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Identification and Characterization of MicroRNAs in Porcine Gametes and Pre-Implantation Embryos

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IDENTIFICATION AND CHARACTERIZATION OF MICRORNAS IN PORCINE GAMETES AND PRE-IMPLANTATION EMBRYOS

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ABSTRACT

MicroRNAs (miRNAs) are short ribonucleic acids that ultimately affect the production of proteins. Although miRNAs are involved in nearly every biological process examined to date, little is known of the identity or function of miRNA in porcine reproductive tissues or their potential involvement in reproductive processes in pigs or other species. The objective of this dissertation research was to determine the presence of miRNAs in porcine gametes and both *in vivo*- and *in vitro*- produced pre-implantation embryos and to identify differences in miRNA expression between normal and aberrant samples. Using a heterologous RT-PCR approach, we demonstrated the presence of a total of 92 miRNAs in porcine oocytes, spermatozoa, and/ or embryos at the 4-cell, 8-cell, 16-cell, and blastocyst stages, with hundreds more predicted by miRNA microarray. Subsequent qRT-PCR analysis showed differential expression of five miRNAs, let-7a, -7d, -7e, miR-15b, and -22, between normal sperm and morphologically abnormal sperm or sperm samples exhibiting low motility. Messenger RNA targets of the differentially expressed miRNAs encode proteins important for spermatogenesis, sperm structure, and/ or sperm cell metabolism. Differential expression was also shown among embryos at various stages in development, demonstrating a temporal expression pattern of specific miRNAs in pre-implantation embryo growth. More interestingly, miR-24 was differentially expressed between *in vivo*- and *in vitro*- produced embryos at the 8-cell and blastocyst stages, supporting the need to characterize aberrant miRNA expression associated with the abnormal embryonic development correlated with assisted reproductive technologies. All of the miRNAs examined demonstrated high sequence

similarity to the corresponding human miRNA sequences, indicative of high conservation among species. Understanding miRNA expression in reproductive processes is critical to comprehending the mechanistic roles miRNAs play in the regulation of all physiological processes.

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CHAPTER ONE

LITERATURE REVIEW

Overview

MicroRNAs (miRNAs) are short ribonucleic acids that ultimately affect the production of proteins by regulating translation of mRNA. Although miRNAs are involved in nearly every biological process examined to date, little is known of the identity or function of miRNA in porcine reproductive tissues or their potential involvement in reproductive processes. MiRNAs have been implicated in diverse physiological processes such as insulin secretion (Poy et al., 2004), adipocyte differentiation (Kajimoto et al., 2006; Pratt, 2010), alcohol tolerance (Pietrzykowski et al., 2008), and carcinogenesis (as reviewed by Cuellar et al., 2005). They have also been shown to play roles in reproductive processes such as oocyte maturation (Tesfaye et al., 2009), spermatogenesis (Maatouk et al., 2008), embryonic development (Houbaviy et al., 2003) and placenta formation (Cui et al., 2009).

Assisted reproduction techniques (ART), such as *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI), and somatic cell nuclear transfer (SCNT) frequently lead to aberrant gene expression and are implicated for the failure of the resulting embryos to establish and/or maintain pregnancy following transfer (Khosla et al., 2001; Vajta, 2007; Young et al., 1998). The underlying cause for decreased survival of *in vitro* produced embryos is unknown but it is highly possible that the expression of miRNAs is altered during procedures such ICSI, SCNT, and *in vitro* culture affecting the translation of specific messenger RNA and negatively impacting embryonic

development. The goal of this research was to identify miRNAs in porcine sperm samples of varying qualities and in both *in vivo* and *in vitro* produced porcine embryos at varying stages in pre-implantation development. Understanding miRNA expression is critical to comprehending the mechanistic roles miRNAs play in the regulation of reproductive processes.

History

Lee and her colleagues at Harvard University (1993) discovered the first miRNA, lin-4, while studying larval development in the nematode *C. elegans*. They knew that the ablation of lin-4 function caused aberrant stage progression and absence of adult structures (Chalfie et al., 1981) and that lin-4 was a negative regulator of lin-14, which encodes the protein LIN-14. Interestingly, as levels of lin-4 increased, protein levels of LIN-14 decreased, but mRNA transcript levels of lin-14 remained constant (Wightman et al., 1993), leading researchers to suspect a post-transcriptional method of regulation. They determined that the lin-4 RNA sequence did not encode a protein, rather, they identified two short, separate transcripts (the pre-miRNA and the mature miRNA sequence), both of which were complementary to the 3' untranslated region (UTR) of the lin-14 transcript. These results indicated that the translation of lin-14 was being repressed by an antisense mechanism.

Seven years following the discovery of lin-4, a second short, regulatory, miRNA was identified. Let-7 was found to direct the stages of *C. elegans* development in a mode similar to that of lin-4 (Reinhart et al., 2000). It was reported that let-7 was evolutionarily conserved from flies to humans, implicating a fundamental role for these genes in

animals (Lagos-Quintana et al., 2002; Pasquinelli et al., 2000). Interest in miRNAs mushroomed after several laboratories revealed the presence of hundreds of miRNAs in both plant and animal genomes (Bhat et al., 2005; Houbaviy et al., 2003; Lagos-Quintana et al., 2001; Lau et al., 2001; Lee et al., 2001; Lim et al., 2003; Reinhart et al., 2002).

Evolution and conservation

It is believed that most miRNA genes originated from gene duplication events (Maher et al., 2006), although incorporation of repetitive elements (Piriyapongsa et al., 2007), local duplication, and mutation may have lead to the origin of miRNAs as well (Bentwich et al., 2005; Zhang et al., 2007). Not only is the let-7 miRNA sequence conserved among species, but the acquisition of let-7 is believed to have been an essential step of evolution from lower metazoan to higher bilaterians (Pasquinelli et al., 2003). Recent studies have revealed instances of miRNA evolution corresponding with introductions of developmental complexity (Figure 1.1). Major miRNA acquisitions occur at branches leading to vertebrates, placental mammals (Hertel et al., 2006), and primates (Bentwich et al., 2005). It has also been observed that both flies and vertebrates have increased their numbers of cell types in correlation with the acquisition of their respective number of miRNAs (Sempere et al., 2006).

Figure 1.1. Evolutionary acquisition of miRNAs

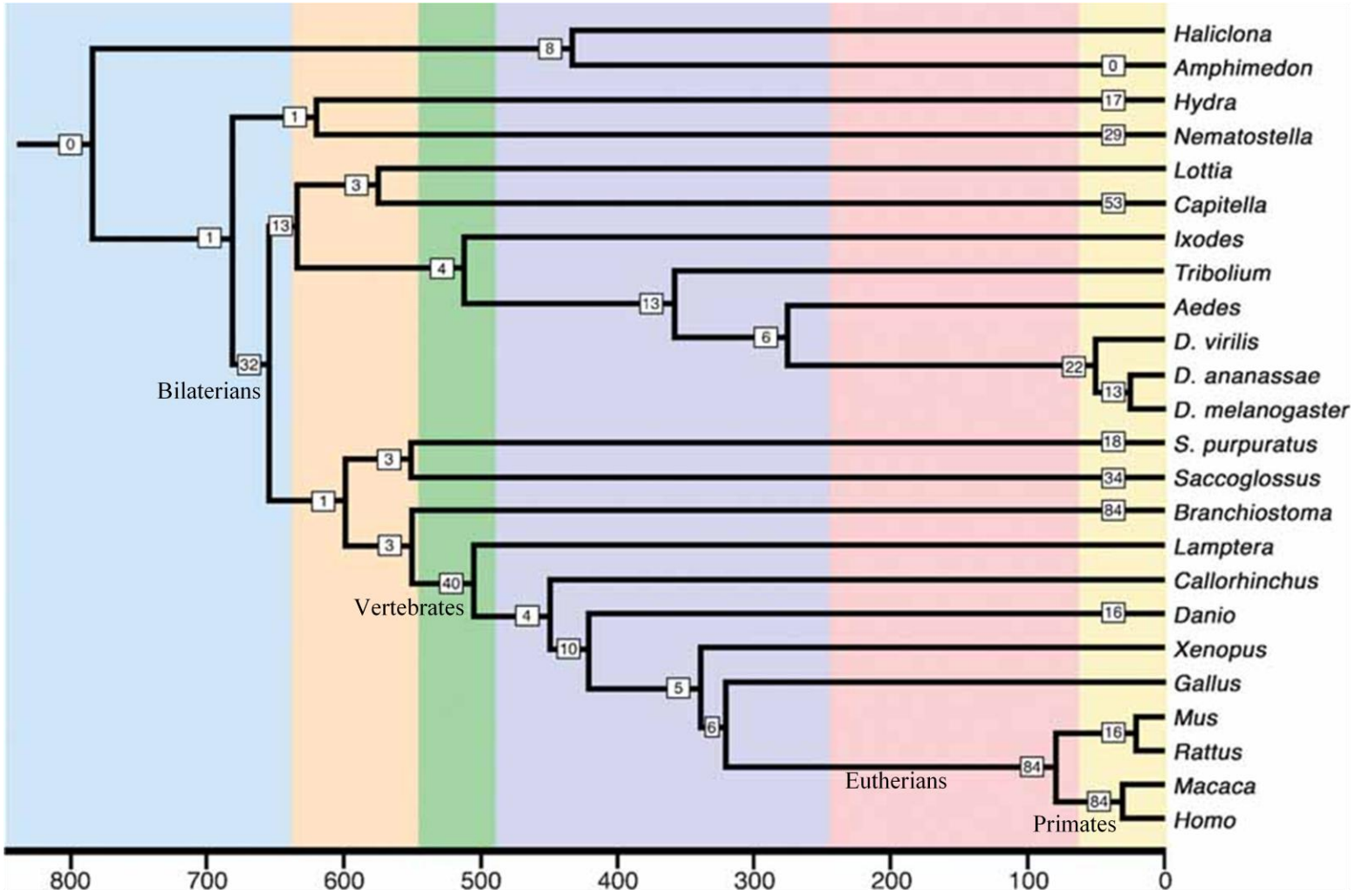

Figure 1.1. Acquisition of miRNAs. Each node is characterized by the addition of at least one new miRNA family and all metazoan lineages acquired at least one novel miRNA family (number of families gained are shown in nodes at each branch). The x-axis measures millions of years. There are at least four instances of a relatively high rate of miRNA family acquisition, one at the base of bilaterians, one at the base of the vertebrates, one at the base of eutherians, and one at the once at the base of primates (Adapted, with modifications, from Niwa et al., 2007; Peterson et al., 2009).

Synthesis and function

Biogenesis

Genes encoding miRNA tend to be highly conserved among species and recent estimates reveal that 60% of human protein-coding genes are under selective pressure to conserve miRNA target sites (Friedman et al., 2009). MiRNAs are estimated to comprise 1- 5% of animal genes (Lim et al., 2003) and can be located within the introns (Fujita et al., 2008; Rodriguez et al., 2004) or exons of mRNAs (as reviewed by Y. Zhao et al., 2007). It has been observed that although miRNA genes are, on average, more frequently located within the introns of long genes, their presence within introns of short genes occurs at a higher frequency than expected by chance (Golan et al., 2010). MiRNA genes tend to be clustered (Lagos-Quintana et al., 2001; Lau et al., 2001; Mourelatos et al., 2002) and may contain their own promoters and enhancers (Fujita et al., 2008).

In the nucleus, miRNAs are transcribed by RNA polymerase II (RNA pol II), creating primary miRNAs (pri-miRNA), which are several kilobases long (Borchert et al., 2006; Cai et al., 2004; Lee et al., 2002). Pri-miRNAs are then processed by a Microprocessor complex composed of the ribonuclease (RNase) III enzyme, Drosha, and an RNA binding protein, Pasha, into a ~70 nucleotide (nt) pre-miRNA (Denli et al., 2004; Gregory et al., 2004; Lee et al., 2003; Lee et al., 2002). The pre-miRNA possesses a 2 nt overhang on its

3’ end, which is recognized by Exportin-5, a GDP-dependent nuclear transmembrane protein which allows for its transport out of the nucleus (Lund et al., 2004; Yi et al., 2003).

In the cytoplasm, the pre-miRNA is processed by Dicer, an RNase III enzyme and the trans-activator RNA (tar)-binding protein (TRBP) in mammals (Haase et al., 2005). Dicer cleaves the pre-miRNA into a ~19- 24 bp double-stranded miRNA (ds-miRNA), of which one strand is the guide strand (the strand with the weakest 5’-end base pairing (Tomari et al., 2004)) and the other is the passenger strand. The ds-miRNA is loaded into a ribonuclear particle (RNP) complex, the RNA-induced silencing complex (RISC), which is a group of proteins including Argonaute2 (Ago2), which cleaves the passenger strand, discarding it (Matranga et al., 2005), and presents the mature miRNA to its mRNA target (Faller et al., 2008).

Figure 1.2. Synthesis of mature miRNAs

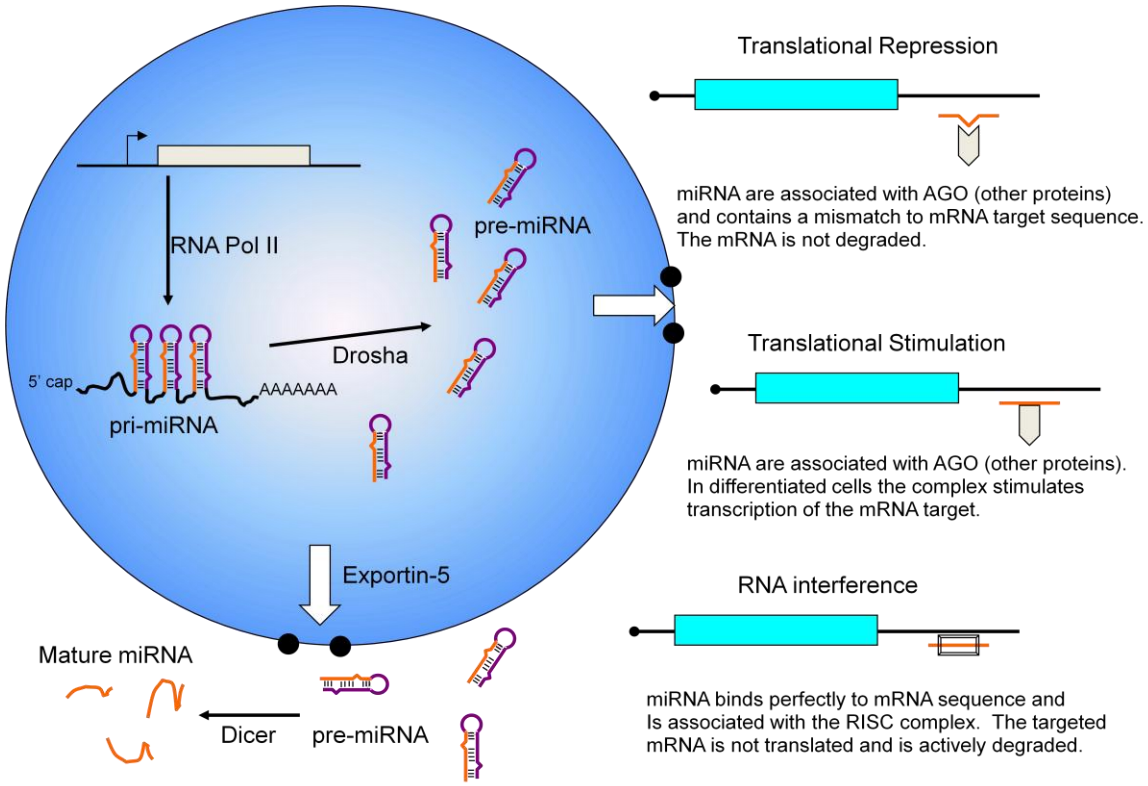

Figure 1.2. Pri-miRNA transcripts are processed into pre-miRNAs by an enzymatic complex that includes the nuclear RNase III enzyme Drosha. The resulting pre-miRNA is transported to the cytoplasm by Exportin-5. Once in the cytoplasm, pre-miRNAs are processed into ~22-nucleotide duplexes by Dicer in association with TRBP. The strand corresponding to the mature miRNA is subsequently loaded onto the RISC. Mature miRNAs bind the 3'-untranslated region of target mRNAs and subsequently destabilize them, block their translation, or, in rare instances, stimulate translation (Tili et al., 2008). (Figure created by Dr. Scott Pratt and used with permission.)

Naming

The Sanger miRBase Registry is an independent intermediary providing miRNA sequence data, annotation, and predicted gene targets (Griffiths-Jones, 2004; Griffiths-Jones et al., 2006). MiRNAs may be submitted to the database after they are confirmed via sequencing or a manuscript depicting their discovery is accepted for publication. The first prefix of the miRNA name denotes the organism (ex. hsa= *homo sapiens*; ssc= *sus scrofa*; mmu= *mus musculus*, etc), while the second indicates whether it is a mature miRNA (i.e. miR) or a hairpin precursor (i.e. mir). The identifiers are assigned in sequential numerical order. Orthologous miRNAs are assigned the same numerical identifier (ex. hsa-miR-16 and ssc-miR-16) while paralogous sequences, those that only differ by one or two nucleotides, are appointed letter suffixes (i.e. mmu-miR-19a and mmu-miR-19b). When two miRNAs result from different arms of the same hairpin precursor, the suffix '-3p' or '-5p' is added to indicate from which arm the mature miRNA is excised. When two or more separate hairpins generate identical miRNAs, a numbered suffix is added (i.e. ssc-miR-105-1 and ssc-miR-105-2). An asterisk indicates a miRNA biogenesis by-product, such as the complementary non-miRNA arm of the hairpin precursor. Exceptions to the standard miRNA annotation rules are the *let* and *lin* families of miRNAs, in which the names were assigned based on their functions, prior to the adoption of the current naming system.

Function

Generally, miRNAs regulate the translation of their mRNA targets at the post-transcriptional level. When a miRNA binds to its mRNA target with perfect

complementarity, mRNA cleavage occurs (Bagga et al., 2005). This is the most common mechanism of miRNA action in plants, but rarely occurs in animals (for exceptions, see Yekta et al., 2004). Animal miRNAs imperfectly bind to their mRNA targets, thereby inducing translational inhibition or repression (Bartel, 2004).

Previously, it was expected that miRNAs were capable only of down-regulating protein production by inhibiting the translation of their mRNA targets, but the expression and function of miRNAs is more complex than originally assumed. Another mechanism for the down-regulation of gene expression by miRNAs was proposed by Wu et al. (2006) who showed that miR-125b and let-7 imperfectly bind to targets in the 3' UTR and reduce mRNA levels by expediting the deadenylation of the poly(A) tail, which leads to mRNA decay. These results were supported when Wakiyama et al. (2007) established that let-7 recruits micro-ribonucleoprotein (microRNP) complexes containing Ago to direct the deadenylation of target messenger RNAs. In Arabidopsis, there is evidence that miRNA interacts with newly transcribed messenger RNA to alter the chromatin state of corresponding mRNA template DNA, affecting methylation of downstream coding sequences (Bao et al., 2004); however, its effects were not determined, nor was this validated in animals.

To complicate issues, miRNAs may additionally up-regulate the production of proteins through different mechanisms. Using serum-starved cells, Vasudevan et al. (2007) demonstrated that miR-369-3p up-regulated the translation of tumor necrosis factor alpha (TNFα) when Ago2 and fragile-X-mental retardation related protein 1 (FXR1) were associated with AU-rich elements (ARE) in the 3'UTR of TNFα mRNA.

They found that, during cell cycle arrest, the ARE were transformed into a translation activation signal that recruited factors associated with the miRNA machinery, microRNPs. Next, they showed that let-7 oscillated between translational repression and activation of its target, high-mobility group AT-hook 2 (HMGA2), in synchronization with the cell cycle. The exact mechanism is unknown and the authors suggested that miRNA translational repression is a property of proliferating cells.

Place et al. (2008) identified target sites for miR-373 within the promoters of E-cadherin and cold-shock domain-containing protein C2. Transfection of pre-miR-373 and miR-373 induced the expression of both proteins and also increased RNA pol II at their transcription start sites. Orom et al. (2008) discovered that, while miR-10a caused translational repression upon binding to its target in the 3' UTR of Ras-related nuclear protein (Ran) and Penicillin-binding protein 1 (Pbp1), it also bound to the 5' UTR of mRNA encoding ribosomal proteins and enhanced their translation during amino acid starvation. Although the precise process is unknown, the authors hypothesized that miR-10a competes with an inhibitory factor that binds downstream from the 5' regulatory motif.

Another study suggested an epigenetic role of miRNAs in embryonic development. Grandjean et al. (2009) noticed that the microinjection of miR-124 into mouse zygotes resulted in increased embryonic growth rates and pups exhibiting a 'giant' phenotype, which was heritable over several generations. qRT-PCR showed that, following microinjection, miR-124 levels quickly returned to the basal level of the controls, but Sox9 (SRY (sex determining region Y)-box 9), which has high sequence homology to

miR-124, showed a significant increase in the microinjected embryos. Sox9 is a transcription factor known for its role in embryo growth and proliferation of various organs. The researchers theorized that exposure of embryos to miR-124 resulted in a change to the chromatin structure of the Sox9 promoter.

Other small RNAs

Aside from miRNAs, there are other classes of small, non-coding RNAs in mammals which are due mention (Figure 1.3). Small nuclear RNA (snRNA) are associated with a protein complex called a small nuclear ribonucleoprotein (snRNP or "snurps") complex that are involved in RNA splicing and telomere maintenance. Small nucleolar RNAs (snoRNAs) are found in the nucleus and Cajal bodies and have a role in RNA synthesis by guiding modifications of rRNAs and tRNAs. Short, interfering RNA (siRNA) are 20-25 bp double-stranded RNA involved in RNA interference. siRNAs originate from long exogenous or endogenous dsRNA molecules, while miRNAs are synthesized from endogenous transcripts that form local hairpin structures. Piwi-interacting RNA (piRNA) is a large class of small RNAs that form interactions with Piwi proteins. The functions and biogenesis of piRNAs are still being elucidated but they have been shown to be testes-specific in mammals (Houwing et al., 2007), generating much interest in their potential roles in spermatogenesis (Aravin et al., 2006).

Figure 1.3. Classes of mammalian RNA

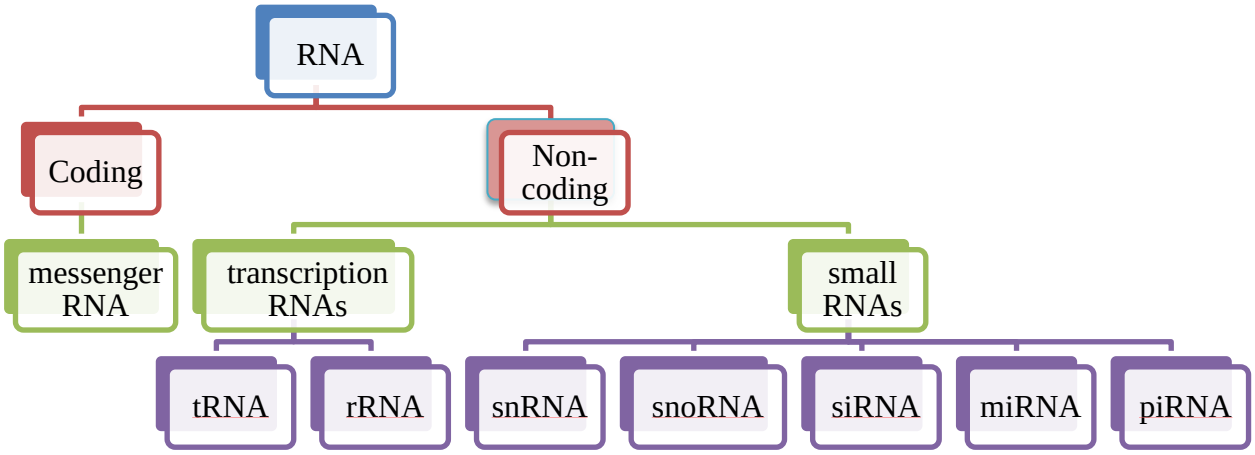

Figure 1.3 shows the different groups of coding and non-coding RNA present in mammals (adapted, with modifications, from Buckingham, 2003).

Target prediction

Unlike plant miRNAs, which bind with perfect complementary to their mRNA targets, animal miRNAs are not completely complementary, rather animal miRNAs contain a seed sequence which must bind to the mRNA target. The seed sequence (Figure 1.4) usually encompasses bases 2- 7 from the miRNA 5' end (Lewis et al., 2005). Bulges are frequently noted between positions 9 and 14 and may be essential for miRNA function or target recognition (Brennecke et al., 2003; Johnston et al., 2003; Vella et al., 2004). The seed region was first identified using bioinformatic analysis as the only consistent region of miRNAs that retrieved more evolutionarily conserved complementary target sites than expected by chance (Lewis et al., 2003). Introduction of mutations into the seed region of a presumed miRNA-mRNA duplex may provide experimental target site validation.

Figure 1.4. Binding of a miRNA molecule to its messenger RNA target

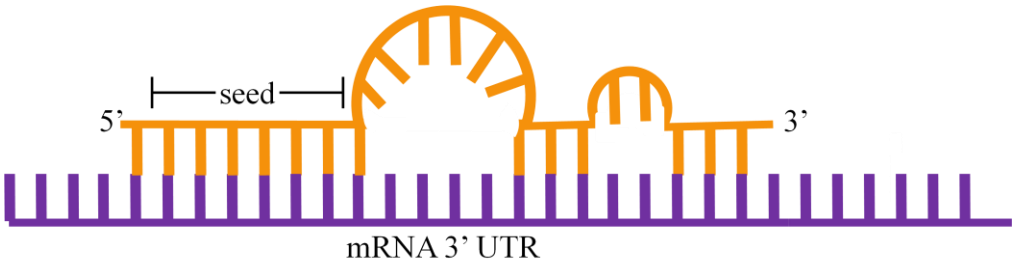

Figure 1.4 illustrates the complementary binding of a miRNA's seed sequence to its mRNA target, while the non-seed region may contain mismatches and bulges.

Not surprisingly, it has been experimentally validated that some miRNAs use non-seed sites in addition to seed-type target sites (Ha et al., 1996; Reinhart et al., 2000) and that GU wobble pairs in the seed region may be tolerated (Didiano et al., 2006; Johnston et al., 2003). A human cytomegalovirus has been shown to generate a miRNA that uses non-seed targeting to repress a major histocompatibility complex-related gene, thus evading destruction by the host's immune system (Stern-Ginossar et al., 2007). Further explorations to illuminate the complexities of mRNA target recognition by miRNAs are warranted.

