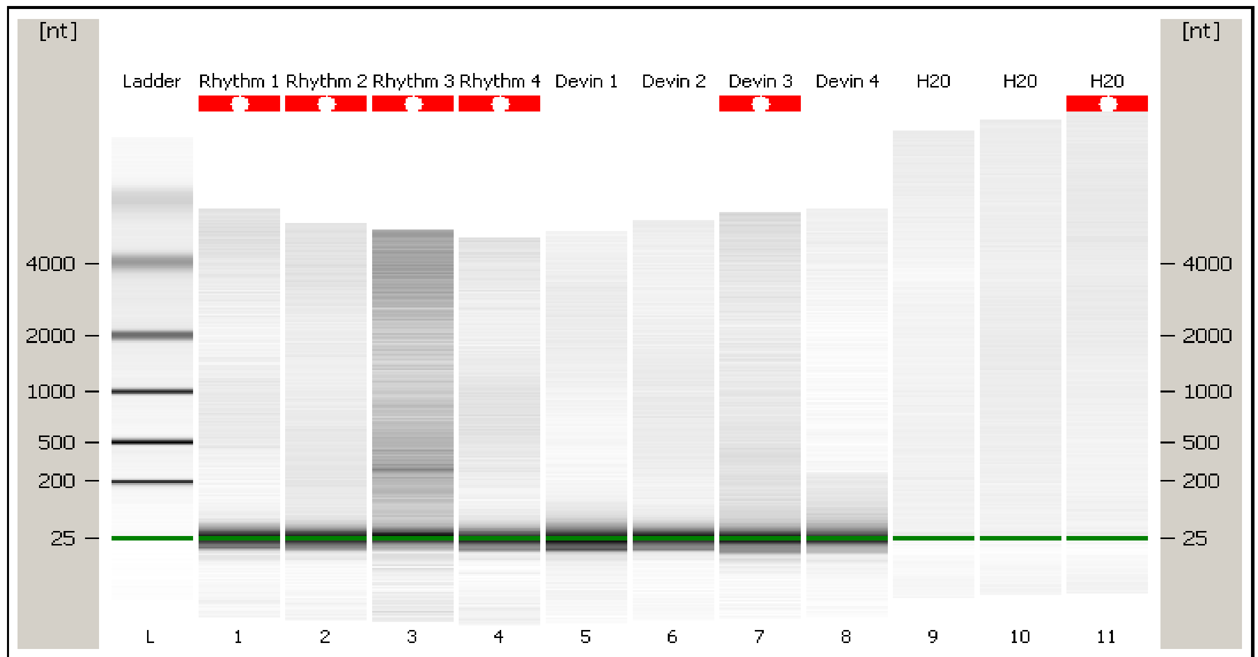

Figure 4.4 Bioanalyzer Gel

CHAPTER 5

CONCLUSIONS

RNA molecules have several functions including catalytic activity and the ability to act as structural components. There are also non-coding RNAs that play essential roles in biological systems without encoding proteins such as tRNA and rRNAs. Similarly, small RNAs do not encode proteins, rather they guide Argonaute proteins to target molecules, which leads to gene silencing.

The silencing mechanism of small RNAs has led to much research towards understanding different aspects of the small RNAs. The most well understood small RNAs are piRNAs, miRNAs, and siRNAs. Many studies have observed the importance of small RNAs, showing that without them there is chaos within the genome.

PiRNAs are one of the newest small RNAs that are being studied. They are mostly found in spermatogenic cells. The mechanism of piRNAs is Dicer-independent; whereas miRNAs and siRNAs are Dicer-dependent mechanisms. Studies have shown that miRNAs and siRNAs are very much alike. Various studies have shown that miRNAs play important roles in development. Without Dicer development and the formation of mature miRNAs, chromosomes fail to align properly. Furthermore, siRNA has been shown to play important roles in disease regulation. These small RNAs have

greatly impacted the RNA world and will continue to influence the way we view genomics.

Understanding the roles of spermatozoa miRNAs will aid in understanding the critical elements of fertilization. Spermatozoa do not only provide DNA but they also release enzymes and proteins that are vital for fertilization and embryonic development. Nevertheless, DNA contribution is very important for fertilization. A study was done to investigate DNA-damaged sperm in a bovine IVF model and to follow the consequences of introducing paternal damaged DNA into oocyte for embryonic development (Fatehi et al., 2006). They expected that the embryo damage will affect embryo development at the stage where paternal gene expression is initiated. The researchers saw that despite DNA damage the sperm cells were capable of fertilizing the oocyte as normal. However, embryonic development was completely blocked. This blockage occurred despite blastocyst formation. After the second and third cleavage, the embryonic cells began to undergo apoptosis which caused failure of blastocyst formation, nuclear fragmentation into apoptotic bodies, and failure in spindle formation (Fatehi et al., 2006).

In our study, we examined spermatozoa miRNA, which serve as critical elements in bovine fertilization. We suspected that miRNAs were present in bovine spermatozoa and they have critical roles in early embryogenesis. We compared the spermatozoa of high fertility and low fertility bulls. High fertility refers to those bulls that need more sperm to reach the maximum fertilization rate. The low fertility bulls are those bulls with fertilizing sperm present in abnormal ejaculates (Saacke, 2008).

We have found that miRNAs are abundant in the spermatozoa of bovine. However, only seven of theses miRNAs were significantly expressed in high and low

fertility bulls. We saw that they were more highly expressed in the low fertility bulls than the high fertility bull. Because there are no annotations, the miRNA functions are unknown. Nevertheless, the higher expression levels of the miRNA in the low fertility bulls could mean that miRNAs might be down regulating the expression of genes whose products play important roles in fertilization and early embryonic development.

Determining the function of miRNAs in bovine spermatozoa will aid in understanding exactly how miRNAs affect fertilization. In future studies, the miRNA will be used to develop a model for the biological roles of miRNAs in mammalian development. By doing this, we will shed light on ways to improve fertility, development, and reproduction.

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