A single gene may be targeted by multiple miRNAs (Ambros, 2003; Reinhart et al., 2000; Vella et al., 2004), of which some target sites may overlap (Doench et al., 2004), and a single miRNA may target multiple genes. Target sequences may be conserved or non-conserved and those genes with non-conserved target sites tend to be expressed in tissues lacking the corresponding miRNA, i.e- conserved sites are generally present in genes that are co-expressed with the miRNAs by which they are targeted (Farh et al., 2005). Mammalian-specific miRNAs have fewer conserved targets than those miRNAs conserved more broadly (Friedman et al., 2009).

TargetScan was the first algorithm developed to identify targets of vertebrate miRNAs (Lewis et al., 2003) and combines thermodynamics-based modeling of RNA- RNA interactions with comparative sequence analysis to predict phylogenetically conserved matches between miRNA seed sequences and 3'UTRs. Results are returned as a ranking by the number of predicted target sites present on each 3' UTR. Many other target prediction servers and databases have been developed, a few of which are described here: miRanda (John et al., 2004) is based on the alignment of miRNAs with their projected targets, with scores based upon complementary pairing between the seed region and the mRNA target and a calculation of binding energy; no targets without a perfect seed match will be found. miRBase (Grun et al., 2005) and microRNA.org both utilize the miRanda algorithm with modifications. miRBase allows predictions for all species in Ensemble (www.ensembl.org) and provides a P-value for each predicted interaction, whereas microRNA.org does not require a perfect seed match, but does not provide P-values. PicTar (Krek et al., 2005) calculates the hybridization energy between the whole miRNA and the mRNA target and the likelihood that a transcript is regulated by two or more miRNAs in combination. It cannot find targets without perfect seed match. RNAhybrid (Rehmsmeier et al., 2004) determines the lowest free energy hybridization between two RNA molecules (i.e. most stable binding site of a miRNA on a mRNA) and allows parameters to be set by the user. DIANA-microT (Maragkakis et al., 2009) is an algorithm based on several parameters calculated individually for each microRNA and it combines conserved and non-conserved microRNA recognition elements into a final prediction score. The MAMI server and database (meta mir: target inference;

http://mami.med.harvard.edu/) is unique in that it provides a composite prediction of target genes from five independent target prediction tools: TargetScan, miRanda, DIANA-microT, miRtarget, and picTar. MAMI also accommodates miRNA lists, rather than a single miRNA search, and allows for sensitivity and specificity to be predefined by the user.

Regulation of miRNA expression

The mechanisms underlying miRNA regulation are still being elucidated. Microarray profiling studies have shown that expression patterns of some miRNAs can be attributed to regulatory sequences in their promoters (Barad et al., 2004; Calin et al., 2004; Liu et al., 2004; Sempere et al., 2004). MiRNAs located within the introns of their host genes can be transcribed along with their host and will exhibit the same patterns of expression (Bartel, 2004; Rodriguez et al., 2004). Fontana et al. (2007) demonstrated a negative feedback loop of miRNA action, in which miRNAs 17-5p, -20a, and -106a down-regulate the translation of acute myeloid leukemia-1 (AML1) which, in turn, binds the promoters of these miRNAs, inhibiting their transcription. Kedde et al. (2007) showed that an RNA-binding protein, dead end 1 (Dnd1) binds to miRNA target sites thereby preventing miRNA binding.

The first indication that hormones may regulate miRNA expression was elucidated by Sempere et al. (2002; Sempere et al., 2003), who showed that, in *Drosophila*, the steroid hormone ecdysone (20-hydroxyecdysone), along with the ecdysone-inducible gene Broad-Complex, is required for the expression of let-7. Bethke et al. (2009) demonstrated a hormone-mediated regulatory mechanism of let-7a in *C. elegans*. The nuclear receptor

DAF-12 regulates developmental progression in response to the environment. In favorable environments, steroid ligands bind to the DAF-12 nuclear receptor, initiating development into the next larval stage. In unfavorable conditions, ligands were suppressed and DAF-12 repressed miRNA expression which led to developmental arrest. Estrogen and the estrogen receptor, ERα, have also been shown to play an elaborate role in miRNA activity, as they can be both mediators of miRNA transcription (Cohen et al., 2008; Lowery et al., 2009; Macias et al., 2009; Maillot et al., 2009) and may be regulated by miRNAs (Adams et al., 2007; Castellano et al., 2009).

Obernosterer (2006) discovered that miRNA expression can also be regulated post-transcriptionally and in a tissue-specific manner. The researchers studied miR-138, which is considered to be a brain-specific miRNA. Surprisingly, they found that the miR-138 precursor was present in all tissues and cells analyzed, while the mature form was found in only the cerebrum, cerebellum, and midbrain, as expected. They hypothesized that the export of pre-miR-138 from the nucleus was impaired in all tissues except the brain, which would prevent it from processing by Dicer; however, northern blot analysis of cytoplasmic RNA showed that pre-miR-138 is effectively transported to the cytoplasm. The authors then tested an activator model in which an activating agent produced only in the brain allowed for pre-miR-138 processing. This theory was dismissed through the observation that a recombinant Dicer protein was still able to process pre-miR-138 *in vitro*. Finally, the group added increasing amounts of cytoplasmic extracts which abolished processing of pre-miR-138 by Dicer. This observation led researchers to favor the presence of an inhibitory factor which binds miR-138, thereby preventing its

processing by Dicer. The processing of other miRNAs was unaffected by titrating increasing amounts of cytoplasmic extracts. Following Obernosterer's discovery, other groups also identified and confirmed examples of post-transcriptional regulation of miRNAs (Thomson et al., 2006; Viswanathan et al., 2008; Wulczyn et al., 2007; Zhang et al., 2009).

At least two studies have shown that components of developmental signaling pathways may control miRNA expression. Sweetman et al. (2006) showed that fibroblast growth factor- mediated signaling negatively regulated the transcription of miR-206 in chickens. Other researchers showed that Oct4 and Sox2, transcription factors required for pluripotency, bind to the promoter region of miR-302, a cluster of miRNAs specifically expressed in embryonic stem cells (ESCs) and pluripotent cells. MiR-302a was shown to repress the translation of cyclin D1, an important G1 regulator (Card et al., 2008).

Roles in reproduction

Testicular and sperm miRNAs

Studies have shown that, in humans, the amount of total RNA in normal spermatozoa is greater than the amount of RNA in non-motile sperm (Roudebush et al., 2004) and less than the amount of RNA in morphologically abnormal sperm (Wild et al., 2000). Ostermeier et al. (2005a) suggested that stable RNAs could be useful for male infertility prognosis and specific male infertility factors may be identified using genomic profiling of spermatozoa. Ostermeier et al. (2002) also proposed that spermatozoa mRNA profiling could be used to generate genetic fingerprint of normal, fertile men. Miller et al. (1994) suggested that spermatozoa mRNAs are remnants of untranslated stores, "providing a

historic record or fingerprint of spermatogenesis". The literature described indicates that sperm RNA, likely to include miRNAs, may contribute to spermatogenesis, sperm fertilization capacity, and/ or early embryonic development. It is evident that miRNA are involved in the production of sperm and that their overabundance or absence in mature sperm could be indicative of aberrant development, function and/or fertility.

Review of spermatogenesis

Mammalian spermatogenesis is the process of the production of mature spermatozoa from spermatogonial cells and can be divided into spermatocytogenesis and spermiogenesis (Figure 1.5). During spermatocytogenesis, mitotic divisions allow spermatogonial renewal while meiotic divisions give rise to primary spermatocytes, secondary spermatocytes, and finally, haploid spermatids. Following meiosis, histones are replaced by transition proteins, which are later replaced by protamines, allowing for chromatin compaction. Spermiogenesis involves morphological changes such as nuclear condensation, acrosome formation, cytoplasmic reorganization, and development of flagella. It is generally agreed that sperm cells are transcriptionally silent due to their tight chromatin compaction, so any RNA (or miRNA) present is likely a result of spermatogenesis, supporting the statements of Miller et al. (1994). In the mouse, transcription ceases at the transition from round to elongating spermatids, before the completion of spermiogenesis (as reviewed by Braun, 1998). In haploid germ cells, approximately two thirds of messenger RNAs are stored in mRNA ribonucleoprotein particles (mRNPs), which are translationally inactive (Kleene, 1993; Schmidt et al.,

1999). Various genes and proteins have been identified as molecular markers of sperm fertility (Muratori et al., 2009), alluding to much opportunity for miRNA regulation.

Another class of small RNAs has also recently attracted attention for its potential role in spermatogenesis. Piwi-interacting RNAs (piRNAs) are slightly larger than miRNAs (~25- 35 nt) and exhibit both tissue-restricted and abundant expression in the mammalian testis (Houwing et al., 2007; Kim, 2006). piRNAs associate with members of the Piwi (P-element wimpy testis-induced) protein family (Aravin et al., 2006; Girard et al., 2006).The Piwi proteins are a subfamily of the Argonaute proteins and it has been shown that ablating specific members of the Piwi family results in a block in spermatogenesis at different arrest points depending on which member has been ablated (Deng et al., 2002; Kuramochi-Miyagawa et al., 2004). Both the biogenesis of piRNAs and their exact function remain to be elucidated. It has been estimated that approximately one million piRNA molecules exist per spermatocyte or round spermatid (Aravin et al., 2006).

Figure 1.5. Mammalian spermatogenesis.

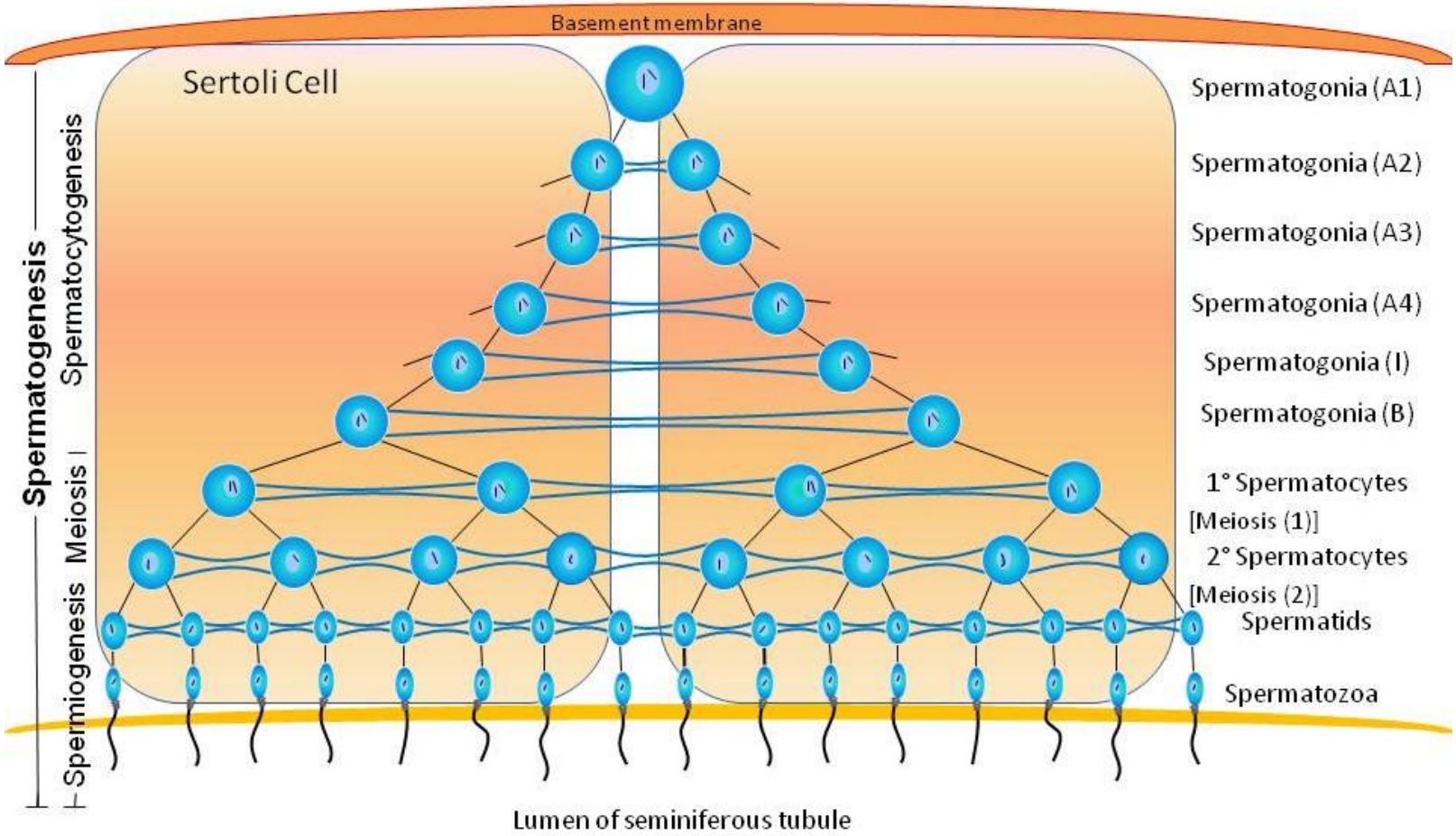

Figure 1.5 illustrates the process of spermatogenesis, the production of mature haploid spermatozoa from diploid spermatogonial cells. Spermatogenesis is divided into two phases: spermatocytogenesis and spermiogenesis.

Testicular miRNAs

There is compelling evidence that miRNAs are imperative for spermatogenesis. Studies have demonstrated that testicular miRNA profiles change during development and puberty (Barad et al., 2004; Yan et al., 2009; Yu et al., 2005). Barad et al. (2004) showed that miR-34b expression is significantly higher in adult mouse testis than in prepubertal mouse testis, implicating a possible role of miRNAs in the differentiation of male germ cells. Using a cloning method, Ro et al. (2007a) identified 141 miRNAs in mouse testis, six of which were testis-specific. Mice lacking Dicer1 have morphologically abnormal elongating spermatids with low motility and are usually infertile (Maatouk et al., 2008), indicating that Dicer1 and miRNA play crucial role in spermatogenesis. Lian et al. (2009) found differential miRNA expression profiles of testes from patients with non-obstructive azoospermia versus normal controls: 154 were down-regulated while 19 were over-expressed, again suggesting a role of miRNAs in regulating spermatogenesis.

Using prepubertal and adult mouse testis, Yu et al. (2005) showed that miR-122a targets mRNA levels of transition protein 2 (Tnp2), a nuclear protein that is synthesized only in round spermatids and stored for translation toward the end of spermatogenesis. They demonstrated that miR-122a reduced the levels of Tnp2 messenger RNA activity (via luciferase assay). This observation suggests a role of miR-122a in the regulation of the expression of proteins which are required for the chromatin condensation process

occurring in the post-meiotic germ cells. The same study also demonstrated that testicular miRNAs show different developmental patterns of expression. Yan et al. (2007) found that 14 miRNA were up-regulated and 5 were down-regulated in immature mouse testis compared to adult testis. Using microarray profiling, Yan et al. (2009), discovered differences in the expression of 26 miRNAs in immature vs. mature rhesus testis samples, some of which are predicted to target genes involved in spermatogenesis.

Sperm miRNAs

An unabridged review of miRNAs identified in the male gamete is a short one. Ostermeier (2005b) used a microarray system to identify 68 small RNAs in human spermatozoa, at least one of which was a known human miRNA (miR-182). Kotaja et al. (2006) used *in situ* hybridization to demonstrate the presence of four miRNAs plus Dicer and Ago2 in the chromatoid bodies of murine haploid germ cells. Amanai et al. (2006) and Yan et al. (2008) detected miRNAs (n= 54 and n= 28, respectively) in mouse sperm using PCR methods, although Amanai and colleagues predicted a few hundred more via miRNA microarrays.

Amanai et al. (2006) injected mature mouse sperm with miRNA inhibitors against five endogenous sperm miRNAs and allowed them to fertilize via IVF. They found no effects of inhibiting these five miRNAs (miR-16, -30c, -145, -191, and -222) on fertilization capacity or early embryonic development. Interestingly, miR-16 initially was reduced but then showed increased levels after 24 h, possibly suggesting *de novo* miR-16 gene transcription or precursor processing in the mature sperm.

Role of sperm RNA in fertilization and embryonic development

Although sperm messenger RNAs previously have been thought to play a negligible role in fertilization and early embryonic development, both paternal messenger RNAs and miRNAs are delivered to the oocyte at fertilization (Amanai et al., 2006; Ostermeier et al., 2004). There is also evidence that sperm messenger RNA can affect the phenotype of the resulting offspring. Rassoulzadegan et al. (2006) reported that sperm from male mice carrying a *Kit* mutation can deliver the messenger RNA transcripts derived from the mutant *Kit* allele into the oocytes during fertilization, causing the offspring to display a mutant white spot phenotype. If sperm messenger RNA can affect the resulting offspring, it is conceivable that sperm miRNA may influence embryo physiology as well. McCallie et al. (2010) detected aberrant embryo miRNA expression from human blastocysts generated from patients with male factor infertility compared to blastocysts produced from normal control males, although the authors did not examine the miRNA profiles of the sperm used.

Ovarian miRNAs

Addressing female physiology, many studies have examined the expression and influence of miRNAs in ovary, with recent interest in the role of miRNAs in ovarian cancer (Bendoraite et al., 2010; Dahiya et al., 2008; Shen et al., 2010). In 2006, a computational analysis of the pig genome predicted 58 miRNAs and northern blot analysis confirmed the expression of two (miRs-31 and -92) within the porcine ovary (Kim et al., 2006). Ro et al. (2007b) used a cloning technique to identify miRNAs expressed in the ovaries of 2 wk old and adult mice, discovering a total of 122 miRNAs

from whole ovaries. Choi et al. (2007) examined miRNA expression levels in the newborn mouse ovary and the effects of knocking down the Nobox transcription factor required for oocyte differentiation and survival. One hundred seventy-seven miRNAs were identified in the newborn ovary and four were found to decrease ~2 fold in the Nobox-/- ovaries (let-7d, miR-346, -699, and -801). In all of these studies, whole ovarian tissue was used and the stage of the estrous cycle of the adult ovaries was unknown.

Recent studies demonstrated that Dicer1 is required for normal ovarian function (Otsuka et al., 2007; Otsuka et al., 2008). A murine Dicer1 hypomorph (*Dicer1d/d*) was constructed, which resulted in a ~75% loss of Dicer1 messenger RNA levels. Transplantation of wild type ovaries into *Dicer1d/d* females resulted in live offspring, but wild type females transplanted with *Dicer1d/d* ovaries failed to establish pregnancies, indicating that the fertility defect was inherent to the ovary. Further analysis of *Dicer1d/d* mice showed that the mice ovulated normally and the ova were fertilized and continued to undergo the first embryonic cell division. The researchers then examined the vasculature of the corpus luteum (CL) in *Dicer1d/d* mice and found a decrease in the amount and length of the blood vessels, which correlated with the upregulation of tissue inhibitor of metalloproteinases 1 (Timp1), an anti-angiogenic factor. MiRNA-17-5p and let-7 regulate Timp1 expression and their loss in *Dicer1d/d* mice was hypothesized to cause the reduction in angiogenesis. Knockdown of miR-17-5p and let-7 in wild type mice reduced CL angiogenesis and decreased serum progesterone levels. Injection of miR-17-5p and let-7 into the ovarian bursa of the Dicer deficient mice restored CL angiogenesis, increasing the level of progesterone; however, subsequent pregnancies

were not maintained, indicating that other ovarian miRNAs play a crucial role (Yang et al., 2005).

Toloubeydokhti et al. (2008) correlated the expression of specific miRNAs to the expression levels of their target messenger RNA in follicular cells collected from women undergoing ovarian stimulations to overcome fertility problems. The researchers examined miRNAs involved in the estrogen (E_2) biosynthesis pathway, miRs-17, -211, -542, and -23b, along with their respective targets: steroidogenic acute regulatory protein (StAR), cytokine IL-1b, Cox-2, and aromatase (CYP19A1). They found that the expression levels of miRs-17, -211, and -542 were inversely correlated to the messenger RNA expression levels of StAR, IL-1b, and Cox-2 while a higher expression of miR-23b was directly correlated to higher CYP19A1 messenger RNA expression levels when compared to healthy controls undergoing ovarian stimulation. This study did not measure proteins encoded by the messenger RNA of interest.

In vitro studies of ovarian cell cultures have shown differences in miRNA expression in response to hormonal treatment. Fiedler et al. (2008) treated murine granulosa cells with human chorionic gonadotropin (hCG) and found that thirteen miRNAs were differentially expressed. Three were up-regulated and ten were down-regulated between 0 h and 4 h post-hCG. MiR-132, which has been shown to be transcriptionally regulated by cAMP (Vo et al., 2005) was up-regulated. Interestingly, miR-132 has been shown to post-transcriptionally regulate co-repressor C-terminal binding protein (Klein et al., 2007), a protein recently exhibiting the ability, along with steroidogenic factor-1, to regulate adrenal steroidogenesis (Dammer et al., 2008).

Because miRNAs play a vital role in cell differentiation events, it is likely that defects in the regulatory control of specific miRNAs can result in abnormal folliculogenesis, cystic ovaries due to an anovulatory state, and pregnancy loss due to CL insufficiency. While it has been demonstrated that miRNAs show altered expression in ovarian cancer and other non-malignant pathologies, the normal ovarian miRNA profile throughout the estrous [or menstrual] cycle has not been described. It is highly likely that the changes in cell proliferation, hormone receptor expression, apoptosis, and steroidogenesis that occur on and within the ovary throughout the estrous cycle are caused, in part, by post-transcriptional gene regulation.

Oocyte miRNAs

Whereas spermatozoa are considered to be transcriptionally dormant, immature oocytes exhibit a high level of mRNA production, crucial for the production of proteins required for maturation of the oocyte and support of the early embryo (Wassarman et al., 1992). A high rate of transcription also creates the prospect for a high degree of transcriptional regulation. Tang et al. (2007) found dynamic changes in miRNA expression in immature versus mature murine oocytes. They next deleted Dicer from maturing oocytes and observed that miRNA biogenesis was blocked, a finding which was further investigated by Murchison et al. (2007), who demonstrated that Dicer is required for meiotic spindle integrity and completion of meiosis I. Aside from mice, miRNAs have been identified in the mature and immature oocytes of a few other species, including bovine (Tesfaye et al., 2009; Tripurani et al., 2010), *Drosophila* (Nakahara et al., 2005),

and *Xenopus* (Watanabe et al., 2005). To date, no studies have investigated the presence of miRNAs in porcine oocytes.

Embryonic miRNAs

Bernstein et al. (2003) demonstrated that the ablation of *Dicer1* in mice was embryonic lethal, suggesting a critical role for miRNA in early embryonic development. Houbaviy et al. (2003) identified embryonic stem (ES) cell-specific miRNAs, a cluster that was not detected in differentiated ES cells or adult tissues. Mineno et al. (2006) detected 390 miRNAs in mouse embryos using massively parallel signature sequencing (deep sequencing) and also showed temporal expression profiles of specific miRNAs. Using high throughput pyrosequencing, it has been estimated that there are 110,000 miRNA transcripts per murine embryonic stem cell (Calabrese et al., 2007). Tesfaye et al. (2009) investigated the expression patterns of six miRNAs during bovine pre-implantation development. Using pools of 10- 100 *in vitro* produced embryos from oocyte to blastocysts stages, qRT-PCR results showed highly variable trends in miRNA expression (Figure 1.6). Giraldez (2006) showed that zebrafish miR-430 not only regulates several hundred mRNAs, but also accelerates the deadenylation and clearance of maternal mRNAs during the shift to zygotic transcription. This has not yet been reported in any mammalian system.

Figure 1.6. Expression profiles of six miRNAs during early bovine embryo development

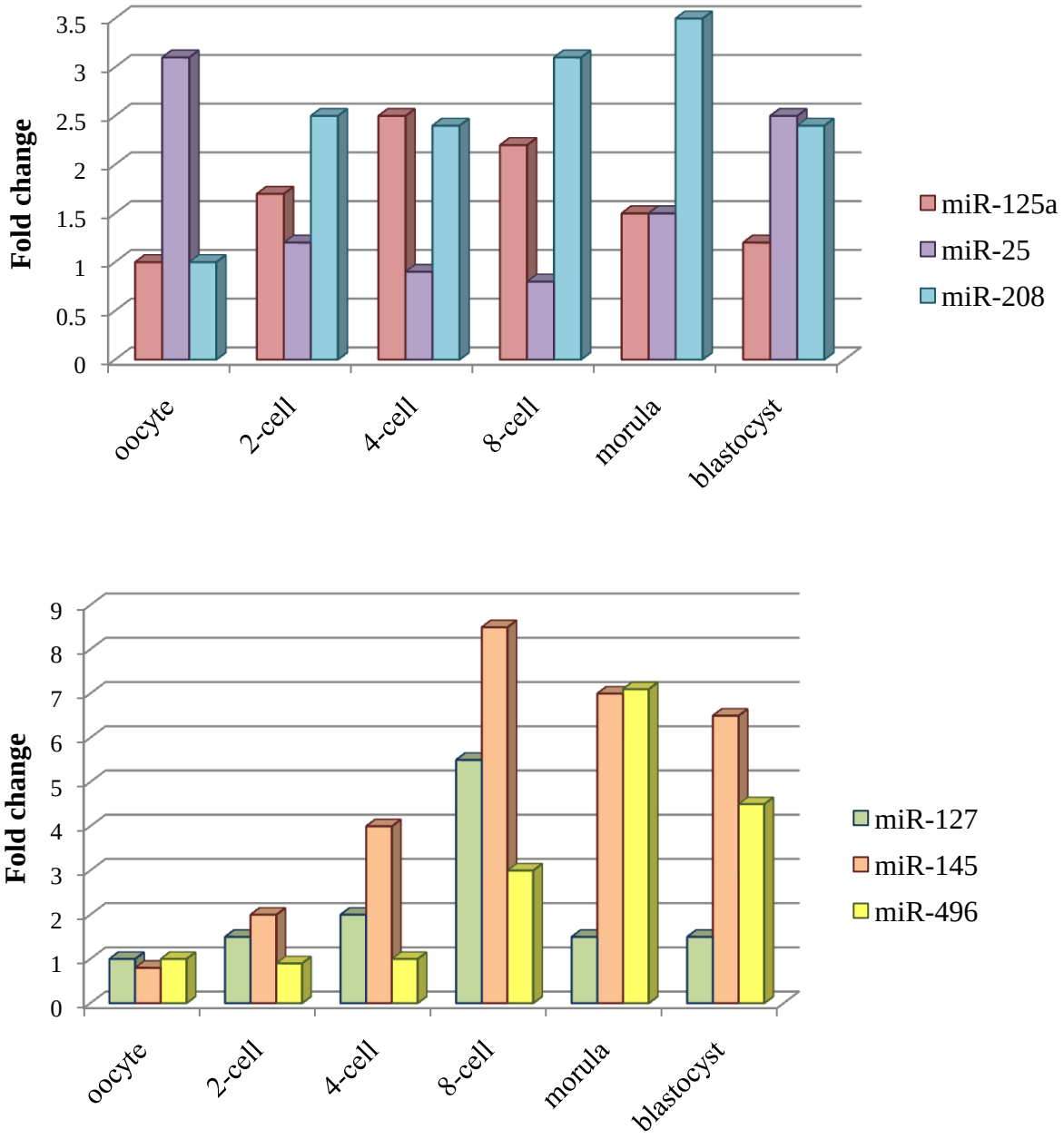

Figure 1.6 shows the capricious expression profile of six miRNAs in six stages of bovine pre-implantation development. Adapted from Tesfaye et al. (2009).

Assisted reproductive technologies

Assisted reproductive technologies (ART) such as *in vitro* fertilization (IVF), embryo transfer (ET), and intracytoplasmic sperm injection (ICSI) are used in both human fertility treatments and in the livestock industry, whereas somatic cell nuclear transfer (SCNT) is reserved mainly for livestock, niche markets, and research. Although these techniques are invaluable with regards to managing infertility and maximizing genetic gain in animal agriculture, research has demonstrated that embryos produced via ART often exhibit aberrations in development, including epigenetic defects (DeBaun et al., 2003; Gicquel et al., 2003; Maher et al., 2003; Moll et al., 2003; Orstavik et al., 2003), chromosomal abnormalities (Hyttel et al., 2000b), actin filament disorganization (Wang et al., 1999), nucleolar-related proteins defects (Bjerregaard et al., 2004; Hyttel et al., 2000a) and even disruptions in lipid content (Romek et al., 2010).

Kikuchi et al. (2004) examined the effect of *in vitro* maturation (IVM) on porcine oocytes, followed by IVF and either immediate ET, ET after two days of culture, or ET after six days of culture. Of the embryos that were immediately transferred into a recipient, 37% developed to the blastocyst stage, whereas those that were cultured for two or six days had a 5% and 20% blastocyst rate. Next, the researchers examined the effect of *in vitro* culture on reaching the fetal stage of development. They performed IVF on IVM oocytes and either transferred the embryos immediately, after 24 h of culture, or after 48 h of culture. Those that were immediately transferred resulted in 6.7% fetal development, whereas those that were cultured for 24 or 48 h resulted in 1.7% or 2.0%, respectively. These results indicated that *in vitro* conditions were inadequate for optimal

embryo development. Machaty et al. (1998) examined the effects of embryo culture on porcine pre-implantation embryos. They collected *in vivo* fertilized embryos at the zygote or 2-cell stage, cultured them for four days, and compared them to embryos allowed to mature *in vivo*. The cultured embryos had lower nuclear numbers and a lower ratio of inner cell mass to trophectoderm nuclei than the *in vivo* matured embryos ($P < 0.001$).

Using whole genome microarrays, Jones et al. (2008) compared gene expression between *in vivo* and *in vitro* matured oocytes and found that the IVM oocytes expressed over 2000 genes at $\geq$ 2-fold higher levels than the *in vivo* matured oocytes, 162 of which were expressed at 10-fold or greater levels. Another group used small amplified RNA-serial analysis of gene expression to compare global gene expression patterns from *in vivo* versus *in vitro* produced porcine embryos (Miles et al., 2008) and detected 938 transcripts that were differentially expressed, suggesting significant aberrations in IVF embryos. Next, using bioinformatic resources, they categorized the mis-expressed genes into functional groups, discovering that they were involved in biological processes including cellular metabolism, organization, and response to stress. Suboptimal culture conditions have been attributed to oxygen concentration (Goto et al., 1993; Yang et al., 1998), gonadotropin levels, including LH, FSH, and hCG, and epidermal growth factors (Akaki et al., 2009).

Methodologies to detect miRNA

Microarrays

Commercially available miRNA arrays were developed (Ambion, Austin, TX; (Shingara et al., 2005)) and microarray procedures allow for the identification of specific

miRNA expression in various tissues (Barad et al., 2004; Bentwich et al., 2005). While most miRNA arrays are generated from human and rodent miRNA sequences, at the commencement of these studies, no information was available for the efficacy of using a commercial array in cross-species hybridizations. Many miRNAs exhibit high conservation among species; however, when using cross species microarrays, failure to detect 100% of miRNAs due to sequence mismatches at hybridization should be assumed. Because there are only ~1000 known miRNAs, an entire "miRNAome" can be identified on a single chip, in triplicate; however, a major drawback of microarray technology is the inability to discover novel miRNA sequences.

Deep sequencing

Next Generation pyrosequencing, also known as deep sequencing, allows for whole transcriptome sequence determination, including small RNAs. Deep sequencing overcomes many of the disadvantages inherent to microarrays in that it allows for measurement of absolute abundance and is not limited to previously known sequences. There are several tools available for analyzing the miRNA transcriptome information resultant of deep sequencing: miRDeep (Friedlander et al., 2008), miRExpress (Wang et al., 2009), SeqBuster (Pantano et al., 2010), miRanalyzer (Hackenberg et al., 2009), and deepBase (Yang et al., 2010).

Northerns

Northern blotting allows for the detection of a particular miRNA or miRNA precursor of interest within a sample of RNA (Grimm et al., 2006; Varallyay et al., 2008). Total RNA is first separated by size via denaturing gel electrophoresis and then blotted onto a

membrane. A labeled probe complementary to the miRNA sequence of interest is allowed to hybridize and, if the sequence of interest is present, the probe will bind and detection may occur. A shortcoming of northern blotting is that a large quantity of RNA is required, which renders this technique ineffective for the detection of miRNAs in samples of limited RNA template, such as embryos.

miRNA inhibition

The practice of inhibiting or 'knocking down' miRNAs mimics the ablation/ replacement studies historically used to study endocrinology and the effects that the presence, or absence, of hormones had on tissues and systems. At the molecular level, many techniques do not require the permanent ablation of a gene or pathway; rather, its deletion can be conditionally induced in tissues of interest. Anti-microRNAs are chemically modified, single stranded, oligonucleotide analogs complementary to either the mature miRNA or its precursors, which can be used either *in vivo* or *in vitro* to inhibit the action of an endogenous miRNA. Commercially available anti-miRNAs are available (Anti-miR™ miRNA Inhibitors; Ambion) that may be injected for *in vivo* studies or transfected or electroporated into cells *in vitro* to allow for the study of the biological effects of specific miRNA.

RT-PCR

To address the shortcomings of northern blotting methodologies for miRNA detection, investigators developed PCR-based technologies for miRNA identification. First attempts at RT-PCR detected the larger miRNA precursors prior to cleavage by Drosha and/or Dicer (Schmittgen et al., 2004). While effective, debate arose over whether it

directly corresponded to the expression of mature miRNA. Because miRNAs are approximately the same size as traditional PCR primers, it was necessary to modify methods to detect mature miRNA (Chen et al., 2005; Shi et al., 2005; Tang et al., 2006).

Ambion's (Austin, TX) *mir*Vana microRNA qRT-PCR kit utilizes primers that bind to the 5' and 3' ends of mature miRNA sequences and contain a 28 or 38 nt overhang which function as stabilization sequences (Figure 1.7). The resulting product is ~85- 89 bp in length, depending on the size of the miRNA.

Figure 1.7. *mir*Vana miRNA primer structure

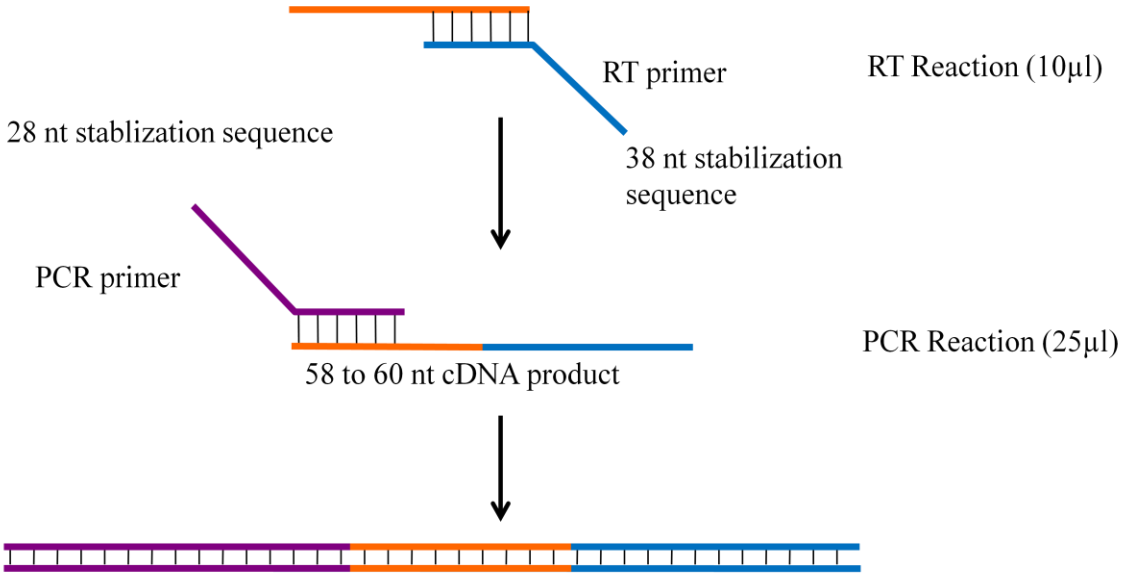

Figure 1.7 shows the stabilization sequence on Ambion's miRNA primers. The result is an 85- 90 bp product containing the 19-24 miRNA sequence flanked by 38 bp from the RT primer and 28 bp from the PCR primer.

The Taqman® miRNA detection system (Applied Biosystems, Foster City, CA) exploits the 5'-3' exonuclease activity of the Taq polymerase, which results in cleavage of fluorescent dye-labeled probes during the primer extension step of PCR (Figure 1.8). The

system includes forward primers, stem-loop reverse primers and a Taqman probe which binds to an internal site on the sequence of interest. The Taqman probe is attached to two fluorescent tags, one of which is a reporter dye (6-carboxyfluorescein or FAM) that has its emission spectra quenched when in close proximity to the second fluorescent dye, 6-carboxy-tetramethyl-rhodamine (TAMRA). When Taq polymerase degrades the Taqman probe, FAM is released from the quenching activity of TAMRA and emits fluorescence proportional to the amount of PCR product formed.

Figure 1.8. Taqman miRNA primer structure

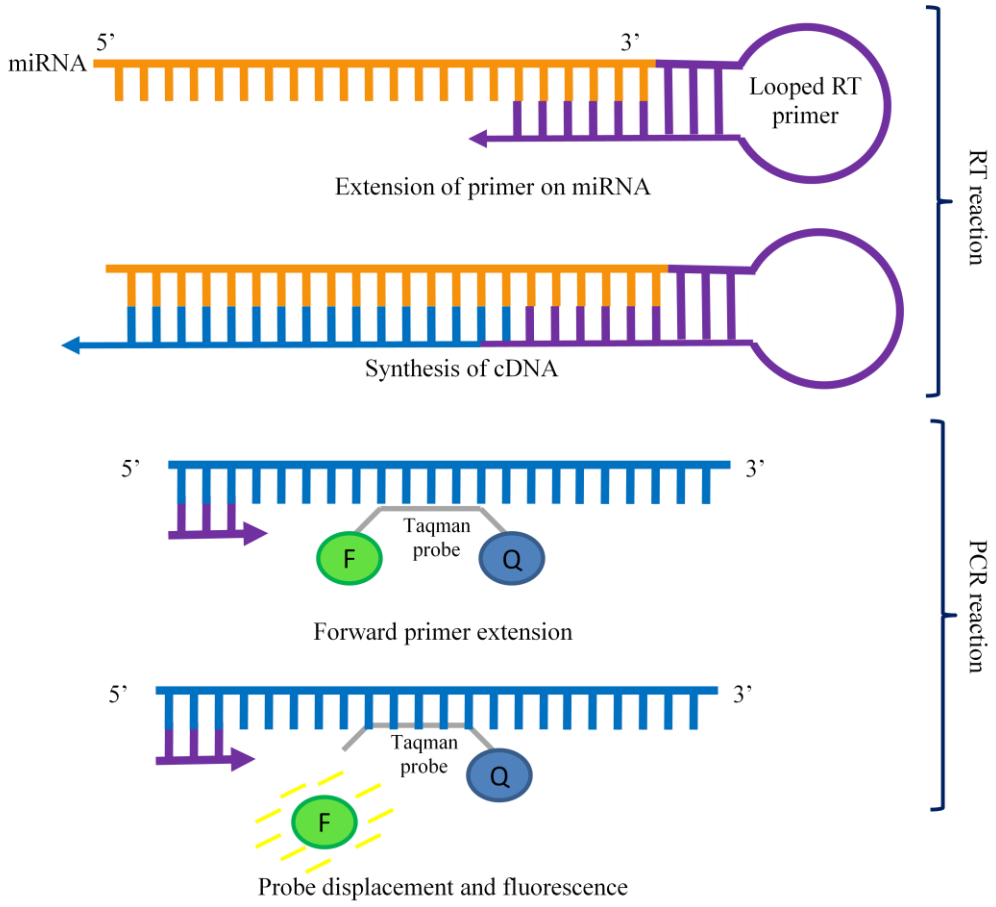

Figure 1.8 illustrates Applied Biosystem's stem-loop reverse primers for miRNA detection and the structure of the Taqman probe.

The normalization nightmare

With emerging insights into miRNA expression and regulation, data normalization for miRNA expression studies presents a challenge. Reviews of the literature unveil inconsistent methods in the normalization of miRNA gene expression as measured by qRT-PCR. The purpose of normalizing data is to reduce technical variation within datasets. An optimal housekeeping gene (also referred to as normalization gene, internal control, standard, or endogenous control), is a single nucleic acid that is invariantly expressed across all samples, is expressed with the target in the cells of interest, and that demonstrates equivalent storage stability, purification properties, and quantification efficiency as the target of interest (Peltier et al., 2008). At least five normalization schemes can be identified in the literature:

1) traditional housekeeping gene. These gene products are typically needed for maintenance of the cell and are not expected to change in response to treatment or experimental conditions. Traditional and frequently used housekeeping genes include glyceraldehyde-3-phosphate dehydrogenase (GAPDH), actins, tubulins, ribosomal RNAs, and ubiquitin; however, their expression has been shown to be regulated due to treatment (Foss et al., 1998; Schmittgen et al., 2000) and, due to their larger size, may not exhibit the same extraction properties as smaller RNAs.
2) other small RNA. It has been suggested that small RNAs, such as nuclear and nucleolar RNA, may better mirror miRNAs in extraction efficiency than larger mRNAs and so are often used as normalization factors. However, U6, a small nuclear RNA, and 5S, a ribosomal RNA which is transcribed by RNA pol III (most

miRNAs are transcribed by RNA pol II), were shown to be poor reference genes for miRNA expression in both normal and cancerous human tissues (Peltier et al., 2008). Davoren et al. (2008) examined the expression of three small nucleolar RNAs (RNU19, RNU48, and Z30) along with five miRNAs for use as endogenous controls across malignant, benign, and normal breast tissue and found certain miRNAs to be more stable than the small RNAs under scrutiny.

3) ubiquitously expressed miRNA. Liang et al. (2007) characterized 345 miRNAs in 40 normal human tissues. Fifteen miRNAs were universally expressed at comparable levels in all tissues examined, based upon their Ct values and Ct variations. The authors recommended the use of these miRNAs as universal reference candidates in which to normalize miRNA expression. Ro et al. (2007a) found that miR-16 was evenly and abundantly expressed in murine testes, spermatocytes, and spermatids and suggested using miR-16 as a housekeeping miRNA gene.
4) more than one stably expressed gene/ miRNA. Some researchers recommend the use of more than one housekeeping gene to further mask technical variation (Andersen et al., 2004; Pfaffl et al., 2004; Szabo et al., 2004; Vandesompele et al., 2002). While attractive in theory, it results in effectively doubling the number of required PCR reactions if interested in examining only a handful of miRNAs.
5) Vandesompele/ Mestdagh method. This group profiled 18 small RNA controls, along with 430 miRNAs, in 147 samples from five human tissues. They assessed the use of the geometric mean of all expressed miRNAs in a given sample as a

normalization factor. They then compared this method to an alternate approach of using one or two small RNA controls, including three previously proposed universal reference miRNAs. Using geNorm software (Vandesompele et al., 2002), they showed that the geometric mean was ranked highest with regards to expression stability and resulted in an adequate reduction in technical variation, as measured by the CVs of normalized expression values (Mestdagh et al., 2009). This method is not appropriate for smaller profiling studies, nor would it be suitable for miRNAs that are clustered or co-regulated, as the mean would undoubtedly be swayed.

In summary, the rules for choosing a gene(s) to use as a normalization factor for miRNA expression analysis remain unresolved. BestKeeper (Pfaffl et al., 2004), Normfinder (Andersen et al., 2004), and geNorm (Vandesompele et al., 2002) are all local software tools useful for the analysis of candidate reference genes: no differences have been found between these tools (Spinsanti et al., 2006; Willems et al., 2006).

Why pigs?

In addition to their obvious role in the food chain, pigs are an important model for biomedical research. Pigs and humans share similarities in their physiology, biochemistry, pathology, and pharmacology and, evolutionarily, pigs are closer than mice to humans (Gorodkin et al., 2007; Wernersson et al., 2005). Pigs have been recognized as advantageous models for the study of numerous areas including: nutrition, toxicology, dermatology, diabetes, cancer, eye diseases, cardiovascular diseases, degenerative joint diseases, and skeletal growth (as reviewed by Matsunari et al., 2009). The emergence of miRNA information was based on studies in non-mammalian species such as *Drosophila*,

C. elegans, and *Xenopus*. Currently, humans have the most miRNAs identified (n= 1048), followed by mice (n= 672), cattle (n= 662), and chimpanzees (n= 601). To date, the presence of only 211 miRNAs has been reported in the domestic pig (*Sus scrofa*) by the miRBase Registry (v. 16.0; September 2010), although more have been predicted (Curry et al., 2009; Huang et al., 2008; Kim et al., 2006). The smaller number of porcine miRNAs predicted using the computational approach is most likely due to the unavailability of the complete pig genome database.

Figure 1.9. Phylogenetic trees showing evolutionary relationships among pigs, mice, and humans.

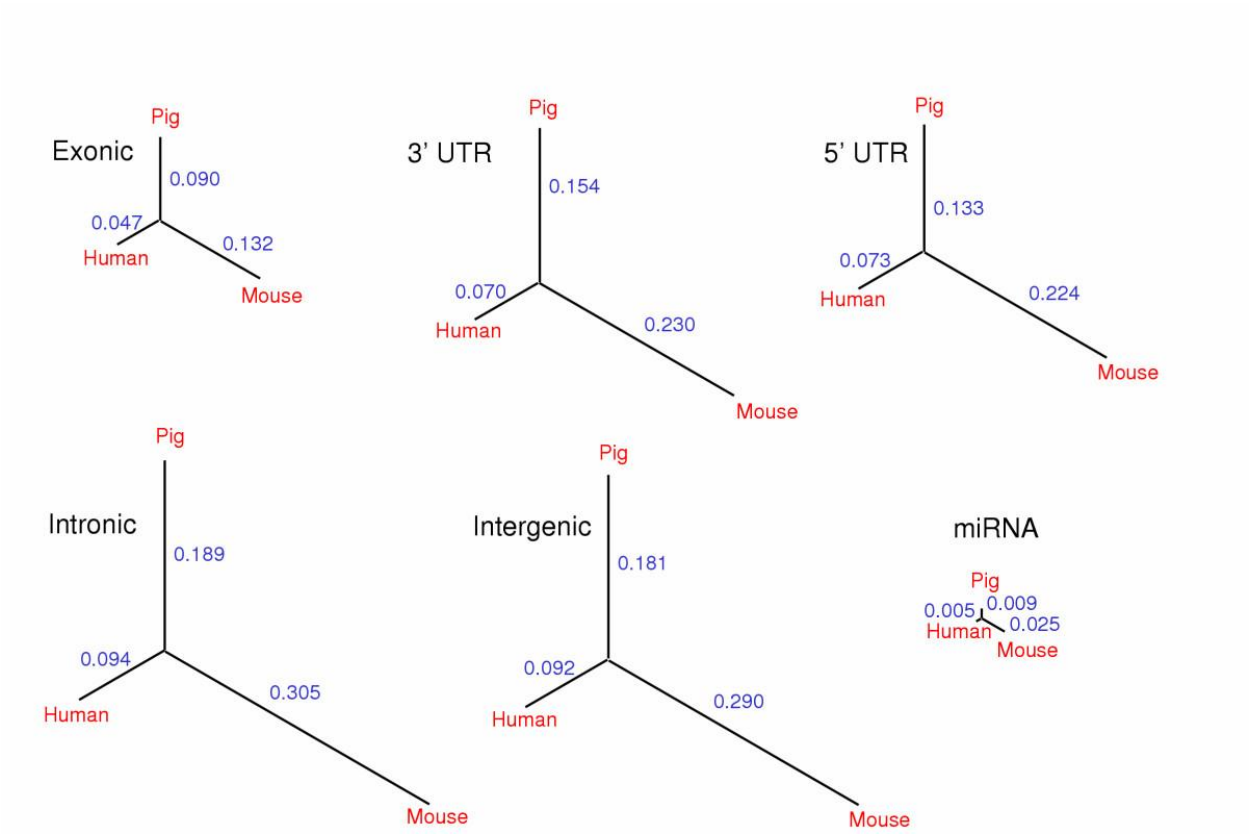

Figure 1.9. Due to a generally lower rate of nucleotide substitutions in the pig and human lineages, the porcine sequences are more similar to the human than to the mouse sequences. Overall, the miRNA sequences show the slowest evolution, followed by exonic, 5' UTR, 3'UTR, intergenic and intronic regions, reflecting different levels of selective constraint on these domains (Wernersson et al., 2005). The blue numbers represent branch lengths determined via the HKY substitution model, a model of DNA sequence evolution. Smaller numbers (and therefore shorter branches) indicate a lower rate of nucleotide substitutions.

The first porcine miRNAs (n= 54) were identified by the analysis of 3.84 million shotgun sequences from a total of 5 pig breeds as part of the Sino-Danish pig genome project (Wernersson et al., 2005). These data led to the identification of the first porcine miRNA cluster (miR-17-92) and to the first porcine miRNA expression analysis, using PCR and northern blot (Sawera et al., 2005). They also demonstrated that the expression of pre-miRNAs does not reflect the expression profile of mature miRNAs. Kim and colleagues (2006) queried human and murine miRNAs against the pig genome to identify 58 potential miRNAs orthologs, six of which were experimentally verified via northern blot analysis. Two years later, another group identified 19 new miRNAs in a cDNA library generated from porcine fibroblast cells and demonstrated tissue-specific expression (Kim et al., 2008). Using a novel concatameric cloning technique combined with sequencing and PCR, Sharbati-Tehrani et al. (2008) identified 10 new miRNA in various tissues from 31-day old piglets. Reddy et al. (2009) pooled and sequenced RNA from pig heart, liver, and thymus to identify 120 conserved miRNA homologs. Twenty-two of the miRNAs were then examined in 14 different tissues by northern blot analysis. Five miRNAs were ubiquitously expressed in all tissues, four miRNAs were highly expressed in 13/ 14 tissues, and 10 showed distinct tissue-specific patterns.

Various studies have also been conducted examining miRNA expression in porcine muscle. Using a human, mouse, and rat microarray, Huang et al. (2008) predicted the expression of 296 miRNAs in the skeletal muscle of fetal pigs (d33 and d65) and adults and found that 255 of them had not yet been reported in pigs. One-hundred forty miRNAs were differentially expressed > 2-fold between developmental stages ($P < 0.001$) and 51 changed > 10-fold. Five were chosen for PCR validation and four of those correlated with the microarray data. McDaneld et al. (2009) measured global miRNA abundance by examining transcriptome profiles of biceps femoris skeletal muscle from six sample types, including cultured cells, during fetal development, and adult. Results provided developmental profiles of seven miRNAs known to be involved in myogenesis, as well as identified twelve potential novel miRNA in pigs. Maak et al. (2010) found no correlation between the expression of ZDHHC9 (zinc finger, DHHC-type containing 9), a gene encoding a protein involved in porcine congenital splay leg syndrome, and two miRNAs predicted to target it; however, the authors neglected to examine protein expression.

With the development of deep sequencing technologies, three more papers have recently been published that examined the porcine microRNAome. Nielsen et al. (2010) identified the sequences and relative expression levels of 212 annotated miRNAs in porcine longissimus dorsi. The expression levels, measured by sequence reads, varied from single counts to several million reads. The most abundant miRNA was miR-1 which had 30 million counts, corresponding to 87.1% of the total reads. A total of four miRNAs (miR-1, -206, -133, and let-7) accounted for 94.5% of the total miRNA reads. Another group (Sharbati et al., 2010) utilized deep sequencing followed by a custom microarray

based on the sequences they had identified to decipher the porcine intestinal miRNA transcriptome. They identified 332 miRNAs, of which 201 had not been described previously in pigs.

Finally, Li et al. (2010) examined miRNA expression in ten small RNA libraries corresponding to ten developmental stages: embryonic day 30, 45, 60, 75, 90, and 105, birth, and post-natal day 30, 120, and 180. They detected 771 unique miRNA sequences resulting from 862 miRNA precursors. Seventy-two of the 77 known porcine miRNAs (based on miRBase v. 14.0) were identified. These previous three experiments compared their deep sequencing results to miRBase release v. 14.0 (September 2009), which only recognized 77 miRNAs. The next release of the miRBase will undoubtedly include hundreds of novel porcine miRNAs based on the results of these publications.

CHAPTER TWO

INTRODUCTION

In a decade's time, miRNAs have impacted nearly every field of biology and have challenged established concepts pertaining to gene regulation. A PubMed search (http://www.ncbi.nlm.nih.gov/pubmed/) shows a rapid growth in the number of papers published concerning miRNA since their discovery (Figure 2.1). Since the commencement of these studies, many advances have been made in the understanding of miRNA function, regulation, expression, and target recognition and, accordingly, in the methodologies in which miRNAs are studied. In 2006, the presence of only 54 miRNAs had been reported in *Sus scrofa* by the miRBase Registry v. 8.0 (Griffiths-Jones, 2004; Griffiths-Jones, 2006) . Today, 211 have been validated (Figure 2.2), whereas hundreds more have been predicted.

Figure 2.1. Number of papers published concerning miRNAs per year

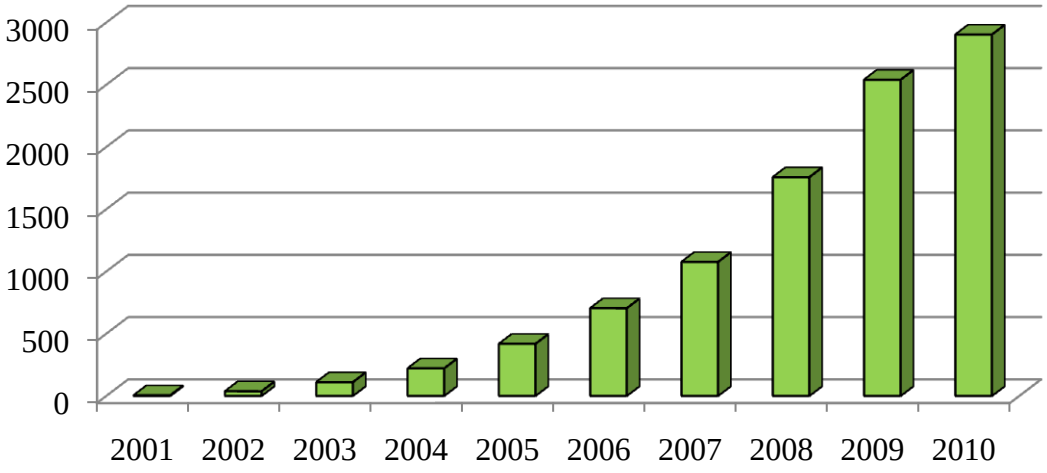

Figure 2.1 shows the number of papers containing the word "microRNA" published in each calendar year. The first year for which data are shown is 2001, which is the year miRNAs were given their name. The data for 2010 represents papers published through October 22, 2010.

Figure 2.2. Total number of miRNAs reported in human, porcine, and murine.

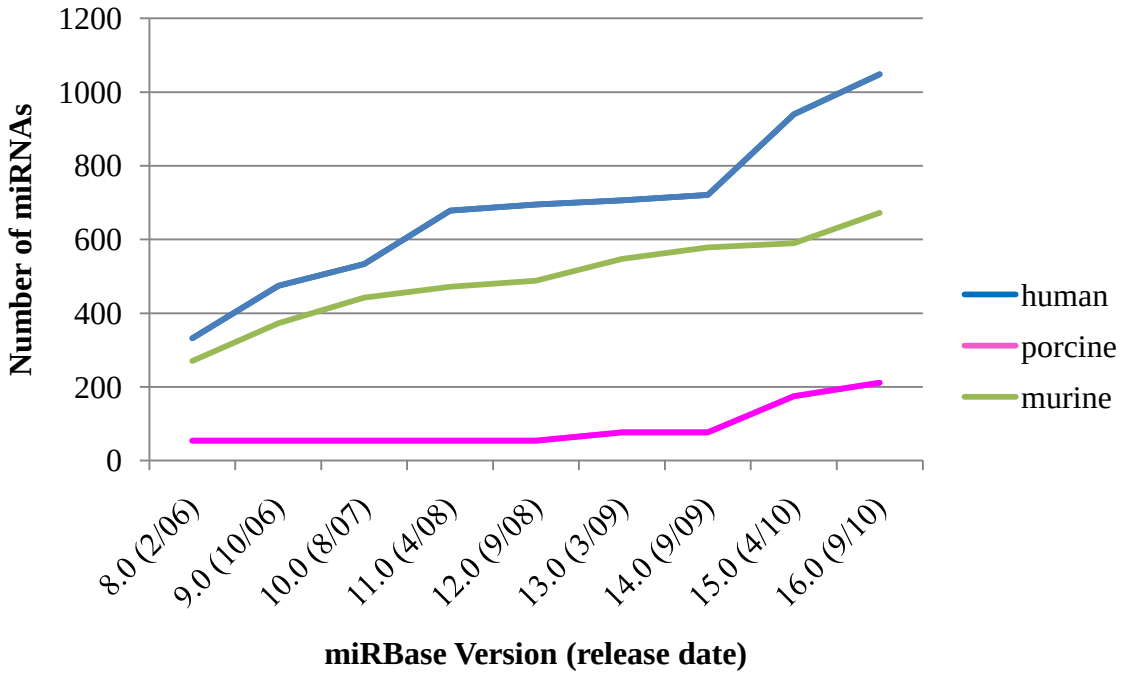

Figure 2.2 shows the growth in miRNA identification in human, porcine, and murine as reported by consecutive miRBase Registry releases.

Objective

There is a need to characterize the abnormal embryonic development associated with ART and caused by aberrant miRNA expression. The objective of this dissertation research was to determine the presence of miRNAs in porcine gametes and pre-implantation embryos and to identify differences in miRNA expression between normal and aberrant samples. We hypothesized that miRNAs are present in porcine reproductive tissues and demonstrate high sequence similarity when compared to human miRNA

sequences. Further, samples of compromised quality (abnormal sperm, *in vitro* produced embryos), may exhibit aberrant miRNA expression when compared to normal samples.

CHAPTER THREE

DETECTION OF PORCINE SPERM MICRORNAS USING A HETEROLOGOUS MICRORNA MICROARRAY AND REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION

Introduction

miRNA exist in mammalian sperm (Amanai et al., 2006; Kotaja et al., 2006; Ostermeier et al., 2005b; Yan et al., 2008), although no reports describe miRNA (or RNA) in porcine sperm. Commercially available miRNA microarrays have been developed (Ambion, Austin, TX; LC Sciences, Houston, TX; (Shingara et al., 2005)) and microarray procedures have been reported for identification of specific miRNA expression in various tissues (Barad et al., 2004; Bentwich et al., 2005; Ostermeier et al., 2005a). Most microarrays are generated from human and rodent miRNA sequences and no information is available for the efficacy of using a commercial array in cross-species hybridizations. Many miRNAs exhibit high conservation among species indicating that cross-species microarrays would be effective; however, failure to detect 100% of miRNAs due to sequence mismatches at hybridization should be assumed. The objectives of this study were to survey the miRNA present in boar sperm while evaluating a heterologous miRNA microarray for the detection of miRNAs in porcine tissue.

Materials and methods

RNA isolation

For the microarray, total RNA was isolated from mature spermatozoa obtained from commercial sources (Swine Genetics International, Eldora, Iowa) using TRIzol® Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. A plethora of techniques, kits, and reagents were evaluated to determine those which provided maximum RNA yield and acceptable quality. For subsequent RT-PCR assays, total RNA enriched for small RNAs was isolated from sperm pellets using the *mir*Vana^{TM} miRNA Isolation Kit (Ambion Inc., Austin, TX) with some modifications. Upon removal from storage, 1 mL of 0.5% SDS (Mallinckrodt Baker, Inc, Phillipsburg, NJ)/ 0.1% Triton-X (Roche, Mannheim, Germany) was added to the sperm pellet and homogenized using a 26 g needle. Next, 6 mL lysis buffer was added and the mixture incubated at 65 ºC for 30 min. A 1:10 vol of miRNA homogenate additive was mixed with the sample and incubated on ice for 20 min. A volume of acid-phenol: chloroform was added equal to that of the lysis buffer, the solution vortexed for 45 sec, followed by a 10 min centrifugation at 10,000 x g. The supernatant containing the total RNA was removed and precipitated with 1.25 vol ethanol (99.5%). The solution was passed through a filter cartridge using vacuum-mediated suction, washed, and RNA was extracted with 100 µl elution solution, preheated to 95 °C. Sample concentration and quality were determined by using the Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

Microarray

Microarray analysis was performed using a service provider (LC Sciences, LLC, Houston, TX) to identify the miRNA profile present in porcine sperm cells. Five µg sperm tcRNA pooled from multiple boars was size fractionated using a YM-100 Microcon centrifugal filter (Millipore, Bedford, MA) to isolate small RNAs. Small RNAs less than 300 nt were 3'- extended with a poly(A) tail using poly(A) polymerase. An oligonucleotide tag was ligated to the poly(A) tail for later fluorescent dye staining.

Hybridization was performed overnight on a µParaflo microfluidic chip using a micro-circulation pump (Atactic Technologies, Houston, TX). On the microfluidic chip, each detection probe consisted of a chemically modified nucleotide coding segment complementary to a known miRNA target (based on Sanger miRBase Release 9.0) or control RNA and a spacer segment of polyethylene glycol to extend the coding segment away from the substrate. MiRNA probes (n= 1260, in duplicate) were complementary to known miRNAs from 19 different species, including 55 porcine probes. The detection probes were made by *in situ* synthesis using photogenerated reagent chemistry. The hybridization melting temperatures were balanced by chemical modifications of the detection probes. Hybridization used 100 µL 6x SSPE buffer (0.90 M NaCl, 60 mM Na_2HPO_4, 6 mM EDTA, pH 6.8) containing 25.0% formamide at 34 °C.

Tag detection was determined using fluorescence labeling with tag-specific dyes. Images were collected using the GenePix® 4000B laser scanner (Molecular Device, Inc, Sunnyvale, CA) and digitized using Array-Pro image analysis software (Media Cybernetics, Silver Spring, MD). Data were analyzed by first subtracting the background

and then normalizing the signals using a LOWESS filter (Locally-weighted Regression) to compensate for the intensity difference between Cy3 and Cy5.

The signal intensities of quadruplicate reactions were averaged and, although the microarray service provider recommended including all samples with detectable signal greater than 30 relative fluorescent units (RFU) in analysis, a more conservative threshold was adopted. Detectable transcripts included those with average signal intensity greater than or equal to 100 RFU and were further divided into subcategories: low expression (100- 999); moderate expression (1000- 9999), and; high expression (> 10,000).

PCR

RT-PCR was conducted using the *mir*Vana™ qRT-PCR miRNA Detection Kit (Ambion, Austin, TX) and was used to verify the presence of 21 specific miRNAs: let-7a, -7d, -7e, -7f, -7i, miR-9, -15b, -16, -21, -22, -24, -27a, -31, -92, -124a, -132, -150, -181a, -182, -212, and -345. Human miRNA primer sets (*mir*Vana™ qRT-PCR Primer Set, Ambion) were used to amplify an 85 to 90 bp product containing the 19 to 24 nt miRNA sequence flanked by 28 nt from the PCR primer and 38 nt from the RT primer. The reverse transcriptase reaction (10 µl) was incubated at 37 ^{o}C for 30 min then at 95 ^{o}C for 10 min. The PCR reaction (25 µl) was initiated with a cycle of 95 °C for 3 min followed by 35 cycles of denaturation at 95 °C for 15 sec and primer annealing at 60 °C for 30 sec, and then a final hold at 4 ºC. Ten µL of each reaction was subjected to non-denaturing slab gel electrophoresis on a 50 mL 3.2% agarose gel containing ethidium bromide (Fisher Scientific, Fair Lawn, NJ). Gels were electrophoresed in 5X Tris/ Borate/ EDTA

buffer at 85 V and product was visualized by exposure to ultraviolet light and photography.

Sequence analysis

PCR products were ligated into the pDrive cloning vector and ligation reactions were used to transform competent *E. coli* cells (Qiagen PCR Cloning Kit; Qiagen, Valencia, CA). Clones containing the insert were propagated and the plasmids were isolated using QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). Plasmids were subjected to dideoxynucleotide sequencing at the Clemson University Genomics Institute (Clemson, SC) using M13 primers and sequences were compared to the corresponding human sequence reported by the miRBase Registry Release 9.0 or 14.0, depending on when results were received.

Results

Microarray

Microarray results showed that of the 1260 known miRNA probes used, 316 produced a detectable signal (intensity ≥ 100 RFU). Nine hundred thirty nine had non-detectable hybridization (74.8%), 162 had low detection (12.9%), 94 were moderately expressed (7.5%), and 60 were highly expressed (4.8%) (Figure 3.1). A dye bias was identified on five transcripts (0.4%) and these samples were deleted from analysis. Of the *Sus scrofa* miRNA sequences listed in the miRBase Registry (n= 55), all were probed on the array, and 23 were detected (41.8%). Sixteen probes complementary to the *let* family of miRNAs produced a detectable signal. Significant hybridization signals were detected for

293 target sequences that have not been previously reported previously in *Sus scrofa*. For a list of detectable transcripts, see Appendix A.

Figure 3.1. Sperm miRNA per expression category

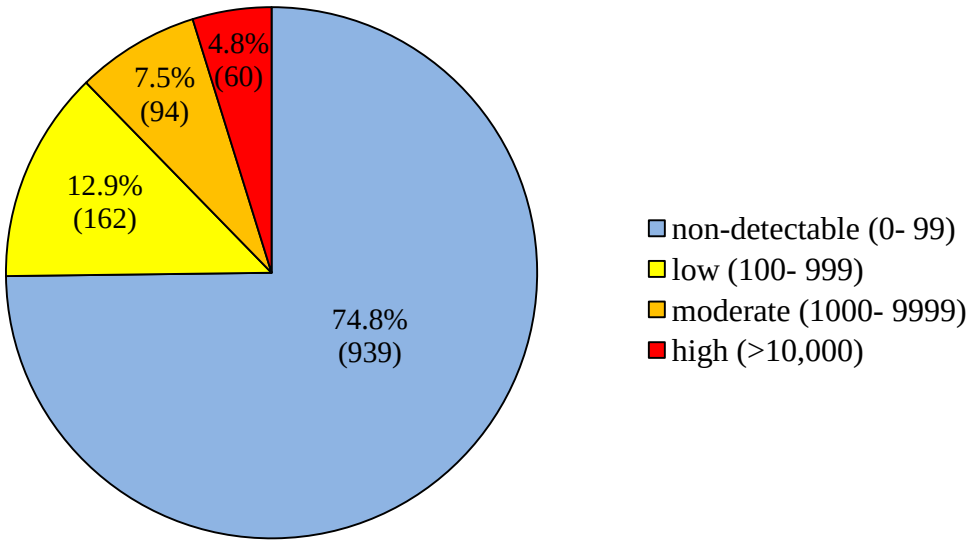

Figure 3.1 shows the percentage of sperm miRNAs included on the microarray that were non-detectable and that exhibited varying levels of expression according to relative fluorescence units. The numbers in parenthesis represent the total number in each expression category.

PCR

RT-PCR and gel analysis were used to confirm the presence of 21 specific miRNAs in porcine sperm cells (Figure 3.2). The PCR results supported the microarray data with four exceptions (Table 3.1): the miR-124a, -345, and -9 primers produced faint bands on the gel, but were not detected in the array and, although miR-150 showed moderate expression in the array, it was not detected by RT-PCR in RNA from three separate sperm samples.

Figure 3.2. MiRNA RT-PCR products

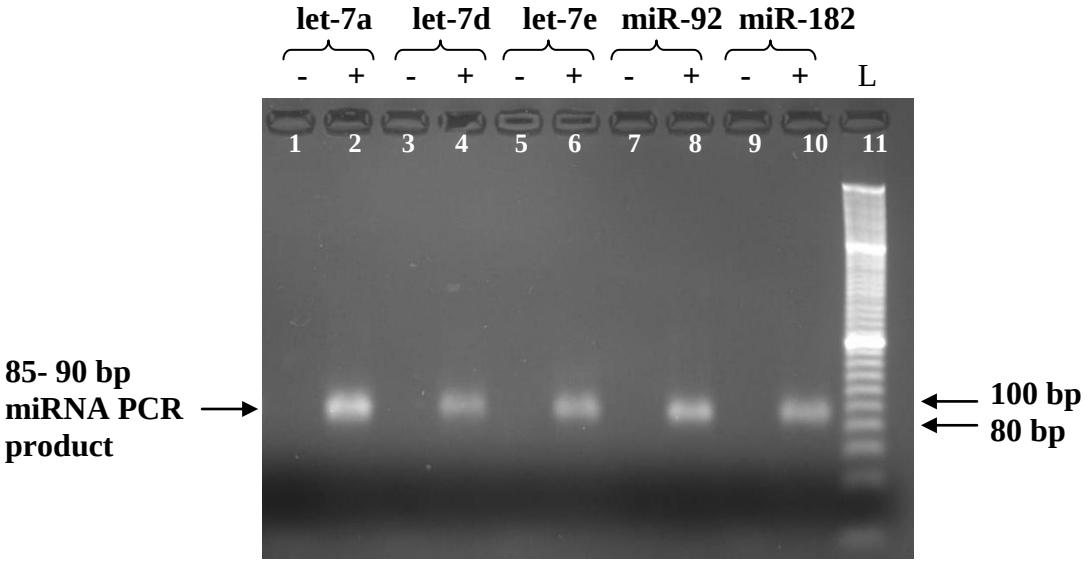

Figure 3.2 is an image of a 3.2% agarose gel showing a selection of 85- 90 bp PCR products stained with ethidium bromide and viewed under UV fluorescence. Lanes 1- 10 show specific miRNAs next to their corresponding negative controls and lane 11 shows the 20 bp ladder (Sigma-Aldrich).

Table 3.1. *Sus scrofa* sperm miRNAs identified by microarray, RT-PCR, and sequencing

miRNA	Length (nt)	Array Expression	RT-PCR
ssc-let-7a[1*]	22	high	+
ssc-let-7d[1,2]	22	high	+
ssc-let-7e[1, 2, 3*]	22	high	+
ssc-let-7i[4]	19	high	+
ssc-let-7f	22	high	+
ssc-miR-124a[3]	22	n.d.	+
ssc-miR-132[1]	22	low	+
ssc-miR-150[1]	22	mod	-
ssc-miR-15b	22	high	+
ssc-miR-16[1*]	22	high	+
ssc-miR-181a[5]	23	mod	+
ssc-miR-182[1]	24	low	+
ssc-miR-21	22	high	+
ssc-miR-212[1,2]	21	low	+
ssc-miR-22[1*]	22	low	+
ssc-miR-24	22	mod	+
ssc-miR-27a	21	mod	+
ssc-miR-31[1]	21	n.d.	-
ssc-miR-345[1,5*]	22	n.d.	+
ssc-miR-9	23	n.d.	+
ssc-miR-92[1*]	22	high	+

[1]miRNAs not previously reported as being identified in pigs by the miRBase Registry. An asterisk (*) indicates miRNAs that have been reported since the commencement of these studies.
[2] Sequence differs from reported human sequence.
[3] Sequence differs from reported pig sequence.
[4] Sequence differs from human sequence but is identical to reported pig sequence.
[5] Sequence differs from reported pig sequence but is identical to human sequence.

Sequencing

All miRNAs examined were successfully subcloned and sequenced. Those that were not detected in sperm by RT-PCR were sequenced from porcine kidney RNA. The majority of the sequences (85.7%) were identical to the human and/ or pig miRNA sequences of the same identity listed in the miRBase Registry (Figure 3.3). Of those that differed, it was only by one (n= 4) or two (n= 1) nucleotides. MiR-212 had a one nucleotide change (A to C) at the tenth position from the 3' end compared to the human sequence. Two miRNAs, let-7d and let-7e, had a U to C substitution at the 3' end when compared to the human sequence. MiR-181a was identical to the human miR-181a, but conflicts with reported pig sequence (the reported ssc-miR-181a has an extra uracil at the 3' position). The reported porcine miR-124a does not have a second uracil at the 5'end, although nine other species do (the human miR-124a was removed from the miRBase Registry after these assays were conducted for unknown reason). The miR-345 sequence was identical to the reported human sequence, but had two nucleotides different from the reported pig sequence, which has a G to U substitution at the fifth position from the 3' end and a C deletion at the 3' end.

Figure 3.3. Porcine miRNA multiple sequence alignment

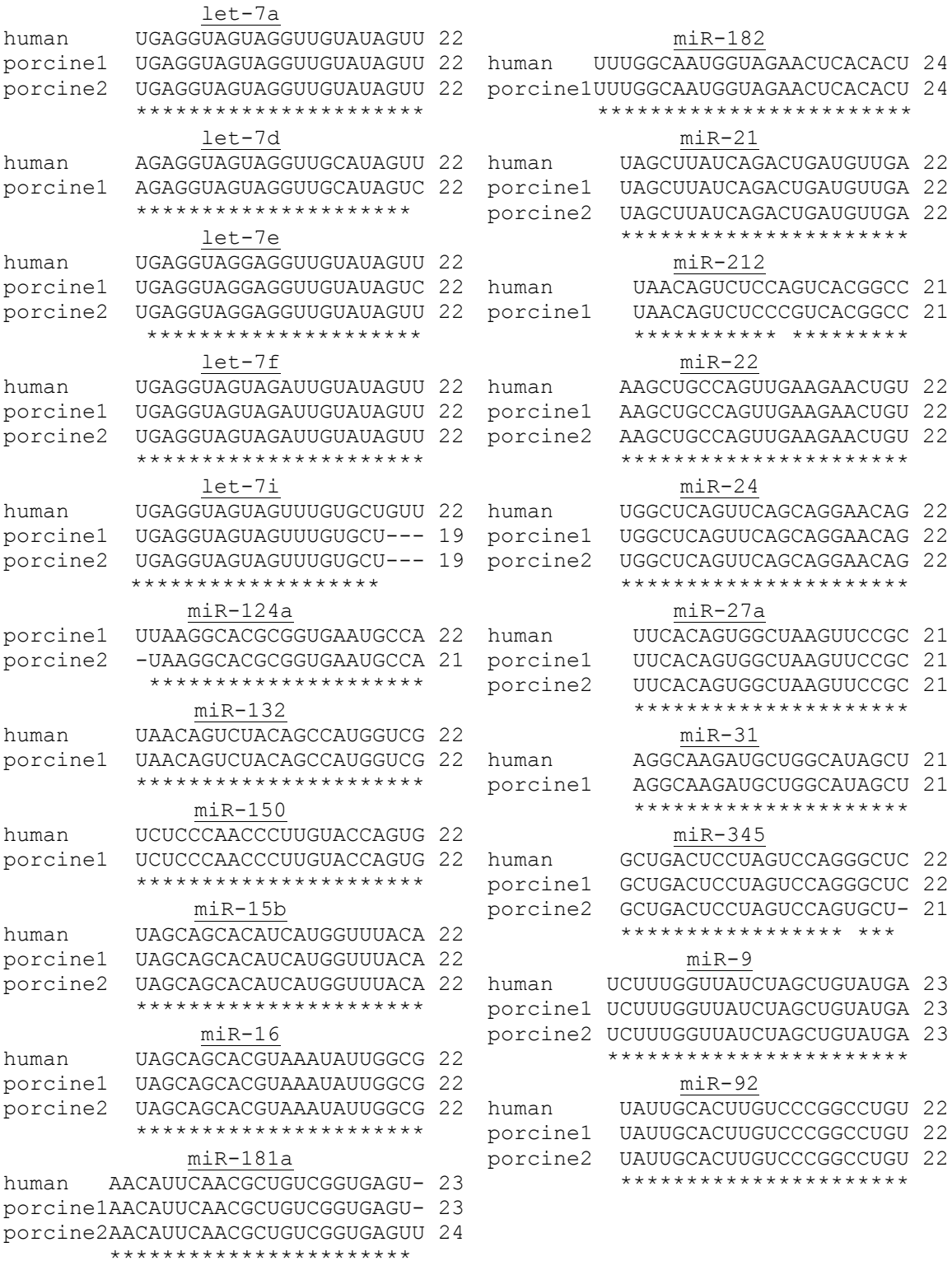

Figure 3.3. Multiple sequence alignments were performed using ClustalW at default settings to compare the pig sequence data generated in this experiment (porcine1) to the corresponding reported human sequences (human) and to the reported porcine sequence (porcine2). Asterisks indicate agreement among nucleotides at corresponding positions among sequences.

Discussion

The RNA sample used in this study was isolated from collected ejaculates pooled from multiple boars of the same breed, which is industry standard for swine production facilities. Multiple boars were used to account for individual variation. The microarray showed that 316 known miRNA probes hybridized to RNA sequences present in porcine sperm RNA. The array results suggest the presence of 293 miRNAs that have not been previously reported in *Sus scrofa*. Although porcine sequences hybridized to 316 of the probes, it is possible that binding to multiple species of same miRNA caused the results to appear inflated (e.g. miRNA, presumably all ssc-miR-16, bound to the hsu-miR-16, the bta-miR-16, and the lca-miR-16 probes). Two hundred thirteen of the 316 detectable signals were unique by assigned miRNA name, regardless of species.

Array results were confirmed via RT-PCR using 21 human miRNA primer sets, which largely demonstrated the conservation of mature miRNAs between species. miRs-124a, -345 and -9 were present following the RT-PCR but not in the microarray. Because the sensitivity of the PCR reaction allows for logarithmic amplification, it is possible that they are present in porcine sperm cells in copy numbers too low to be detected by the microarray hybridization. It should be mentioned that miR-124a was detected in one of the two spots using Cy3 on the microarray and also that the human miR-124a was removed from the miRBase Registry after assays were conducted (between the release of

version 9.0 and 14.0) for unknown reasons. The sequence data conflicts with the previously reported porcine miR-124a sequence (Reddy et al., 2009) in that the reported ssc-miR-124a does not have an extra uracil on its 5' end.

Along with miR-124a, miR-181a also conflicts with previously reported porcine miR-181a sequence in that the reported ssc-miR-181a does not have a uracil on its 3' end. Both miR-124a and miR-181a were described previously by Reddy et al. (2009) as the result of pyrosequencing. Possible explanations for these discrepancies include primer artifact, the existence of possible paralogs of miR-182, or contamination of porcine samples with human miRNA. Let-7i, while sharing 100% identity to the reported porcine let-7i, differs from the human sequence due to a lack of three nucleotides at its 3' end (GUU). A recent analysis of small RNA transcripts from porcine fetuses revealed that both "isomiRs" are present in pig tissues, with the longer (22 nt) variant being much more prevalent (Li et al., 2010). Although miRNAs tend to be conserved among species over their entire length, no specific function has been allocated to the 3′ end of miRNAs, as they do not appear to have a role in mRNA recognition or binding. The miR-345 sequence, although identical to the reported human miR-345 sequence, disagreed with the reported pig sequence at two nucleotides.

Subsequent Real-time qRT-PCR analysis on miR-345 revealed an abnormal trendline resembling a double sigmoid-curve (see Chapter IV), warranting its removal from analysis. Although miR-150 did not produce a visible PCR product when analyzing sperm RNA, the array showed moderate expression, and the product was visible when RNA samples from other porcine tissues were examined. MiR-150 is present in pigs, as it

was identified in other porcine tissue samples and sequenced in kidney (Appendix D). A sequence very similar to miR-150 that is present in porcine sperm may have bound to the miR-150 probe in the array. MiR-212 showed low expression in the array, but analysis revealed that the sequence was not identical to the human or mouse sequences, differing by one nucleotide. It is likely that the array hybridization signal would be higher if the porcine sequence was probed rather than the heterologous sequences.

Results of this study verified that miRNAs are present in porcine sperm cells and that, due to a high degree of sequence conservation among species, heterologous miRNA microarrays and PCR primers are effective for porcine miRNA expression profiling in sperm cells. Comparative data emphasized that, while a miRNA microarray provides a suitable survey to ascertain which miRNAs are present in samples, it is obligatory to verify microarray results by RT-PCR and sequencing.

CHAPTER FOUR

DIFFERENTIAL EXPRESSION OF PORCINE SPERM MICRORNAS AND THEIR ASSOCIATION WITH SPERM MORPHOLOGY AND MOTILITY

Introduction

The literature suggests that miRNAs are involved in spermatogenesis and that their presence or absence in mature sperm could be indicative of aberrant development, function and/or fertility (Amanai et al., 2006; Curry et al., 2009; Lian et al., 2009; Maatouk et al., 2008; Yan et al., 2008). Mice lacking *Dicer1*, an enzyme required for global miRNA synthesis, have morphologically abnormal elongating spermatids, exhibit low motility, and are usually infertile (Maatouk et al., 2008). Lian et al. (2009) revealed differential miRNA expression profiles of testes from patients with non-obstructive azoospermia versus normal controls. Amanai et al. (2006), Yan et al. (2008), and, as described in Chapter III, Curry et al. (2009) validated the presence of miRNAs (n= 54, n= 28, and n= 17, respectively) in mouse or boar sperm using RT-PCR, although more were predicted based upon miRNA microarrays (n= 191 (Amanai et al., 2006) and n= 293 (Curry et al., 2009)).

Ostermeier et al. (Ostermeier et al., 2005b) proposed that RNAs could be useful for male infertility prognosis and that specific male infertility factors may be identified using genomic profiling of spermatozoa. Although sperm RNAs previously were thought to play a negligible role in fertilization and early embryonic development, both paternal mRNAs and miRNAs are delivered to the oocyte at fertilization (Amanai et al., 2006; Ostermeier et al., 2004; Rassoulzadegan et al., 2006) and could affect the phenotype of

the resulting offspring (Rassoulzadegan et al., 2006). If sperm messenger RNA can affect the resulting offspring, it is conceivable that sperm miRNA may influence embryo physiology as well. McCallie et al. (2010) detected aberrant embryo miRNA expression from human blastocysts derived from patients with male factor infertility compared to blastocysts produced from normal controls, suggestive of a contribution from sub-fertile sperm affecting the phenotype of the resulting embryo.

Very little information as to the identity of miRNAs in porcine reproductive tissues and their potential involvement in reproductive processes is known. Only a handful of studies have characterized miRNAs in sperm (Amanai et al., 2006; Curry et al., 2009; Yan et al., 2008) and no studies have investigated aberrant miRNA expression in mature sperm. While male infertility may be caused by a variety of factors, the identification of specific miRNAs that are associated with sperm structure and/or motility or that are responsible for sperm fertility could lead to the development of a microarray-based diagnostic assay to provide an assessment of male fertility. The objective of this study was to identify differences in miRNA expression between normal porcine sperm samples and those exhibiting morphological abnormalities or low motility.

Materials and Methods

Samples and semen preparation

Boar semen samples of normal motility and morphology from individual boars (average motility= 92.8%, SD= 5.2; < 15% abnormal morphology) were used as controls (n= 7). Samples of abnormal morphology (AB; n= 7) contained $\geq$ 15% primary and/or secondary abnormalities, while low motility samples (LM; n= 8) exhibited $\leq$ 68% motile

sperm (average motility= 47.5%, SD= 15.2). All samples were from single ejaculates collected from commercial Duroc boars and were characterized at the boar stud at the time of collection. Upon arrival, samples were centrifuged at 10,000 x g for 10 min. The sperm pellets were washed twice in PBS, centrifuged, and the supernatant removed. The pellets were snap-frozen in liquid nitrogen and stored at -80 °C until RNA isolation.

RNA isolation

Total cellular RNA enriched for small RNA was isolated from sperm pellets using the *mir*Vana™ miRNA Isolation Kit (Ambion Inc., Austin, TX) with some modifications. Upon removal from storage, 1 mL of 0.5% SDS (Mallinckrodt Baker, Inc, Phillipsburg, NJ)/ 0.1% Triton-X (Roche, Mannheim, Germany) was added to sperm pellet and homogenized using a 26 g needle. Next, 6 mL lysis buffer was added and the mixture incubated at 65 ºC for 30 min. A 1:10 vol of miRNA homogenate additive was mixed with the sample and incubated on ice for 20 min. A volume of acid-phenol: chloroform was added equal to that of the lysis buffer, the solution vortexed for 45 sec, followed by a 10 min centrifugation at 10,000 x g. The supernatant containing the miRNA was removed and precipitated with 1.25 vol ethanol (99.5%). The solution was passed through a filter cartridge using vacuum-mediated suction, washed, and RNA was extracted with 100 µl elution solution, preheated to 95 °C. Sample concentration and quality were determined by using the Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

Quantitative real-time PCR

MiRNAs chosen for analysis were found to be differentially expressed by microarray analysis (Appendices B and C), previously identified in boar sperm (Curry et al., 2009) and/ or predicted to target genes that code for proteins involved in sperm structure, motility, or metabolism (Table 4.1). Quantitative real-time PCR (qRT-PCR) was conducted using the Realplex Mastercycler epgradient **(**Eppendorf**,** Hamburg, Germany). Reactions were performed in quadruplicate on 10 ng sperm RNA using the *mir*Vana™ qRT-PCR miRNA Detection Kit (Ambion, Austin, TX) and compared expression levels of 11 specific miRNAs: let-7a, -7d, -7e, -7i, miRs-15b, -16, -182, -22, -24, -345, and -92. Human miRNA primer sets (*mir*Vana™ qRT-PCR Primer Set, Ambion) were used to amplify an 85 to 90 bp product containing the 19 to 24 miRNA sequence flanked by 28 bp from the PCR primer and 38 bp from the RT primer (Figure 3.2). The reverse transcriptase reaction (10 µl) was incubated at 37 ^{o}C for 30 min, then at 95 ^{o}C for 10 min. The PCR reaction (25 µl) was initiated with a cycle of 95 °C for 3 min followed by 35 cycles of denaturation at 95 °C for 15 sec and primer annealing at 60 °C for 30 sec, and then a final hold at 4 ºC. Ct is defined as the point at which fluorescence rises appreciably above the background (threshold) fluorescence and Cts were measured at a constant threshold among plates.

As previously described, sperm miRNA expression levels were normalized to endogenous miR-16 (Amanai et al., 2006; Liang et al., 2007; Ro et al., 2007a). MiR-16 exhibited stable expression among all sperm samples (Pearson correlation coefficient (r)= 0.954) and was validated using BestKeeper© version 1 software (Pfaffl et al., 2004). A

more traditional housekeeping gene, 5S ribosomal RNA, was also evaluated, but expression levels were irregular among samples (see also Peltier et al., 2008) and BestKeeper© analysis showed it to be a poor normalizer (r = 0.597). Standard curves for each miRNA were calculated on 5-fold serial dilutions of input reference RNA ranging from 125 ng to 0.04 ng. Statistical analysis was performed using REST 2005 software (Pfaffl et al., 2002) with significance at $P < 0.05$.

Functional inference of miRNA

The small number of porcine miRNAs and predicted mRNA targets relative to other species is partly due to the incomplete pig genome database. Because miRNAs are highly conserved among species, especially within mammals, it is extremely likely that pigs share evolutionarily preserved miRNA binding sites with humans. In order to elucidate the functions of differentially expressed miRNAs among control, AB, and LM sperm populations, their target genes were extracted using MAMI server and database (meta mir: target inference; http://mami.med.harvard.edu/) which provides a composite prediction of target genes from five independent target prediction tools: TargetScan (Lewis et al., 2005), miRanda (John et al., 2004), DIANA-microT (Maragkakis et al., 2009), miRtarget (Grun et al., 2005) and picTar (Krek et al., 2005). Targets were predicted for up- and down-regulated miRNAs from either AB or LM compared to the controls. A MAMI sensitivity of 0.46 and a specificity of 1.0 were used to minimize Type I Errors.

The Database for Annotation, Visualization and Integrated Discovery (DAVID v. 6.7; http://david.abcc.ncifcrf.gov; (Dennis et al., 2003; Huang da et al., 2009)) is a free online

bioinformatics resource that provides interpretation of biological themes associated with large gene lists. DAVID was used to annotate MAMI-predicted target genes of differentially expressed miRNAs and to identify significant functional enrichment in the miRNA gene targets relative to the whole genome background. Entrez gene IDs were uploaded to the functional annotation tool and enriched biological themes were analyzed using a modified Fisher's exact test (EASE score; $P < 0.05$), medium classification stringency, minimum enrichment score of 1.3, and a minimum fold enrichment of 1.5. Functional annotation charts and clustering were analyzed to determine enriched molecular functions, biological processes, cellular components, biochemical pathways, and other gene annotations.

Table 4.1. MiRNAs and their predicted mRNA targets involved in sperm function

miRNA	Predicted Gene Target	Official Symbol, Entrez Gene ID	Protein function	Reference(s)
let-7a, let-7d, let-7e, let-7i	High mobility group AT-hook 2	HMGA2, 8091	Binds DNA in AT rich regions; predicted to affect transcription by altering chromatin conformation; important for spermatogenesis.	(Chieffi et al., 2002)
let-7i	Carbonic anhydrase II	CA2, 760	Responsible for the maintenance of adequate intraspermatozoal bicarbonate concentration; altered in low-motility samples.	(Tajima et al., 1987; C. Zhao et al., 2007)
miR-15b	Isocitrate dehydrogenase 3 (NAD+) alpha	IDH3A, 3419	Involved in the TCA cycle; necessary for sperm energy metabolism; expressed at lower levels in low-motility patients.	(C. Zhao et al., 2007)
miR-15b, miR-182	Rho GDP-dissociation inhibitor 1	ARHGDIA, 396	Associated with sperm structure; expression was decreased in low-motility patients.	(Huang et al., 2008)
miR-182, miR-24	Glycogen synthase kinase 3 alpha	GSK3A, 2931	Found in the flagellum and in the anterior portion of the sperm head. The phosphorylation of GSK3A may initiate motility in the epididymus and the expression was high in motile compared to immotile sperm.	(Vijayaraghavan et al., 2000)
miR-22	Outer dense fiber protein 1	ODF1, 4956	Necessary for sperm structure; aberrant expression causes tail abnormalities in humans; used as a marker for diagnosis of male factor infertility.	(Haidl et al., 1991; Mortimer, 1997; Petersen et al., 1999)
miR-24	Fascin homolog 1, actin-bundling protein	FSCN1, 6624	Actin-bundling protein involved in cell motility; decreased expression associated with morphologic abnormalities.	(Adams, 2004; Cheng et al., 2007)

Table 4.1 shows sperm miRNAs of interest and information regarding their predicted mRNA targets involved in sperm function. It is important to note that a single miRNA usually targets many genes and a single gene may be targeted by multiple miRNAs.

Results

Quantitative real-time PCR

Expression profiles of ten probe sets (let-7a, -7d, -7e, -7i, miRs-15b, -16, -182, -22, -24, and -92) were obtained from each sample. MiR-345, although chosen for Real-time analysis, showed a peculiar trend line resembling a double sigmoid-curve and was not analyzed further (data not shown). There were differences in the expression of five miRNAs in AB or LM groups when compared to controls, all of which are predicted to target mRNA that encode proteins responsible for sperm function. qRT-PCR revealed significant increases in the expression of four miRNAs, let-7a, -7d, -7e, and miR-22, in the AB group when compared to controls (Figure 4.1). Let-7a had nearly a 22-fold increase in expression, whereas let-7d and -7e were 5.6-fold and 3.5-fold higher than controls, respectively. miR-15b displayed a significant decrease in expression in the AB group, with a 2.5-fold decrease compared to controls. miR-92 was not significantly different between AB and controls, but the P-value was 0.10, and so should be considered a potential sperm miRNA of interest. Two miRNAs, let-7d and let-7e, were increased in the LM group when compared to controls ($P < 0.05$), with 2.5 and 6.2-fold increases in expression, respectively. Although the expression of miR-24 was not significantly different between LM and controls, a possible trend of increased expression was observed ($P= 0.09$).

Figure 4.1. Relative expression of AB and LM sperm miRNAs compared to controls

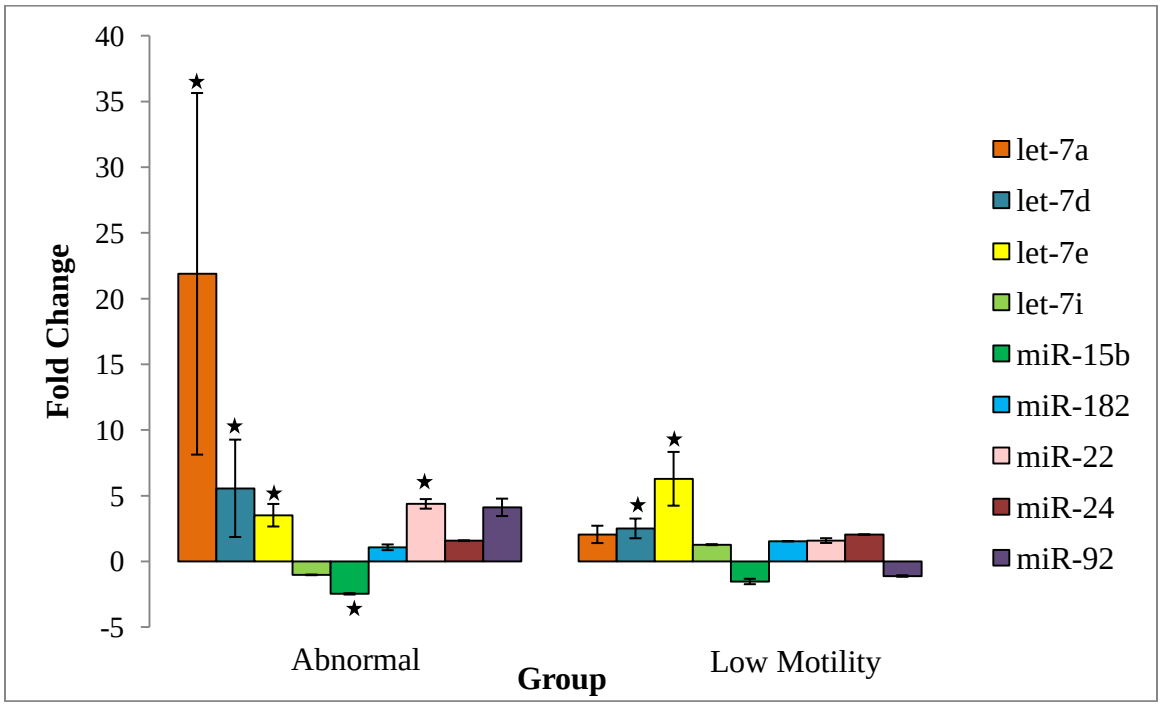

Figure 4.1 shows fold changes (efficiency^$^{-\Delta\Delta Ct}$) in miRNA expression in AB and LM as compared to controls. Error bars represent standard error (SEM). Stars indicate significance ($P < 0.05$).

Functional inference of miRNA

The bioinformatic analyses not only provided potential gene targets for the miRNAs of interest, but also allowed inferences to be deduced by grouping the genes into functional categories. When the differentially expressed miRNAs between the C and AB groups were subjected to MAMI analysis, MAMI predicted 105 miRNA-gene interactions, 62 of which were unique gene targets. DAVID analysis showed no significant enrichment of any gene ontology (GO) category meeting the requisite significance or enrichment scores. MAMI determined 55 targets of miR-15b, the only miRNA down-regulated in the AB group, all of which were unique genes. Interestingly,

DAVID results showed enriched GO categories of reproduction, spermatogenesis, male gamete generation, and cell differentiation. Since qRT-PCR analysis failed to reveal miRNAs that were down-regulated in the LM group, only the miRNAs that were up-regulated, let-7d and let-7e, were utilized in the MAMI analysis. MAMI yielded 54 miRNA-gene interactions, with 38 unique gene targets. DAVID analysis showed GO terms enriched for apoptosis and regulation of cellular and biological processes, both of which, plausibly, would affect cell motility.

Discussion

Let-7a, -7d, and -7e are all predicted to target *HMGA2* and were up-regulated in the AB samples, with let-7d and -7e also exhibiting over-expression in the LM group ($P < 0.05$). *HMGA2* is presumed to serve an essential function in spermatogenesis, as *HMGA2* null mice produce no mature spermatozoa (Chieffi et al., 2002). Over-expression of let-7a by transfection of let-7a precursor has been shown to decrease HMGA2 protein production, whereas decreasing let-7a via anti-miRNA oligonucleotides resulted in an increase in HMGA2 expression (Lee et al., 2007). In addition, *HMGA2* has been validated as a target of both let-7d and let-7e (Shell et al., 2007). HMGA2 expression is highest from spermatocyte to spermatid stage (Chieffi et al., 2002), a period during which cell division has ceased yet morphological changes ensue. It is possible that high levels of let-7 expression during the spermatogenic process alter the production of proteins required for normal spermatogenesis, potentially affecting proteins associated with morphology and motility. MiR-22 was also up-regulated in the AB group and is

predicted to target *ODF1*. ODF1 is a protein required for normal sperm structure and is over-expressed in asthenozoospermic (low motility) patients (C. Zhao et al., 2007).

MiR-15b was the only miRNA found to exhibit a significant decrease in expression in this study in either group compared to controls. MiR-15b is predicted to target the mRNA encoding ARHGDIA and IDH3A, both of which exhibited lower expression in asthenozoospermic patients (C. Zhao et al., 2007). IDH3A is an enzyme involved in the TCA cycle and the authors hypothesized that low expression of IDH3A may disrupt sperm motility by altering sperm energy metabolism. As sperm cells are terminally differentiated, it is possible that miRNAs are actually up-regulating protein production. Because miR-15b was differentially expressed in AB samples and is predicted to target statistically overrepresented genes in GO categories involved in sperm production, further investigation of its role in spermatogenesis and sperm function is warranted.

The phenotype of specific morphological abnormalities and low motility can be generated by scores of factors or conditions, both genetic and environmental. It should be acknowledged that the individual sperm samples examined in this study did not contain identical sperm cells, but were diluted with normal sperm; so technically, the AB and LM samples were contaminated with controls and vice versa. The probability of acquiring an ejaculate of homogenous sperm cells is unlikely, so laser capture microdissection could potentially be employed to isolate populations of sperm cells of a single phenotype. A sample of uniform cells would perhaps uncover differences in miRNA expression currently masked by high standard deviation due to incongruent cell types.

McCallie et al. (2010) found differential miRNA expression from blastocysts produced from healthy donor eggs and sperm from patients with non-specified male-factor infertility, which can be due to decreased sperm count, poor morphology and/ or low motility. They found that both miR-24 and let-7a were significantly down-regulated in the male-infertility factor blastocysts when compared to controls. Interestingly, our results showed no difference in miR-24 expression and a significant *increase* in the expression of let-7a in AB sperm.

The results of GO groupings were due, in part, to the established sensitivity parameters. In the MAMI analyses, a high specificity was used to avoid false positives, resulting in a smaller pool of miRNA-mRNA interactions. An increase in predicted targets (higher sensitivity) would affect DAVID enrichment scores, but would likely sacrifice accuracy. Because miRNA target predictions are essentially educated speculation, messenger RNA targets and the translation of the encoded protein should be experimentally tested to validate miRNA function. Although the precise role of miRNA in sperm remains to be elucidated, it is feasible that they are remnants of spermatogenic processes, accumulated for a role in oocyte fertilization, delivered to the oocyte to influence events in early embryonic development, or a combination thereof.

CHAPTER FIVE

IDENTIFICATION OF MICRORNAS IN PORCINE OOCYTES AND *IN VIVO*-PRODUCED EMBRYOS

Introduction

In mammals, miRNAs have been identified in the oocytes of mice (Tang et al., 2007) and cows (Tesfaye et al., 2009; Tripurani et al., 2010) and in the embryos of mice (Cui et al., 2009), cows (Castro et al., 2010; Coutinho et al., 2007), and humans (McCallie et al., 2010; Tzur et al., 2009). No research has been reported regarding the presence or function of miRNA in swine pre-implantation embryonic development. As demonstrated in other mammalian species, I predict that miRNAs are present in porcine oocytes and pre-implantation embryos. The objective of this study was to confirm that miRNAs are present in porcine oocytes and embryos at the 8-cell and blastocyst stages and to characterize trends in the expression levels of specific miRNAs at different stages of early embryonic development.

Materials and Methods

Oocyte maturation

Cumulus oopherus complexes (COCs) aspirated from abattoir gilt ovaries were obtained from Applied Reproductive Technologies, LLC (Madison, WI) and subjected to a two-step maturation process as described by Sherrer et al. (2004). Oocytes were matured in a maturation medium (tissue culture medium 199 (TCM199); Gibco, Grand Island, NY) supplemented with 0.1% polyvinylalcohol, 3.05 mM glucose, 0.91 mM

sodium pyruvate, 75 µg/ mL of potassium penicillin, 50 µg/ mL of streptomycin sulfate, 0.5 µg/ mL of ovine LH, 0.5 µg/ mL of porcine FSH, and 10 ng/ mL of murine EGF for 20- 22 h. They were the transferred to hormone-free TCM199 and incubated at 37 °C in 5% CO_2 under sterile mineral oil for 24 h. Upon removal from media, oocytes were washed once in PBS and vortexed in hyaluronidase (0.1%) to remove cumulus cells. To confirm maturation, a subset of oocytes was stained using Hoechst 33342 (Sigma-Aldrich, St. Louis, MO) to visualize the extruded second polar body. Those oocytes subjected to staining were discarded and the rest were combined into three pools, snap-frozen in liquid nitrogen, and stored at -80 °C.

Embryo collection

Cycling gilts (Landrace x Yorkshire) were subjected to estrous synchronization via two injections of dinoprost tromethamine (10 mg, i.m.; Lutalyse™, Pfizer Animal Health, New York, NY) twelve hours apart, with evaluation of estrus occurring twice daily (see Appendix E). Artificial insemination was performed at standing estrus and 12 h post-standing estrus using commercially available Duroc boar semen (Swine Genetics International, Cambridge, IA). Reproductive tracts were harvested at 4 or 7 days post-insemination and each uterine horn was flushed twice using 60 mL PBS containing bovine serum albumin (4%; Sigma, St. Louis, MO), which was passed through a 75 micron filter to recover embryos. Embryos were staged, washed twice in PBS, and transferred to 1.5 mL centrifuge tubes containing ~20 µL RNAlater® (Ambion, Austin, TX). Samples were then snap-frozen in liquid nitrogen and stored at -80 °C until RNA

isolation. All animal research was approved by the Clemson University Institutional Animal Care and Use Committee (IACUC protocol #ARC2007-41).

RNA isolation

Total RNA enriched for small RNAs was isolated from embryos using the *mir*Vana™ miRNA Isolation Kit (Ambion Inc., Austin, TX) from three pools of matured oocytes (n= 200, 50, and 5), three pools of *in vivo* produced 8-cell embryos (Day 4; n= 4, 3, and 4), and three pools of *in vivo* produced blastocysts (Day 7- 7.5; n= 5, 4, and 5). Upon removal from storage, 600 µL Lysis buffer was added to each sample and vortexed for 30 sec. A 1:10 vol of miRNA homogenate additive was mixed with each sample and incubated on ice for 10 min. Six hundred µL acid-phenol: chloroform was then added and the solution vortexed for 45 sec, followed by a 10 min centrifugation at 10,000 x g. The supernatant containing the RNA was removed and precipitated with 1.25 vol ethanol (99.5%). The solution was passed through a filter cartridge, washed, and RNA was extracted with 100 µl elution buffer, preheated to 95 °C. Sample concentration and quality were determined by spectrophotometry using the Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

PCR array

A total of 275 ng total cellular RNA from each pool of oocytes and embryos was subjected to qRT-PCR probing for 88 mature human miRNAs (Table 5.1) that have been correlated with development (Cell Differentiation & Development RT² miRNA PCR Array; SABiosciences; Frederick, MD). Because many miRNAs are highly conserved among species, the heterologous PCR array allowed for efficient cross-species

amplification. Specific miRNA abundance was classified according to the manufacturer's recommendations and miRNAs that exhibited a Ct of $\geq$ 35 were categorized as non-detectable, while detectable miRNAs were categorized as displaying low expression (Ct= 33- 34.9), moderate expression (Ct= 30- 32.9), or high expression (Ct= 25.5- 29.9) (Figure 5.1).

Figure 5.1. MiRNA expression categories

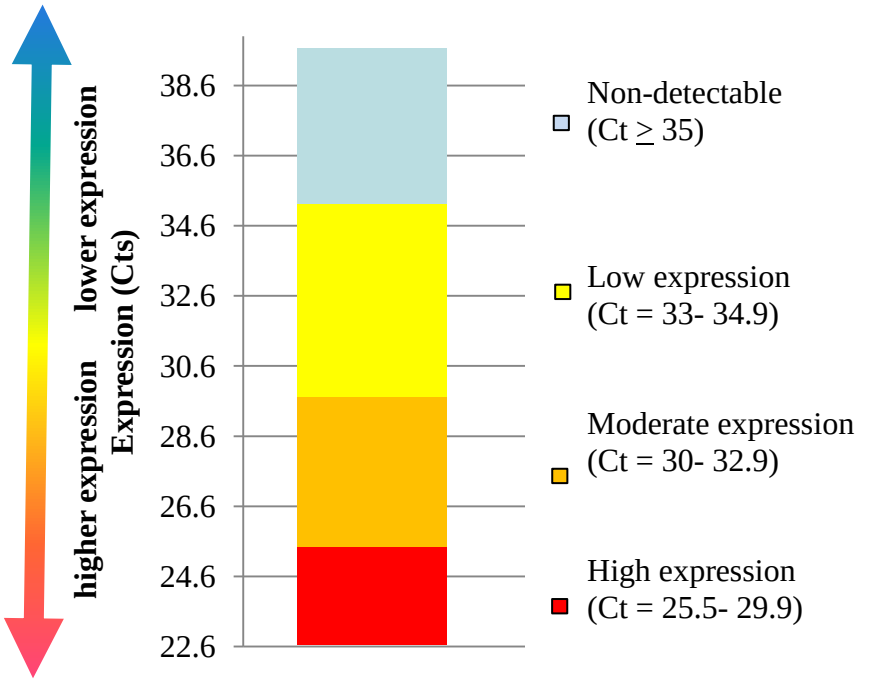

Figure 5.1 depicts the categorization of miRNA expression as determined by Ct. Ct is defined as the point at which fluorescence rises appreciably above the background (threshold) fluorescence. Cts were measured at a constant threshold among plates.

Data were analyzed using the RT^2 Profiler PCR Array Data Analysis web-based tool (SABiosciences; http://www.sabiosciences.com/pcrarraydataanalysis.php). Expression levels were normalized using the Vandesompele method (Mestdagh et al., 2009) and Chi-square analysis was used to detect differences in proportions of the number of miRNAs detected per expression category per group. P-values < 0.05 were deemed significant.

Functional inference of miRNAs

In order to elucidate the functions of differentially expressed miRNAs among oocytes, 8-cell embryos, and blastocysts, target genes were determined using MAMI server and database (meta mir: target inference; http://mami.med.harvard.edu/). A MAMI sensitivity of 0.46 and a specificity of 1.0 were used to minimize Type I Errors. DAVID v. 6.7 (http://david.abcc.ncifcrf.gov; (Dennis et al., 2003; Huang da et al., 2009)) was used to annotate MAMI-predicted target genes of differentially expressed miRNAs and to identify significant functional enrichment in the miRNA gene targets relative to the whole genome background. Entrez gene IDs were uploaded to the functional annotation tool and enriched biological themes were analyzed using a modified Fisher's exact test (EASE score; $P < 0.05$), medium classification stringency, minimum enrichment score of 1.3, and a minimum fold enrichment of 1.5. Functional annotation charts and clustering were analyzed to determine enriched molecular functions, biological processes, cellular components, biochemical pathways, and other gene annotations.

Results

PCR array

A total of 86 miRNAs were detected in at least one of the stages examined (Table 5.1). Seventy-six miRNAs (86.4% of the 88 miRNAs probed) were detected in oocytes, 63 (71.6%) in 8-cell embryos, and 84 (95.5%) in blastocysts.

Table 5.1. MiRNAs examined using the SABiosciences qRT-PCR miRNA array

miRNA	Accession	oocytes	8-cell	blasto-cysts	Porcine Accession
let-7a	MIMAT0000062	+	+	+	MI0013085
let-7b	MIMAT0000063	+	+	+	n/a
let-7c	MIMAT0000064	+	+	+	MI0002445
let-7d	MIMAT0000065	+	ND	+	n/a
let-7e	MIMAT0000066	+	+	+	MI0013086
let-7f	MIMAT0000067	ND	ND	ND	MI0002446
let-7g	MIMAT0000414	+	+	ND	MI0013087
let-7i	MIMAT0000415	+	+	+	MI0002447
miR-1	MIMAT0000416	ND	ND	+	MI0010682
miR-100	MIMAT0000098	+	+	+	MI0013128
miR-101	MIMAT0000099	+	+	+	MI0010678
miR-103	MIMAT0000101	+	+	+	MI0002448
miR-106b	MIMAT0000680	+	+	+	n/a
miR-10a	MIMAT0000253	+	+	+	MI0013101
miR-10b	MIMAT0000254	+	+	+	MI0013102
miR-122	MIMAT0000421	+	ND	+	MI0002413
miR-124	MIMAT0000422	+	+	+	MI0010680
miR-125a-5p	MIMAT0000443	+	+	+	MI0013115
miR-125b	MIMAT0000423	+	+	+	MI0013172
miR-126	MIMAT0000445	+	+	+	n/a
miR-127-5p	MIMAT0004604	+	ND	+	MI0013144
miR-128a	MIMAT0000424	+	+	+	MI0002451
miR-129-5p	MIMAT0000242	+	+	+	MI0013169
miR-130a	MIMAT0000425	+	ND	+	MI0008217
miR-132	MIMAT0000426	+	+	+	n/a
miR-133b	MIMAT0000770	ND	ND	+	MI0013089
miR-134	MIMAT0000447	+	+	+	n/a
miR-137	MIMAT0000429	ND	ND	+	n/a
miR-141	MIMAT0000432	+	+	+	n/a
miR-142-3p	MIMAT0000434	ND	+	+	MI0013134
miR-142-5p	MIMAT0000433	ND	ND	+	n/a
miR-146a	MIMAT0000449	+	+	+	n/a
miR-146b-5p	MIMAT0002809	+	ND	+	MI0010685
miR-150	MIMAT0000451	+	+	+	n/a
miR-155	MIMAT0000646	+	ND	ND	n/a
miR-15a	MIMAT0000068	+	ND	+	MI0008211
miR-15b	MIMAT0000417	+	+	+	MI0002419
miR-16	MIMAT0000069	+	+	+	MI0008213
miR-17	MIMAT0000070	+	+	+	MI0008214

miR-181a	MIMAT0000256	+	+	+	MI0010686
miR-182	MIMAT0000259	+	+	+	n/a
miR-183	MIMAT0000261	+	+	+	MI0002439
miR-185	MIMAT0000455	+	+	+	MI0008218
miR-18a	MIMAT0000072	+	+	+	MI0002455
miR-18b	MIMAT0001412	+	ND	+	n/a
miR-192	MIMAT0000222	+	+	+	MI0013127
miR-194	MIMAT0000460	+	+	+	n/a
miR-195	MIMAT0000461	+	+	+	MI0013141
miR-196a	MIMAT0000226	+	ND	+	MI0002457
miR-205	MIMAT0000266	+	+	+	MI0002440
miR-206	MIMAT0000462	+	ND	+	MI0013084
miR-208	MIMAT0000241	+	+	+	n/a
miR-20a	MIMAT0000075	+	+	+	MI0002423
miR-20b	MIMAT0001413	+	+	+	n/a
miR-21	MIMAT0000076	+	+	+	MI0002459
miR-210	MIMAT0000267	+	+	+	MI0008220
miR-214	MIMAT0000271	+	+	+	MI0002441
miR-215	MIMAT0000272	ND	ND	+	MI0010687
miR-218	MIMAT0000275	+	+	+	n/a
miR-219-5p	MIMAT0000276	+	+	+	n/a
miR-22	MIMAT0000077	+	ND	+	MI0014770
miR-222	MIMAT0000279	+	+	+	MI0013151
miR-223	MIMAT0000280	+	+	+	n/a
miR-23b	MIMAT0000418	+	+	+	MI0013112
miR-24	MIMAT0000080	+	+	+	MI0002428
miR-26a	MIMAT0000082	+	+	+	MI0002429
miR-301a	MIMAT0000688	+	+	+	MI0002432
miR-302a	MIMAT0000684	+	+	+	n/a
miR-302c	MIMAT0000717	+	+	+	n/a
miR-33a	MIMAT0000091	+	+	+	n/a
miR-345	MIMAT0000772	+	ND	+	MI0013117
miR-370	MIMAT0000722	+	+	+	n/a
miR-371-3p	MIMAT0000723	+	+	+	n/a
miR-375	MIMAT0000728	+	+	+	n/a
miR-378	MIMAT0000732	+	+	+	MI0013088
miR-424	MIMAT0001341	+	+	+	MI0013135
miR-452	MIMAT0001635	+	ND	+	n/a
miR-488	MIMAT0004763	ND	ND	+	n/a
miR-498	MIMAT0002824	ND	ND	+	n/a
miR-503	MIMAT0002874	ND	ND	ND	MI0010684
miR-518b	MIMAT0002844	ND	ND	+	n/a

miR-520g	MIMAT0002858	ND	ND	+	n/a
miR-7	MIMAT0000252	+	+	+	MI0002435
miR-9	MIMAT0000441	+	+	+	MI0002462
miR-92a	MIMAT0000092	+	+	+	MI0013125
miR-93	MIMAT0000093	+	+	+	n/a
miR-96	MIMAT0000095	+	+	+	n/a
miR-99a	MIMAT0000097	+	+	+	MI0013114

Table 5.1 shows the miRNAs probed for in the PCR array and their presence (+) or absence (ND) in oocytes, 8-cell embryos, and blastocysts. If the miRNA had been identified previously in pigs, the miRBase v. 14.0 accession number is also shown.

Let-7f and miR-503 were the only two miRNAs not detected in any of the samples. Chi-square analysis showed differences in proportions of detectable vs. non-detectable miRNAs between oocytes and 8-cell embryos (P= 0.026) and 8-cell embryos and blastocysts (P< 0.0001), but not between oocytes and blastocysts (P= 0.064) (Figure 5.2).

Figure 5.2. Total number of detectable vs. non-detectable miRNAs at each stage

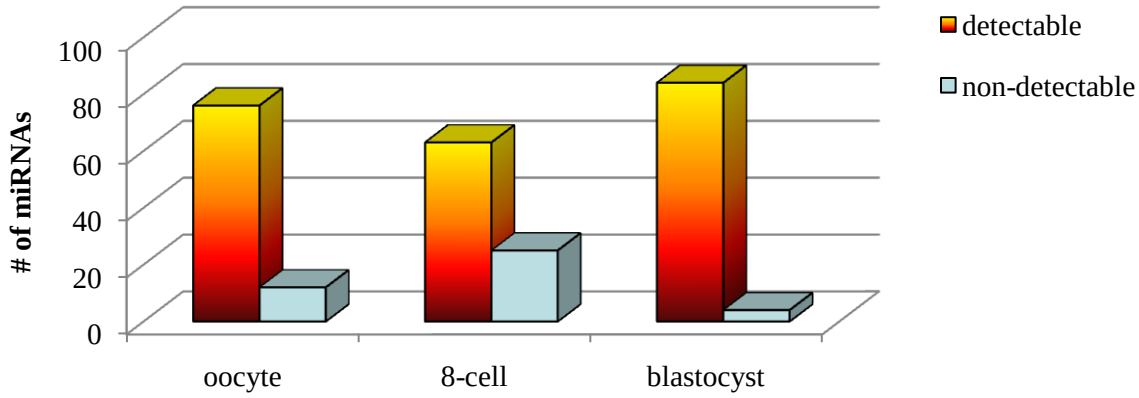

Figure 5.2 shows the total number of detectable and non-detectable miRNAs at the oocyte, 8-cell, and blastocyst stages. Chi-square analysis showed significant differences in the proportions of detectable vs. non-detectable miRNAs between oocytes and 8-cell embryos (P= 0.026) and 8-cell embryos and blastocysts (P< 0.0001).

Figure 5.3. Number of detectable miRNAs at each stage by expression category

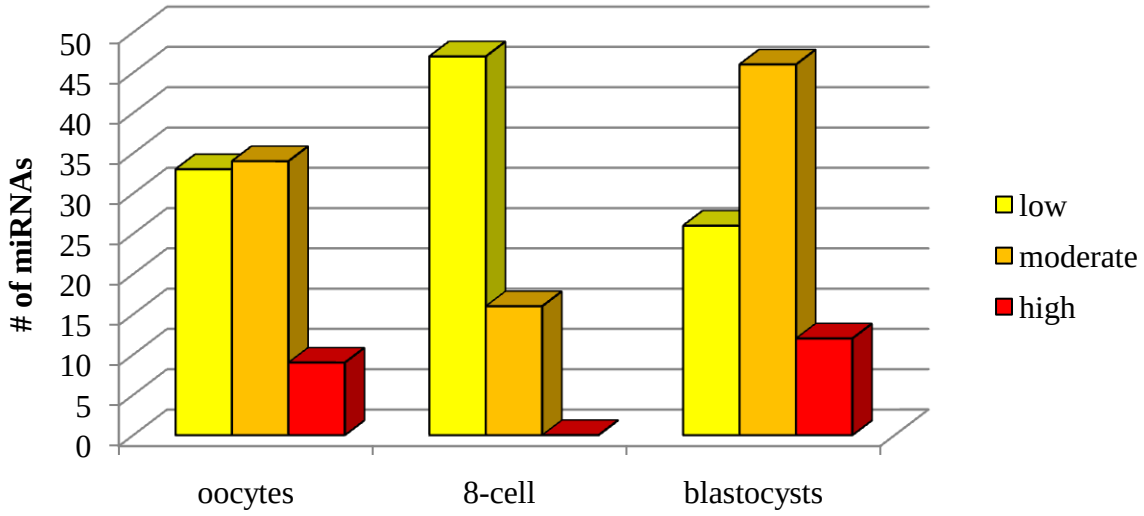

Figure 5.3 shows the total number of detectable miRNAs at the oocyte, 8-cell, and blastocyst stages broken down into expression categories of low, moderate, and high expresssion. Chi-square analysis showed significant differences in the proportions of miRNAs at various expression levels between oocytes and 8-cell embryos ($P < 0.0001$) and 8-cell embryos and blastocysts ($P < 0.0001$).

In analyzing only the detectable miRNAs, Chi-square analysis showed significant differences in the proportions of embryos at various expression levels between oocytes and 8-cell embryos ($P < 0.0001$) and 8-cell embryos and blastocysts ($P < 0.0001$), but not between oocytes and blastocysts (Figure 5.3).

The most highly expressed miRNAs in oocytes were miRs-21, -205, -195, -16, and -23b. The most highly expressed in 8-cell embryos were miR-125b, -205, -128, -17, and -125a-5p, and the most highly expressed miRNAs in blastocysts were miRs-210, -92a, -302a, -129-5p, and -205. Significant differences were found in the expression levels of 21 specific miRNAs among oocyte, 8-cell, and blastocysts stages (Table 5.2).

Table 5.2. Expression levels of individual miRNAs among stages

	Average Δ(Ct) (Ct (miRNA) – Ct (normalizer))			P-value		
mature ID	**oocytes**	**8-cell**	**blastocysts**	**oocytes vs. 8-cell**	**oocytes vs. blastocysts**	**blastocysts vs. 8-cell**
let-7b	-3.287	-1.223	0.400	0.033	0.015	0.265
let-7c	-1.220	0.017	1.107	0.094	0.043	0.002
let-7e	-1.670	0.263	2.053	0.159	0.114	0.005
let-7g	-1.950	0.167	3.043	0.114	0.078	0.001
miR-100	-1.017	-0.490	0.540	0.506	0.052	0.227
miR-101	-0.343	-0.290	1.880	0.982	0.038	0.089
miR-103	-1.637	-0.337	-2.240	0.360	0.812	0.003
miR-125b	-0.853	-3.503	0.143	0.158	0.405	0.042
miR-128	-2.130	-2.507	0.753	0.784	0.150	0.009
miR-142-3p	2.007	0.003	2.980	0.100	0.428	0.002
miR-182	-1.383	0.293	1.713	0.282	0.229	0.042
miR-183	1.783	-0.220	1.157	0.083	0.875	0.050
miR-18a	-1.587	-0.393	-1.093	0.012	0.018	0.152
miR-194	1.447	0.433	2.890	0.336	0.286	0.023
miR-222	1.267	0.147	-0.747	0.158	0.054	0.104
miR-223	1.323	0.120	0.580	0.824	0.850	0.015
miR-24	-3.020	-0.500	-0.957	0.042	0.051	0.425
miR-301a	1.053	0.183	0.860	0.177	0.935	0.042
miR-370	0.840	0.093	-0.973	0.441	0.043	0.656
miR-378	1.637	-0.077	-1.270	0.097	0.032	0.079
miR-99a	-2.130	-1.197	-0.420	0.286	0.181	0.029

Table 5.2 shows the delta Ct of miRNAs that exhibited significant differences in expression ($P < 0.05$). A total of 21 miRNAs (of 88 examined) were differentially expressed between oocytes and 8-cells, 8-cells and blastocysts, or oocytes and blastocysts. Significant P-values are shown in red.

Functional inference of miRNAs

The bioinformatic analyses not only provided potential gene targets for the miRNAs of interest, but also allowed inferences to be deduced by grouping the genes into functional categories. When the differentially expressed miRNAs between the oocytes and the 8-cell embryos were subjected to MAMI analysis, 56 targets were identified, with 57 miRNA-gene interactions (one gene was a target of both let-7b and miR-24). The MAMI results for the differentially expressed miRNAs between the 8-cell embryos and blastocysts identified 188 gene targets with 253 miRNA-gene interactions. The differentially expressed miRNAs between the oocytes and blastocysts yielded 123 possible miRNA-gene interactions, with 95 predicted gene targets.

DAVID analysis of genes predicted to be targeted by miRNAs differentially expressed between oocytes and 8-cell embryos showed enriched gene ontology (GO) categories for regulation of TGFβ receptor signaling, helicase activity, cellular protein localization, and nucleotide binding. Targets of miRNAs differentially expressed between 8-cell embryos and blastocysts were overrepresented in GO categories such as cell motion and migration, helicase activity, chromatin organization, transcriptional activity, and a cellular component enrichment of Golgi cis cistern. Lastly, targets of miRNAs differentially expressed between oocytes and blastocysts had enriched GO categories for nucleotide binding, positive regulation of transcription, epigenetic regulation of gene expression, chromatin remodeling, and response to nitrogen and amines.

Discussion

This study confirmed the presence of miRNAs in porcine oocytes and embryos at the 8-cell and blastocyst stages. A total of 86 miRNAs were detected in at least one of the stages examined and differences in the proportions of detectable vs. non-detectable were found between oocyte and 8-cell embryos and between 8-cell embryos and blastocysts, with oocytes and blastocysts being the most similar. It is generally accepted that mature oocytes of all species accumulate large amounts of RNA (Marello et al., 1992). The 8-cell group had the lowest number of detectable miRNAs, which is consistent with the shift from the maternal to embryonic genome and probably reflects the degradation of maternal miRNAs. These data indicate that, like mRNA, miRNAs from maternal sources may be depleted prior to the 8-cell embryonic stage [in porcine embryos] and that miRNA expression post-8-cell embryonic stage is due to the activation of the fetal genome.

The let-7 family, a highly conserved group of miRNAs shown to play critical roles in developmental timing in non-mammalian species, was present in porcine oocytes and embryos, and, for let-7b, -7c, 7e, and -7g, exhibited significantly higher expression in the oocytes. Let-7 is involved in cell cycle regulation and has been referred to as the ‘master regulator’ of cell proliferation pathways (Johnson et al., 2005), having a role in activating the terminal differentiation of organs, tissues, and specific cell types.

CHAPTER SIX

COMPARATIVE MICRORNA EXPRESSION IN BOTH *IN VIVO*- AND *IN VITRO*-PRODUCED PRE-IMPLANTATION EMBRYOS

Introduction

Assisted reproductive technologies (ART) such as somatic cell nuclear transfer and intracytoplasmic sperm injection frequently lead to abnormal embryonic growth; however, the underlying mechanisms responsible for aberrant development remain largely unknown. The assessment of embryo quality is prone to subjective biases and is generally ill-defined (Ebner et al., 2003). Embryologists consider morphological parameters such as cellular fragmentation, symmetry of blastomere cleavage, and rate of development (Selk, 2009; Steer et al., 1992), but these characteristics can be poor indicators of blastocyst and pregnancy rates (Graham et al., 2000). The measurement of biochemical components in the embryo culture media as a non-invasive method to deduce embryo quality have been studied, such as platelet activating factor (Roudebush et al., 2002) and amino acid consumption (Booth et al., 2007; Brison et al., 2004), both of which resulted in parameters correlated with pregnancy rates and live births.

Recent interest has turned to gene expression studies for a quantitative evaluation of embryo quality and to identify embryos with the highest potential for developmental competency. Van Montfoort et al. (2008) used microarray analysis to determine differentially expressed genes between cumulus cells removed from oocytes that resulted in early embryo cleavage following IVF versus cumulus cells removed from those that did not cleave. They found the most differentially expressed were those involved in

response to hypoxic conditions or delayed oocyte maturation. A similar study (Assou et al., 2008) revealed that the expression levels of specific genes by cumulus cells were significantly correlated with pregnancy outcomes. Although genes have been examined in early embryonic development, data is scarce describing differences in gene expression in *in vivo*- versus *in vitro*-produced embryos.

Small non-coding RNAs have been shown to regulate gene expression during gametogenesis and embryonic development, and could play a role in the abnormal development of embryos produced through ART. The ablation of the miRNA pathway is embryonic lethal (Bernstein et al., 2003), indicating that the miRNA regulatory pathway may contribute to the failure of miRNA-deficient embryos to develop or to establish and maintain pregnancy. We predict that embryonic stress induced by *in vitro* culture systems will lead to altered miRNA expression, when compared to *in vivo*-produced embryos at corresponding stages. The miRNAs differentially expressed would be candidates for markers of embryonic quality. The objective of this study was to identify differences in miRNA expression between *in vivo*- and *in vitro*- produced porcine embryos at specific stages of pre-implantation development.

Materials and Methods

In vivo- produced embryos

Estrous synchronization was achieved by feeding a synthetic progestin, Altrenogest (Matrix^{TM}; 15 mg p.o., Schering-Plough), to cycling gilts (Landrace x Yorkshire) for 14 consecutive days. Estrous detection was initiated 24 h after the discontinuation of Matrix (Appendix E). Artificial insemination was performed at standing estrus and 12 h post

standing estrus using commercially available boar semen (Swine Genetics International, Cambridge, IA). Reproductive tracts were harvested at 2, 4, or 7 days post-insemination and each oviduct or uterine horn was flushed twice using 5 mL or 60 mL PBS, respectively, containing bovine serum albumin (4%; Sigma, St. Louis, MO), which was then passed through a 75 micron filter to recover embryos. Embryos were examined, assigned a developmental stage, washed twice in PBS, and transferred to individual 1.5 mL centrifuge tubes containing ~5 µL RNAlater® (Ambion, Austin, TX). Embryos exhibiting a retarded developmental stage, a mid-mitotic phase, and/ or excessive fragmentation were not analyzed. Samples were then snap-frozen in liquid nitrogen and stored at -80 °C until RNA isolation. All animal research was approved by the Clemson University Institutional Animal Care and Use Committee (IACUC protocol #ARC2007-41).

In vitro- produced embryos

The *in vitro*- fertilized (IVF) embryos in this study were obtained from Drs. Rebecca Krisher and Melissa Paczkowski (University of Illinois, Urbana, IL). All chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) unless specified otherwise. Ovaries of cycling sows and prepubertal gilts were collected from two local abattoirs (Momence Packing Co. and Indiana Packers Corp., respectively) and transferred to the laboratory in 30- 34 °C 0.9% (w/v) NaCl. Prepubertal ovaries were confirmed by the absence of developed corpora lutea. Oocytes were vacuum aspirated from 3- 8 mm follicles using an 18-gauge needle. Oocytes with several layers of unexpanded cumulus cells and uniformly dark cytoplasm were selected and rinsed in Hepes-buffered synthetic oviductal

fluid supplemented with 0.1% BSA (SOF-HEPES) (Gandhi et al., 2000; Tervit et al., 1972). Selected oocytes were matured *in vitro* in TCM 199 medium (Invitrogen Corp.) supplemented with 3.05 mM glucose, 0.91 mM pyruvate, 0.5 mM cysteine, 10 ng/mL EGF, 0.01 units/mL LH and FSH (Sioux Biochemicals, Sioux City, IA), 1% v/v PSA (100 IU/mL penicillin, 100 µg/mL streptomycin, 0.25 ng/mL amphotericin B; MP Biomedicals) and 1 mL porcine follicular fluid, for 42 - 44 h in 7% CO_2 in humidified air at 38.7 °C. Following maturation, oocytes were denuded by vortexing for 3 minutes in SOF-HEPES with 100 µg/mL (80-160 U/mL) hyaluronidase.

Denuded oocytes were washed three times in modified Tris-buffered medium (mTBM) (Abeydeera et al., 1997a; Abeydeera et al., 1997b) supplemented with 2 mM caffeine, 0.2% (w/v) fraction BSA and 1% v/v PSA. Oocytes were placed into 50 µL drops of mTBM under 10 mL mineral oil (20 oocytes/ drop). Sperm preparation was performed by placing 1 mL of chilled, extended (1: 5 dilution, Androhep EnduraGuard, Minitube of America Inc., Verona, WI, USA) semen, warmed for 20 min, onto a gradient of 45%: 90% Percoll^{TM} (GE Healthcare Life Sciences) and centrifuged for 20 min at 700 x *g*. The supernatant was removed and the remaining sperm pellet was washed in 5 mL D-PBS (GIBCO Invitrogen) twice by centrifuging for 5 min at 1000 x *g*. Sperm were then counted, diluted in mTBM, and added to drops (final volume 100 uL) containing oocytes for a final sperm concentration of 250,000 sperm/mL. Gametes were co-incubated for 5 h in 5% CO_2 in humidified air. Following co-incubation, zygotes were washed three times and cultured in 50 µL NCSU-23 (Petters et al., 1993) medium (10 zygotes/ drop) containing 0.4% crystallized BSA (MP Biomedicals) under 10 mL mineral

oil in 5% CO_2, 10% O_2, 85% N_2. Embryos were collected over seven replicates at the 4-, 8-, 16-cell and blastocyst stages, and subsequently frozen at -80 °C until RNA isolation.

RNA isolation

Total RNA enriched for small RNAs was isolated from individual embryos (n= 151) using the *mir*Vana™ miRNA Isolation Kit (Ambion Inc., Austin, TX). Upon removal from storage, 600 µL lysis buffer was added to the sample and vortexed for 30 sec. A 1:10 vol of miRNA homogenate additive was mixed with the sample and incubated on ice for 10 min. A volume of acid-phenol: chloroform was added equal to that of the lysis buffer and the solution was vortexed for 45 sec, followed by a 10 min centrifugation at 10,000 x g. The supernatant containing the RNA was removed and precipitated with 1.25 vol ethanol (99.5%). The solution was passed through a filter cartridge, washed, and RNA was extracted with 100 µl elution buffer, preheated to 95 °C. Sample concentration and quality were determined by spectrophotometry using the Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

Quantitative RT-PCR

Real time quantitative RT-PCR was conducted using the Realplex Mastercycler epgradient (Eppendorf**,** Hamburg, Germany). Reactions were performed using the Taqman® MicroRNA Reverse Transcription Kit and Taqman® MicroRNA Assays[1] (Applied Biosystems, Foster City, CA) and examined the expression levels of four

[1] During the course of the previous sperm studies, the Ambion miRNA qRT-PCR primer sets became unavailable due to the purchase of Ambion by Applied Biosystems and the phasing out of those primers. We chose to use the stem-loop RT-PCR-based TaqMan system (Figure 1.8) because of its specificity, the availability of specific primers, and the growing number of reports describing its use.

specific miRNAs: let-7b, miR-18a, miR-21, and miR-24. The miRNAs evaluated were chosen based on the following criteria: 1) expression as described in Chapter V; 2) bioinformatic analysis identifying messenger RNA targets of interest, and/ or; 3) their validated presence in human pre-implantation embryos at corresponding stages.

A multiplex reverse transcription (RT) reaction was performed for each embryo RNA sample by pooling the RT primers and concentrating them to 5X using a speed vacuum (SVC100H-200, Savant, Thermo Fisher Scientific, Waltham, MA) as recommended by Applied Biosystems technical support. Each RT reaction consisted of 10 ng embryo RNA, 1 µL RT primer pool, 0.2 µL 100 mM dNTPs, 2 µL MultiScribe^{TM} Reverse Transcriptase (50 U/µL), 1 µL 10X reverse transcription buffer, 0.125 µL RNase Inhibitor (20 U/µL), and was brought up to a total volume of 10 µl in nuclease-free H_2O. The RT reaction was incubated on ice for 5 min, at 16 ^{o}C for 30 min, 42 ^{o}C for 30 min, and then at 85 ^{o}C for 5 min.

Due to the limited amount of embryo miRNA, a pre-amplification step was employed to increase the starting amount of cDNA template. Each pre-amplification reaction consisted of 6.25 µL 2X Taqman® PreAmp Master Mix, 4.38 µL of pooled Taqman Assays containing each of the four miRNAs to be examined (0.2X each), and 1.89 µL (1.89 ng cDNA) of RT reaction. The reactions were incubated at 95 ^{o}C for 10 min, 55 ^{o}C for 2 min, 72 ^{o}C for 2 min, then 12 cycles of 95 ^{o}C for 15 sec and 60 ^{o}C for 4 min. Upon completion, reactions were immediately placed on ice and diluted 4-fold by the addition of 37.5 µL 0.1X TE. Samples were stored at -20 ^{o}C until PCR analysis.

Each PCR reaction (20 µl) consisted of 1.33 µL of pre-amplification reaction, 10 µL Taqman 2X Universal PCR Master Mix (No AmpErase® UNG), and 1 µL TaqMan MicroRNA Assay (20X) and was initiated with a cycle of 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 sec and primer annealing/ extending at 60 °C for 60 sec. Ct was defined as the point at which fluorescence rises appreciably above the background (threshold) fluorescence and was measured at a constant threshold among plates. To address potential pre-amplification bias and to validate the linear amplification of cDNA during the pre-amplification process, standard curves were performed comparing cDNA to pre-amplified cDNA. Results were consistent with those of other studies (Chen et al., 2009; Mengual et al., 2008; Mestdagh et al., 2008) and showed an unbiased linear amplification (Figure 6.1).

There was difficulty in determining a suitable normalization gene for this analysis. RNU-48 endogenous control (Applied Biosystems) was evaluated, but was not present in porcine tissues (although was found to be highly expressed in human control RNA tested at equivalent RNA input). Ribosomal protein 18S had been previously reported to be stably expressed in porcine pre-implantation development *in vitro* (Kuijk et al., 2007), but we found the 18S Taqman primer set to demonstrate a high degree of non-specific binding, as it amplified product not only from cDNA, but also from DNA, RNA, and in the no template negative control. Although the undesired resulting bands were much larger (~ 200 bp) than the anticipated 18S product size when viewed following gel electrophoresis, they were indistinguishable from the desired product in real-time

analysis. Let-7b was found to exhibit stable expression across all embryos and between groups, nominating it as a suitable housekeeping gene.

Figure 6.1. Standard curves obtained from cDNA vs. pre-amplified cDNA

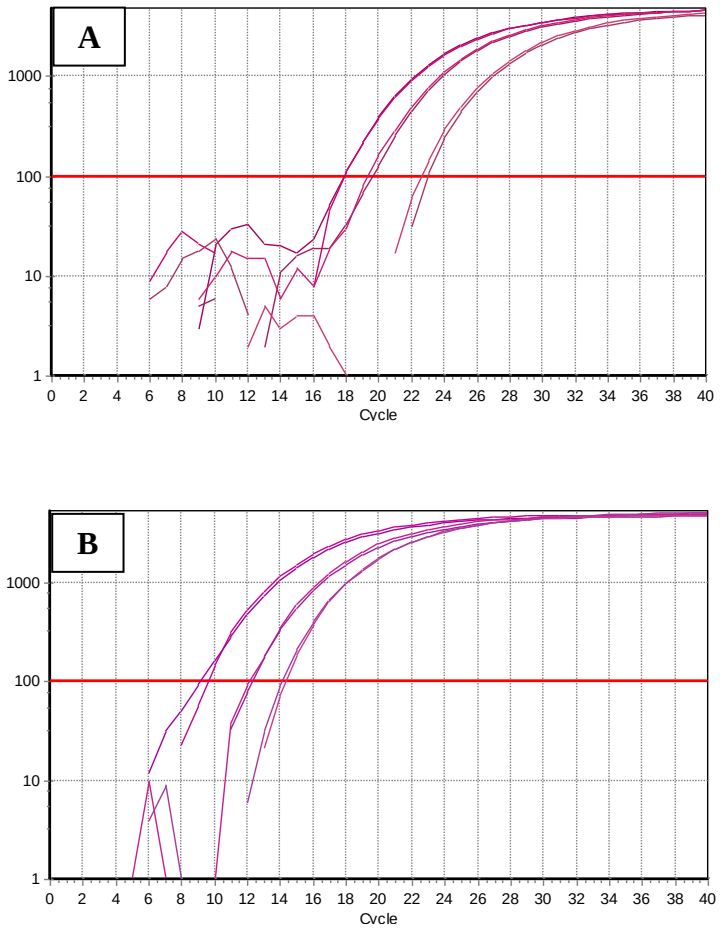

Figure 6.1. Real-time analysis of miR-24 in porcine kidney. Standard curves were performed to compare qRT-PCR reactions on cDNA (Panel A) to pre-amplified cDNA (Panel B). Each resulted in identical slopes, but the Ct values for the pre-amplified cDNA were decreased. These results are representative of all miRNA primer sets examined.

Statistical analysis

Expression levels of miRNAs were normalized to those of let-7b, which exhibited minimal variation among all embryo samples. Normalization to let-7b was verified using

both Normfinder (Andersen et al., 2004) and Bestkeeper (Pfaffl et al., 2004) software. Standard curves for each miRNA primer set were calculated on 5-fold serial dilutions of reference RNA ranging from 25 ng to 0.04 ng. Statistical analyses were performed using JMP (version 8.0; SAS Institute, Inc., Cary, NC) at a 95% significance level and were confirmed with REST-2009 software (Pfaffl et al., 2002). As recommended by Ambion technical support, miRNAs that were non-detectable were assigned a Ct of 40 to allow for appropriate statistical analyses.

Data were analyzed using a completely randomized two-way complete model. The two factors of interest were group (i.e. production type), with levels IVO and IVF, and stage, with levels 4-cell, 8-cell, 16-cell, and blastocyst. Raw Ct values were normalized to let-7b and subjected to ANOVA testing. A test for interaction between the two factors was performed before analyzing the main effects separately. The least squares means were used to determine significant differences with PDIFF at alpha= 0.05.

Functional inference of miRNA

In order to elucidate the functions of differentially expressed miRNAs among embryos from different developmental stages and between those produced via IVO and IVF, their target genes were extracted using MAMI server and database (meta mir: target inference; http://mami.med.harvard.edu/) which provides a composite prediction of target genes from five independent target prediction tools: TargetScan (Lewis et al., 2005), miRanda (John et al., 2004), DIANA-microT (Maragkakis et al., 2009), miRtarget (Grun et al., 2005) and picTar (Krek et al., 2005). MAMI parameters were a sensitivity of 0.66 and a specificity of 0.8.

The Database for Annotation, Visualization and Integrated Discovery (DAVID v. 6.7; http://david.abcc.ncifcrf.gov; (Dennis et al., 2003; Huang da et al., 2009)) was used to annotate MAMI-predicted target genes of differentially expressed miRNAs and to identify significant functional enrichment in the miRNA gene targets relative to the whole genome background. Entrez gene IDs were uploaded to the functional annotation tool and enriched biological themes were analyzed using a modified Fisher's exact test (EASE score; $P < 0.05$), medium classification stringency, minimum enrichment score of 1.3, and a minimum fold enrichment of 1.5. Functional annotation charts and clustering were analyzed to determine enriched molecular functions, biological processes, cellular components, biochemical pathways, and other gene annotations.

Results

Embryos

A total of 151 embryos at 4-cell, 8-cell, 16-cell, or blastocyst stage were collected for analysis. Seventy-seven were collected from gilts and 74 embryos were produced by *in vitro* methods (Table 6.1).

Table 6.1. Total number of embryos examined by group and stage

	4-cell	8-cell	16-cell	blastocyst	total
IVO	24	20	10	23	77
IVF	18	19	18	19	74
Total	42	39	28	42	151

Quantitative RT-PCR

Table 6.2. Slopes and efficiencies of standard curves for each Taqman primer set

miRNA	slope	efficiency
let-7b	-3.667	1.87
miR-18a	-3.726	1.86
miR-21	-3.425	1.96
miR-24	-4.057	1.76

Let-7b

Let-7b was detected in all embryos evaluated. The average Ct for let-7b in the IVO embryos was 26.93 (SEM= 0.13) and the average Ct of let-7b in the IVF embryos was 27.35 (SEM= 0.11). There were no differences in the detection of let-7b by group or among stages (P> 0.05), advocating its use as a suitable reference gene.

Figure 6.2. Average Cts of let-7b by group and stage (non-normalized)

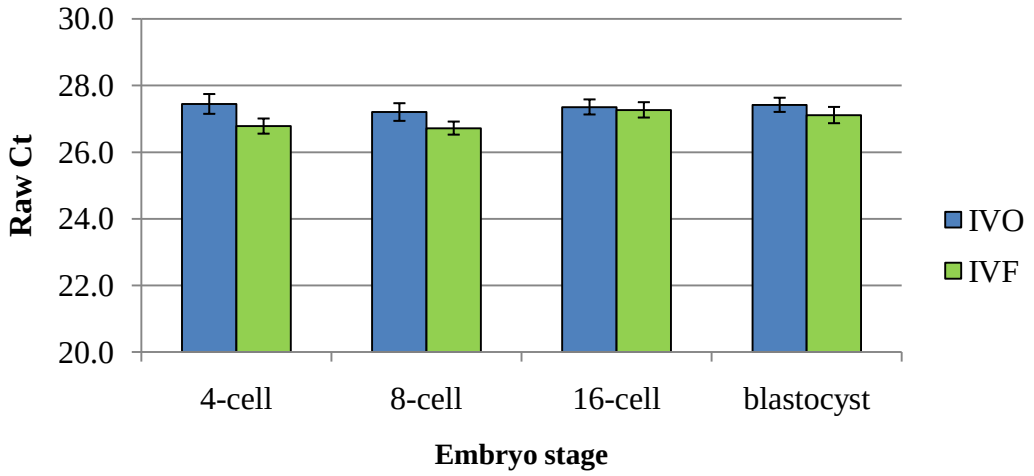

Figure 6.2 shows mean Ct (± SEM) of let-7b in IVO and IVF embryos at different stages.

miR-18a

MiR-18a was detected in 46 of the IVO embryos (59.7%) and in 43 of the IVF embryos (58.1%). Although there were no differences between groups, Chi-square analysis showed a significant difference in the proportions of embryos at different developmental stages in which miR-18a was detectable vs. non-detectable ($P < 0.05$). Differences were found in 4-cell vs. blastocyst ($P < 0.001$), 4-cell vs. 16-cell ($P < 0.01$), and 8-cell vs. blastocysts ($P < 0.001$). Results also showed that, although there were no effects of group or group*stage on miR-18a expression, there was an effect of developmental stage (Figure 6.4).

Table 6.3. Detection of miR-18a in porcine embryos

Group	Detection	4-cell	8-cell	16-cell	Blastocyst	Total
IVO	# present (%)	9 (37.5)	11 (55.0)	7 (70.0)	19 (82.6)	46 (59.7)
	# undetectable (%)	15 (62.5)	9 (45.0)	3 (30.0)	4 (17.4)	31 (40.3)
IVF	# present (%)	5 (27.8)	9 (47.4)	12 (66.7)	17 (89.5)	43 (58.1)
	# undetectable (%)	13 (72.2)	10 (52.6)	6 (33.3)	2 (10.5)	31 (41.9)
Total	# present (%)	14 (33.3)	20 (51.3)	19 (67.9)	36 (85.7)	89 (58.9)
	# undetectable (%)	28 (66.7)	19 (48.7)	9 (32.1)	6 (14.3)	62 (41.1)

Table 6.3 lists the number of embryos by group and by stage in which miR-18a was detected. Although there were no differences by production type, Chi-square analysis showed differences in proportions of detectable vs. non-detectable between 4-cell and blastocyst ($P < 0.001$), 4-cell and 16-cell ($P < 0.01$), and 8-cell and blastocysts ($P < 0.001$).

Figure 6.3. Detection rate of miR-18a in porcine embryos

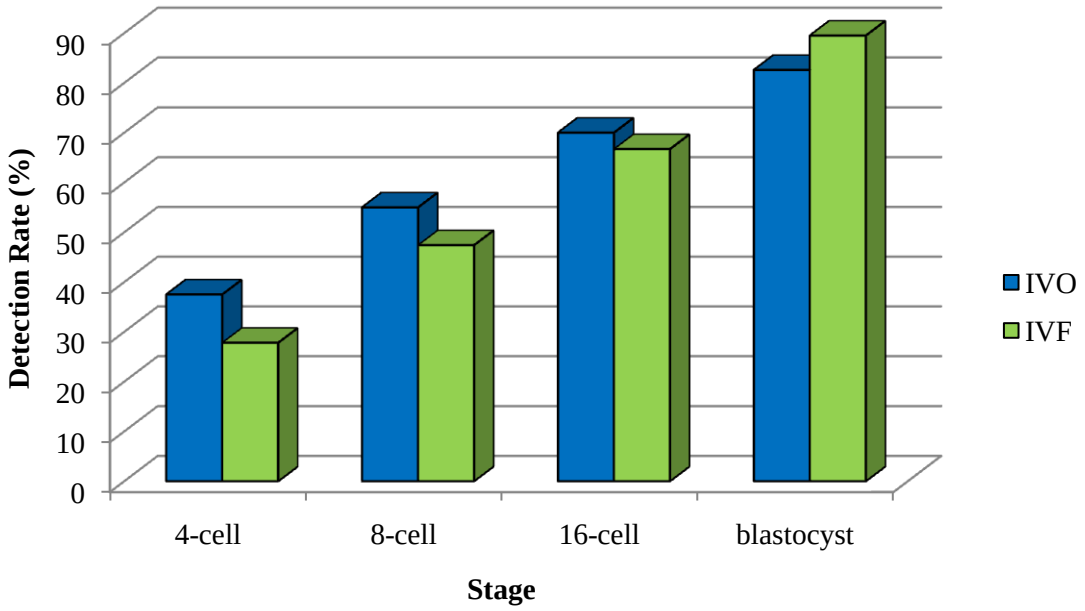

Figure 6.3 depicts the percentage of embryos by group and by stage in which miR-18a was detected. The proportion of embryos in which miR-18a was detectable increased with progressive developmental stages.

Figure 6.4. Normalized miR-18a expression at different stages

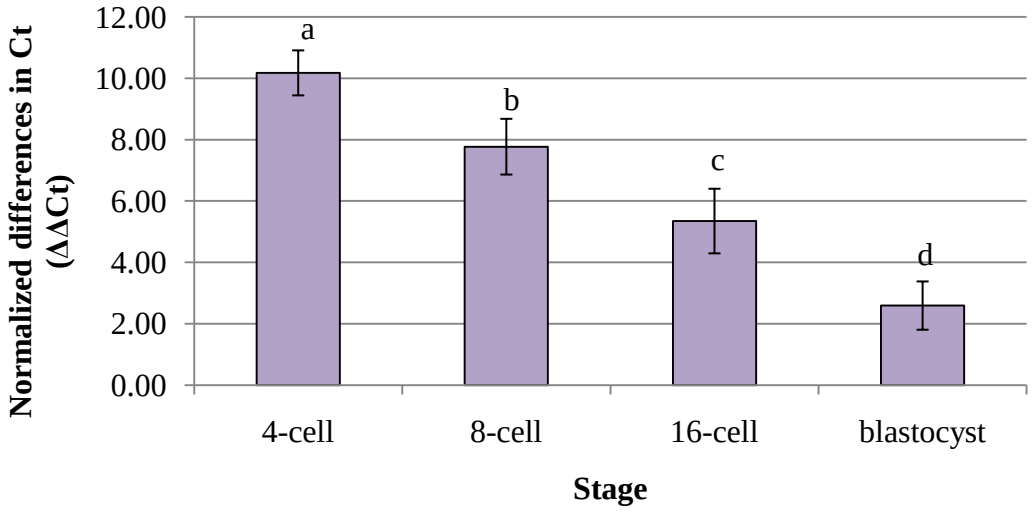

Figure 6.4 shows the normalized differences in Ct for miR-18a for the combined IVO and IVF groups at each stage. Error bars represent the SEM. Bars not sharing letters are statistically different ($P < 0.05$).

miR-21

MiR-21 was detected in 53 of the IVO embryos (68.8%) and in 50 of the IVF embryos (67.6%). Like miR-18a, there were no differences between groups, but Chi-square analysis showed a significant difference in the proportions of embryos at different developmental stages in which miR-21 was detectable vs. non-detectable ($P < 0.05$). Differences were found in 4-cell vs. blastocyst ($P < 0.05$) and 8-cell vs. blastocysts ($P < 0.05$). Results also showed that, as for miR-18a, there were no effects of group or group*stage on miR-21 expression; however, there was an effect of developmental stage (Figure 6.6). Differences were found between 4-cell embryos and blastocysts and between 8-cell embryos and blastocysts ($P < 0.05$).

Table 6.4. Detection of miR-21 in porcine embryos

Group	Detection	4-cell	8-cell	16-cell	Blastocyst	Total
IVO	# present (%)	15 (62.5)	13 (65.0)	8 (80.0)	17 (73.9)	53 (68.8)
	# undetectable (%)	9 (37.5)	7 (35.0)	2 (20.0)	6 (26.1)	24 (31.2)
IVF	# present (%)	10 (55.6)	11 (57.9)	12 (66.7)	17 (89.5)	50 (67.6)
	# undetectable (%)	8 (44.4)	8 (42.1)	6 (33.3)	2 (10.5)	24 (32.4)
Total	# present (%)	25 (59.5)	24 (61.5)	20 (71.4)	34 (81.0)	103 (68.2)
	# undetectable (%)	17 (40.5)	15 (38.5)	8 (28.6)	8 (19.0)	48 (31.8)

Table 6.4 lists the number of embryos by group and by stage in which miR-21 was detected. Chi-square analysis showed a significant difference in the proportions of embryos at different developmental stages in which miR-21 was detectable vs. non-detectable: 4-cell vs. blastocyst ($P < 0.05$) and 8-cell vs. blastocysts ($P < 0.05$).

Figure 6.5. Detection of miR-21 in porcine embryos

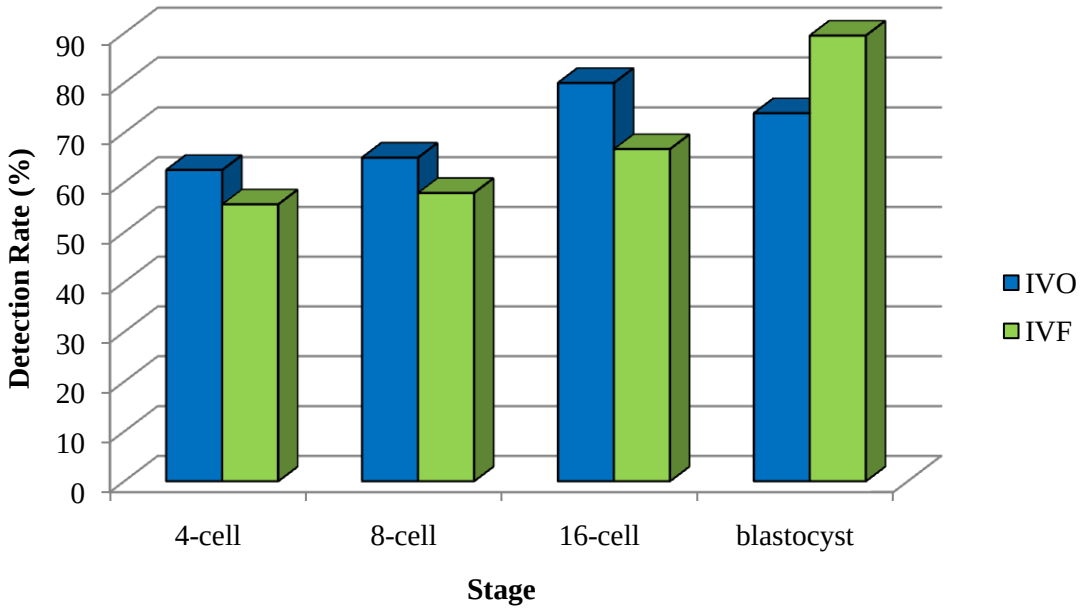

Figure 6.5 depicts the percentage of embryos by group and by stage in which miR-21 was detected.

Figure 6.6. Normalized miR-21 expression at different stages

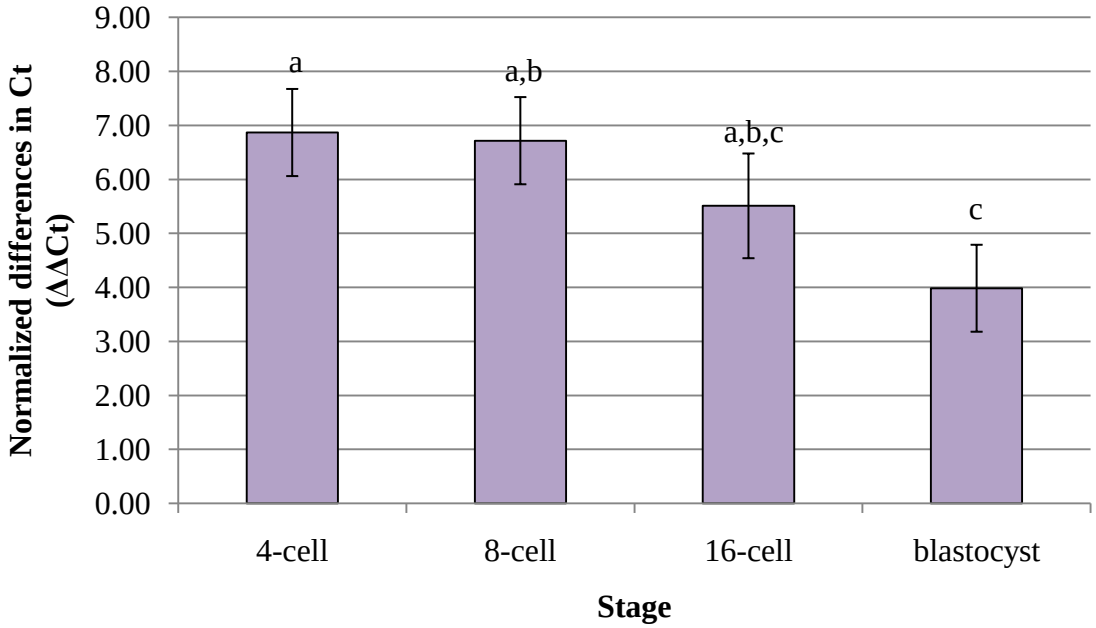

Figure 6.6 shows the normalized differences in Ct for miR-21 for the combined IVO and IVF groups at each stage. Error bars represent the SEM. Bars not sharing letters are statistically different ($P < 0.05$).

miR-24

MiR-24 was detected in 73 of the IVO embryos (94.8%) and in 68 of the IVF embryos (91.9%). Chi-square analysis showed a significant difference in the proportions of IVO vs. IVF embryos at the 8-cell stage in which miR-24 was detectable vs. non-detectable ($P < 0.05$). There were no differences among embryo stages. There were significant effects of both stage (Figure 6.8) and group*stage on miR-24 expression (Figure 6.9). Differences were found between 4-cell and 16-cell, 8-cell and 16-cell, and between 8-cell and blastocyst ($P < 0.05$). In the group*stage analysis, miR-24 was found to exhibit a decrease expression in IVF embryos compared to IVO embryos at the 8-cell, but an increase at the blastocysts stage ($P < 0.05$).

Table 6.5. Detection of miR-24 in porcine embryos

Group	Detection	4-cell	8-cell	16-cell	Blastocyst	Total
IVO	# present (%)	23 (95.8)	20 (100.0)	9 (90.0)	21 (91.3)	73 (94.8)
	# undetectable (%)	1 (4.2)	0 (0.0)	1 (10.0)	2 (8.7)	4 (5.2)
IVF	# present (%)	16 (88.9)	15 (78.9)	18 (100.0)	19 (100.0)	68 (91.9)
	# undetectable (%)	2 (11.1)	4 (21.1)	0 (0.0)	0 (0.0)	6 (8.1)
Total	# present (%)	41 (97.6)	35 (89.7)	27 (96.4)	40 (95.2)	141 (93.4)
	# undetectable (%)	1 (2.4)	4 (10.3)	1 (3.6)	2 (4.8)	10 (6.6)

Table 6.5 lists the number of embryos by group and by stage in which miR-24 was detected. Unlike the previous two miRNAs examined, there were no differences among embryo stages; however, there were significant differences in the proportions of IVO and IVF embryos at the 8-cell and blastocyst stages in which miR-24 was detectable vs. non-detectable ($P < 0.05$).

Figure 6.7. Detection of miR-24 in porcine embryos

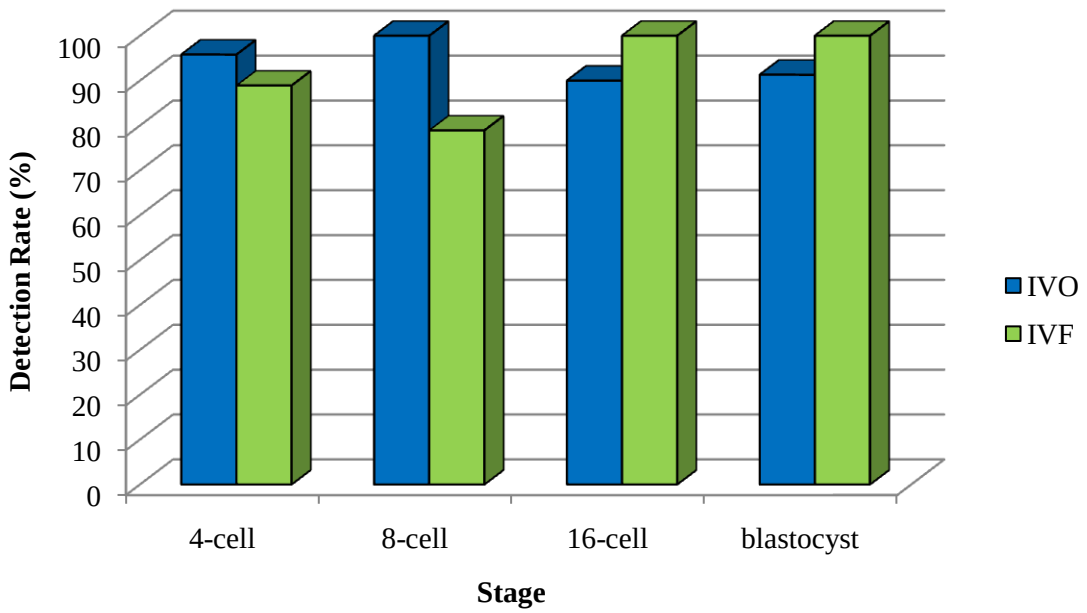

Figure 6.7 shows the percentage of embryos by group and by stage in which miR-24 was detected.

Figure 6.8. Normalized miR-24 expression at different stages

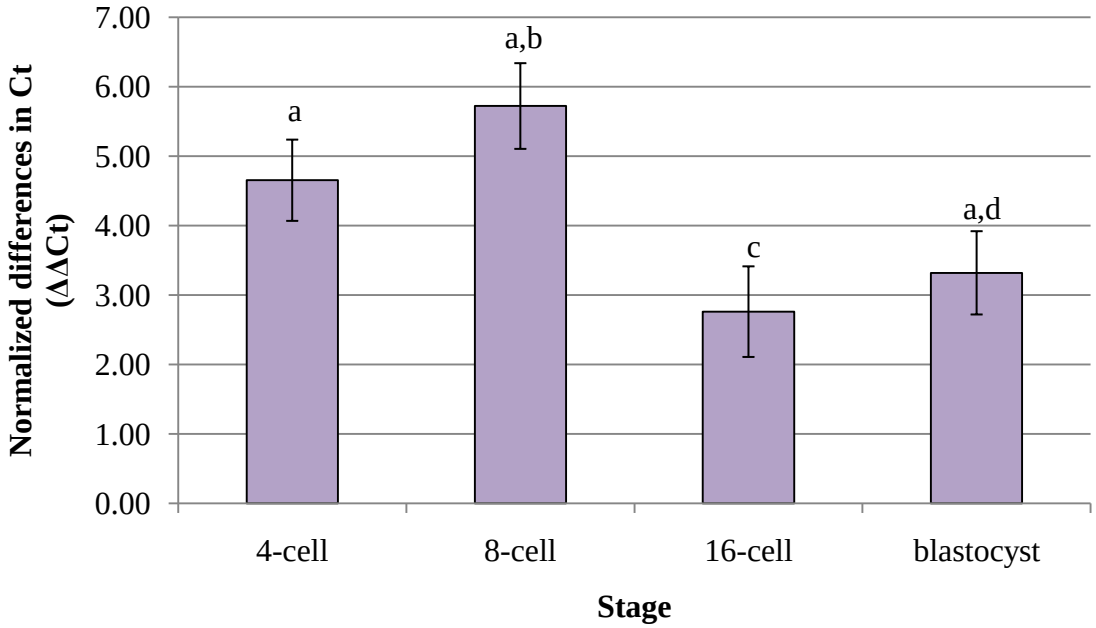

Figure 6.8 shows the normalized differences in Ct for miR-24 for the combined IVO and IVF groups at each embryo stage. Error bars represent the SEM. Bars not sharing letters are statistically different ($P < 0.05$).

Figure 6.9. Normalized miR-24 expression by group and stage

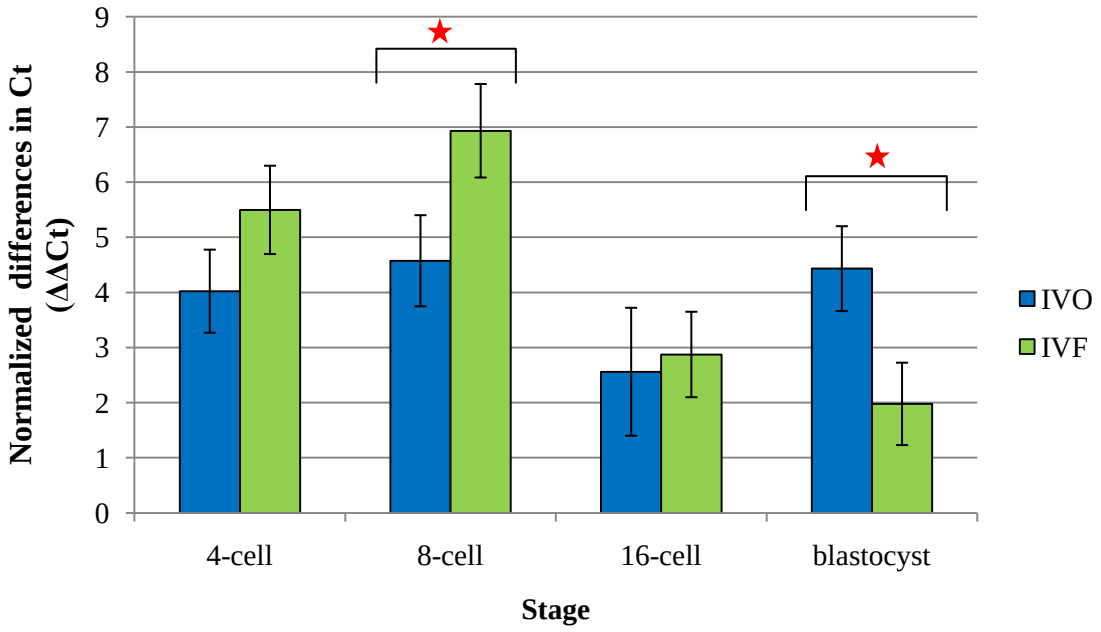

Figure 6.9 shows the normalized differences in Ct for miR-24 by group and stage. Error bars represent the SEM ($P < 0.05$).

Functional inference

The bioinformatic analyses provided potential gene targets for the miRNAs examined and allowed inferences to be deduced by grouping the genes into functional categories. The MAMI-predicted targets of miR-18a included 28 genes. When these genes were subjected to DAVID analysis, enriched GO terms included regulation of transcription, exonuclease, cell migration, and transmembrane receptor protein tyrosine kinase signaling pathway. Targets of miR-21 predicted by MAMI were a list of 37 genes, with DAVID enriched terms of negative regulation of cell differentiation and a cellular

location of nucleoplasm. There were 84 gene targets predicted for miR-24. DAVID showed enriched terms for negative regulation of inflammatory response, regulation of potassium ion transport, and negative regulation of response to external stimulus.

Discussion

This study demonstrated temporal expression of miRNAs in porcine embryos at different stages of pre-implantation development and also between IVO and IVF embryos at corresponding stages. At least three other reports exist describing miRNAs in *in vitro*-produced embryos of bovine and human (Castro et al., 2010; McCallie et al., 2010; Tesfaye et al., 2009), but none compared miRNA expression of IVF embryos to IVO embryos. This is the first report of miRNA identification in porcine pre-implantation embryos and also the first describing differential miRNA expression between IVO and IVF embryos in any species.

Not all of the miRNAs assayed were detectable in every embryo sample, so Chi-square analysis was employed to assess proportions of detectable and non-detectable miRNAs by group and by stage. It is possible that miR-18a and -21 were present in all embryos, but at low levels which were undetectable using this assay. Rather than selectively exclude the embryos in which specific miRNAs were non-detectable, a Ct of 40 was assigned, which was beyond the limits of the standard curve and represented a non-detectable level.

It is conceivable that only competent embryos produced detectable levels of miR-18a and miR-21, so those which developed to the blastocyst stage were more likely to express higher levels of those miRNAs, whereas embryos not expressing miR-18a and -21

underwent growth arrest were discarded for being developmentally retarded. A potential target of miR-18a is hypoxia-inducible factor 1 (HIF-1), a transcription factor involved in a cascade that mediates the effects of hypoxia within the cell. In a study investigating the effects of the copper metabolism gene MURR1 domain (Commd1) on early embryonic lethality in mice, researchers found that HIF-1 protein, along with at least 16 target genes of HIF-1, were significantly upregulated in *Commd1*$^{-/-}$ embryos (van de Sluis et al., 2007). If miR-18a is under-expressed in developmentally incompetent embryos, it could lead to HIF-1 over-expression, which would result in abnormal embryogenesis (as shown by Gnarra et al., 1997; Iyer et al., 1998) leading to embryonic loss in the pig.

Although miR-18a and miR-21 were not different between groups, they showed differential expression among developmental stages. The proportion of embryos in which miR-18a was detectable significantly increased with progressive embryonic stages. The data presented in Chapter V showed that miR-18a was more highly expressed in the porcine oocyte than both the 8-cell embryo and blastocyst. It is possible that oocytes and potentially 2-cell embryos would express miR-18a more highly than in the 4-cell embryos, and that miR-18a expression is lowest at the 4-cell stage. Tang et al. (2007) compared miRNA expression from murine oocytes and embryos from the zygote through the 8-cell stage and found that the total amount of miRNA is down-regulated by 60% between the zygote and 2-cell embryo and that some miRNAs were lost by 95%, suggesting that maternal miRNAs are actively degraded during the first cell division.

MiR-21 showed the same trend as miR-18a, although not as pronounced. Both the 4-cell and 8-cell embryos had significantly lower expression of miR-21 than blastocysts. As

reported by McCallie et al. (2010), there was no difference in miR-21 expression between normal blastocysts and those produced by sub-fertile patients; however, McCallie found that miR-21 was invariantly expressed across all blastocysts examined, while the present study showed that it was undetectable in 19.0% of blastocysts. MiR-21 is predicted to target genes involved in cell differentiation, some of which may be temporally expressed in the early embryo. Because it's impossible to know which embryos were destined to develop to full term, it would be interesting to conduct a study in which individual blastomeres from embryos are biopsied and assayed for miR-18a, miR-21, and other miRNAs of interest. The embryos could then be transferred to recipients and their developmental rate correlated to their miRNA signature to retrospectively determine differences in expression between competent embryos and those that fail to develop.

The only miRNA that was differentially expressed between IVO and IVF embryos was miR-24, which exhibited a significant decrease in IVF embryos at the 8-cell stage, but then increased significantly at the blastocyst stage. MiR-24 is predicted to target Appl2 (adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper), a protein required for the regulation of cell proliferation in response to extracellular signals. The knockdown of Appl2 in zebrafish has been shown to induce apoptosis and early embryonic death (Schenck et al., 2008). Interestingly, miR-24 was also examined in the McCallie study (2010) and was found to be significantly decreased in embryos produced by patients with male-factor infertility or polycystic ovarian syndrome compared to normal donors. Bioinformatic analysis revealed miR-24 targets genes that appear to be

involved in inflammation and stress, which could easily be extended to embryo *in vitro* culture conditions.

This study identified changes in the expression of specific miRNAs across developmental stages and between IVO and IVF porcine embryos at the 8-cell stage and blastocyst stage. The miRNA differentially expressed are potential candidates for markers of embryonic quality. Because there are dozens of predicted gene targets for miRs-18a, -21, and -24, future studies should focus on validating potential targets of interest and identifying differential protein expression of the predicted gene.

CHAPTER SEVEN

CONCLUSIONS

The goals of these experiments were to identify miRNAs in porcine gametes and pre-implantation embryos and to investigate differences in expression in normal versus abnormal samples. Normal samples included sperm cells of standard motility and morphology and *in vivo*- produced oocytes and pre-implantation embryos. Abnormal samples included sperm samples with high percentages of morphological abnormalities or samples exhibiting a high percentage of non-motile sperm, and embryos produced via *in vitro* methods.

Using a heterologous RT-PCR approach, these studies demonstrated the presence of a total of 92 miRNAs in porcine spermatozoa, oocytes, and/ or embryos at the 4-cell, 8-cell, 16-cell, and blastocyst stages, with hundreds more predicted by miRNA microarray. Subsequent qRT-PCR analysis showed differential expression of five miRNAs, let-7a, -7d, -7e, miR-15b, and -22, between normal sperm and morphologically abnormal sperm or sperm samples exhibiting low motility. Messenger RNA targets of the differentially expressed miRNAs encode proteins important for spermatogenesis, sperm structure, and/ or sperm cell metabolism. Differential expression was also shown in embryos at various stages in development, demonstrating a temporal expression pattern of specific miRNAs in pre-implantation embryo growth. More interestingly, miR-24 was differentially expressed between *in vivo* and *in vitro*- produced embryos at the 8-cell and blastocyst stages, supporting the need to characterize aberrant miRNA expression associated with

the abnormal embryonic development correlated with ART. All of the miRNAs examined demonstrated high sequence identity to the corresponding human miRNA sequences, indicative of high conservation among species.

The results of the messenger RNA target predictions were due, in part, to the established sensitivity parameters. In the MAMI analyses, a higher specificity was used to avoid false positives, resulting in a smaller pool of miRNA-mRNA interactions. An increase in predicted targets (higher sensitivity) would affect DAVID enrichment scores, but would likely sacrifice accuracy. Because miRNA target predictions are essentially educated speculation, messenger RNA targets and the translation of the encoded protein should be experimentally tested to validate miRNA function.

To our knowledge, these are the first experiments characterizing miRNA expression in porcine sperm, oocytes, and pre-implantation embryos and the only experiments describing differential expression of miRNAs in aberrant sperm samples. Future directions include examining miRNA expression in pure populations of sperm samples (sperm with very specific phenotypes) or sperm samples of similar phenotypes that exhibit differences in fertilization rates. It would also be interesting to compare miRNA expression of IVO embryos to those produced via intracytoplasmic sperm injection (ICSI) and somatic cell nuclear transfer (SCNT; cloning). It is possible that miRNAs are introduced or left behind during these procedures, which would potentially impact mRNA levels required for normal embryonic development. The characterization of a miRNA signature of normal, competent sperm samples and embryos could aid in choosing which samples are best suited for insemination or embryo transfer.

APPENDICES

Appendix A

Detectable miRNA in boar sperm via microarray

miRNA name	mean signal (RFU)	miRNA name	mean signal (RFU)
hsa-miR-223	59702.06	hsa-miR-30c	16839.65
rno-miR-223	56174.51	mmu-miR-709	16361.51
hsa-miR-923	38318.00	hsa-let-7g	15520.99
mmu-miR-762	37208.12	bta-miR-30a-5p	14934.28
hsa-miR-638	35653.99	hsa-let-7i	14215.54
hsa-miR-26b	34647.68	hsa-miR-92b	14096.81
bta-miR-26b	34365.97	mmu-miR-34b-3p	13860.72
hsa-miR-26a	33810.49	bta-miR-30d	13743.76
hsa-miR-16	32178.77	mdo-let-7g	13719.13
hsa-miR-23a	31295.31	hsa-let-7e	12916.10
bta-miR-21	31178.17	hsa-let-7b	12727.33
bta-miR-16	30959.85	mmu-miR-155	12716.79
hsa-let-7a	30116.74	hsa-miR-195	12608.05
hsa-miR-23b	29179.58	hsa-miR-671-5p	12302.36
bta-miR-23a	28720.92	ssc-let-7i	12063.06
hsa-let-7f	28658.01	hsa-miR-30d	12007.79
ppa-miR-23b	28279.35	hsa-miR-30a	11794.77
mdo-miR-23a	28227.22	hsa-miR-663	11423.54
lca-miR-16	27868.73	bta-let-7e	10911.07
mmu-miR-705	27757.09	hsa-miR-155	10742.41
ggo-miR-30b	25927.70	mdo-let-7i	10652.58
hsa-miR-21	25577.15	hsa-miR-34b	10504.04
hsa-miR-92a	24773.04	bta-miR-15a	10467.61
hsa-let-7d	23617.52	hsa-miR-149*	10429.79
hsa-miR-15b	23550.23	ggo-miR-29a	10246.35
hsa-miR-654-5p	22870.89	hsa-miR-425	10067.79
hsa-miR-574-5p	22866.19	bta-miR-425-5p	9991.11
mmu-miR-92a	22180.42	hsa-miR-15a	9243.57
hsa-miR-25	21967.71	mdo-miR-15a	9118.64
ssc-miR-15b	21406.16	mmu-miR-720	9051.11
hsa-miR-30b	20854.23	mmu-miR-714	9016.40
ssc-miR-15b	20522.24	bta-miR-150	8754.38
hsa-let-7c	20376.62	hsa-miR-150	8505.74
mdo-let-7d	20279.34	bta-miR-29a	8427.13
hsa-miR-936	20080.02	hsa-miR-29a	8358.40
hsa-miR-191	18659.87	bta-miR-107	7500.63
ptr-miR-25	18459.14	hsa-miR-483-5p	6782.70
mdo-miR-191	18040.50	hsa-miR-103	6622.63

miRNA name	mean signal (RFU)	miRNA name	mean signal (RFU)
mmu-miR-711	6455.77	hsa-miR-29c	2320.12
hsa-miR-107	6254.70	hsa-miR-30c-1*	2317.27
mmu-miR-483	6003.50	bta-miR-181a	2281.89
hsa-miR-361-5p	5716.80	hsa-miR-200c	2259.48
bta-miR-30e-5p	5705.37	rno-miR-200c	2215.75
hsa-miR-221	5297.46	hsa-miR-181a	2148.81
bta-miR-221	5266.02	ggo-miR-200c	2145.52
hsa-miR-15b*	5188.56	hsa-miR-148a	2127.00
bta-miR-27a	4818.98	mmu-miR-466f-5p	2062.22
hsa-miR-151-5p	4659.47	hsa-miR-564	1881.33
hsa-miR-146a	4368.38	ppa-miR-128a	1853.29
hsa-miR-765	4334.00	hsa-miR-222	1647.56
hsa-miR-675	4319.34	hsa-miR-374b	1588.78
hsa-miR-98	4058.01	hsa-miR-132	1575.23
hsa-miR-17	3864.96	hsa-miR-342-3p	1548.07
rno-miR-352	3813.40	hsa-miR-27b	1508.60
bta-miR-106	3707.80	hsa-miR-203	1501.77
ggo-miR-17-5p	3629.20	rno-miR-200b	1499.01
hsa-miR-423-5p	3594.14	bta-miR-181b	1486.28
mmu-miR-466h	3579.99	bta-miR-342	1467.97
hsa-miR-939	3572.26	hsa-miR-371-5p	1388.17
hsa-miR-34c-3p	3549.91	mmu-miR-34c*	1361.44
hsa-miR-185	3503.05	hsa-miR-30e	1356.19
hsa-miR-20a	3483.87	mdo-miR-24	1334.12
ggo-miR-27a	3449.97	ggo-miR-181b	1317.50
bta-miR-195	3371.07	hsa-miR-200b	1313.13
hsa-miR-106a	3340.86	bta-miR-200b	1312.48
hsa-miR-93	3325.51	hsa-miR-672	1300.23
hsa-miR-24	3296.01	hsa-miR-186	1276.59
hsa-miR-27a	3076.16	hsa-miR-513-5p	1225.19
ggo-miR-93	3047.23	mdo-miR-181b	1200.33
mmu-miR-685	3003.90	mdo-miR-22	1171.85
hsa-miR-128a	2943.75	hsa-miR-628-3p	1161.45
bta-miR-93	2878.11	hsa-miR-940	1156.93
bta-miR-20b	2874.71	mmu-miR-770-3p	1111.09
hsa-miR-140-3p	2739.17	hsa-miR-20b	1040.63
hsa-miR-106b	2737.88	hsa-miR-449b	1009.75
hsa-miR-320	2656.78	hsa-miR-10b	993.05
ggo-miR-106a	2632.71	mmu-miR-582-5p	978.00
bta-miR-140	2626.90	hsa-miR-188-5p	959.11
rno-miR-466b	2539.01	mmu-miR-721	911.89
mmu-miR-106a	2450.19	hsa-miR-532-5p	900.73
ggo-miR-20	2447.42	mdo-miR-10b	890.08

miRNA name	mean signal (RFU)	miRNA name	mean signal (RFU)
rno-miR-20b-5p	885.11	hsa-miR-425*	352.27
mmu-miR-763	868.50	hsa-miR-362-5p	347.58
hsa-miR-340	853.91	hsa-miR-801	344.15
rno-miR-327	842.41	hsa-miR-296-5p	340.47
bta-miR-423	834.55	rno-miR-10b	338.54
hsa-miR-182	822.35	hsa-let-7b*	335.41
bta-miR-142	792.47	mmu-miR-468	331.38
mdo-miR-375	779.34	hsa-let-7d*	327.38
mdo-miR-203	775.90	ggo-miR-10b	322.96
hsa-miR-28-5p	762.17	mdo-miR-9*	305.59
ppy-miR-182	749.46	hsa-miR-9*	303.57
hsa-miR-28-3p	747.63	hsa-miR-628-5p	303.09
hsa-miR-652	741.73	hsa-miR-484	286.08
hsa-miR-933	701.89	mmu-miR-182	285.18
hsa-miR-449a	691.66	hsa-miR-421	284.69
hsa-miR-148b	691.36	hsa-miR-494	281.76
hsa-miR-768-5p	681.62	mmu-miR-678	279.74
hsa-miR-744	678.20	hsa-miR-500	267.88
ggo-miR-186	664.34	hsa-miR-7-1*	266.52
hsa-miR-363	641.79	ggo-miR-198	264.29
hsa-miR-22	607.64	mmu-miR-466d-5p	259.13
hsa-miR-375	577.13	rno-miR-345-3p	253.73
mmu-miR-680	575.11	hsa-miR-193a-5p	253.47
hsa-miR-122	540.33	bta-miR-99a	247.31
hsa-miR-146b-5p	540.30	mmu-miR-702	243.13
hsa-miR-660	539.44	hsa-miR-505*	236.14
hsa-miR-582-5p	534.38	mmu-miR-665	235.34
hsa-miR-151-3p	517.49	hsa-miR-324-3p	233.78
hsa-miR-142-5p	517.18	mmu-miR-290-5p	228.67
mdo-miR-122	502.33	hsa-miR-99a	226.20
hsa-miR-150*	499.26	hsa-miR-134	225.95
hsa-miR-19b	485.69	bta-miR-7	209.82
ssc-miR-122	480.30	hsa-miR-191*	209.31
hsa-miR-374a	449.57	hsa-miR-454	206.26
hsa-miR-125b	421.16	hsa-miR-149	205.34
hsa-miR-181b	418.35	hsa-miR-874	205.25
bta-miR-363	395.62	rno-miR-290	203.35
hsa-miR-658	395.39	rno-miR-409-3p	193.10
mdo-miR-29a	378.40	mml-miR-198	190.02
hsa-miR-335	375.16	hsa-miR-373*	189.75
mmu-miR-298	368.18	mmu-miR-150*	189.37
hsa-miR-659	367.79	mmu-miR-689	187.63
hsa-miR-637	364.26	rno-miR-494	187.58

miRNA name	mean signal (RFU)
hsa-miR-181d	187.54
hsa-miR-760	183.73
bta-miR-181c	182.35
mmu-miR-712*	178.29
mmu-miR-297a	176.51
mmu-miR-466f-3p	174.67
hsa-miR-612	173.12
rno-miR-324-3p	172.63
hsa-miR-130b	172.20
bta-miR-660	171.26
hsa-miR-650	169.04
hsa-miR-16-1*	163.05
hsa-miR-30e*	159.98
mmu-miR-425*	159.58
rno-miR-7a*	159.57
mmu-miR-327	159.33
hsa-let-7f-1*	157.55
mmu-miR-362-5p	157.37
hsa-miR-23a*	154.74
hsa-miR-331-3p	153.55
hsa-miR-10a	153.14
mmu-miR-207	152.54
hsa-miR-183	151.25
hsa-miR-532-3p	149.37
sla-miR-198	148.32
hsa-miR-30b*	146.86
bta-miR-139	143.27
hsa-miR-921	142.51
mdo-miR-100	141.59
hsa-miR-190b	141.05
hsa-miR-210	139.23
mmu-miR-700	135.93
hsa-miR-574-3p	133.24

miRNA name	mean signal (RFU)
rno-miR-505	131.77
hsa-miR-362-3p	131.13
hsa-miR-625	130.93
hsa-miR-563	130.68
mmu-miR-92a*	128.76
hsa-miR-100	127.79
hsa-miR-223*	127.33
mmu-miR-191*	126.14
rno-miR-345-5p	126.07
rno-miR-214	125.78
hsa-miR-625*	122.82
hsa-miR-602	121.60
hsa-miR-198	121.50
hsa-miR-212	120.55
mmu-miR-675-5p	120.25
bta-miR-200a	119.99
bta-miR-29b	119.71
hsa-miR-877	118.78
hsa-miR-324-5p	116.30
mmu-miR-715	115.99
mmu-miR-801	115.73
hsa-miR-636	115.10
hsa-miR-608	113.20
hsa-miR-365	112.94
mmu-miR-16*	111.98
hsa-miR-18a	111.97
ggo-miR-183	108.35
hsa-miR-885-3p	106.97
hsa-miR-129-5p	105.41
mmu-miR-292-5p	103.68
mmu-miR-483*	103.19
mmu-miR-345-5p	103.12
hsa-miR-500*	101.16
ppa-miR-188	100.00

Appendix B

Comparison of qRT-PCR results to microarray data

Introduction

The objective of this study was to compare results from qRT-PCR data comparing miRNA expression among normal (control), morphologically abnormal (AB), and low motility (LM) sperm miRNA, as reported in Chapter IV, to results obtained from a miRNA microarray.

Materials and methods

Microarray

Five µg sperm total cellular RNA from individual sperm samples representing each group, (C, AB, and LM) was hybridized to commercially available heterologous miRNA microarrays (LC Sciences, LLC; Houston, TX). The RNA samples used were included in the qRT-PCR analysis. The RNA was size fractionated using a YM-100 Microcon centrifugal filter (Millipore, Bedford, MA) to isolate small RNAs. Small RNAs less than 300 nt were 3'- extended with a poly(A) tail using poly(A) polymerase. An oligonucleotide tag was ligated to the poly(A) tail for later fluorescent dye staining. Hybridization was performed overnight on a µParaflo microfluidic chip using a micro-circulation pump (Atactic Technologies, Houston, TX). Each detection probe on the microfluidic chip consisted of a chemically modified nucleotide coding segment complementary to a known miRNA target (based on Sanger miRBase Release 12.0) or control RNA and a spacer segment of polyethylene glycol to extend the coding segment

away from the substrate. MiRNA probes (n= 1097, in triplicate) were complementary to known miRNAs from 8 different species, including 64 porcine probes. The detection probes were made by *in situ* synthesis using photogenerated reagent chemistry. The hybridization melting temperatures were balanced by chemical modifications of the detection probes. Hybridization was carried out in 100 µL 6x SSPE buffer (0.90 M NaCl, 60 mM Na2HPO4, 6 mM EDTA, pH 6.8) containing 25.0% formamide at 34 °C.

Tag detection was determined using fluorescence labeling with tag-specific dyes. Images were collected using the GenePix® 4000B laser scanner (Molecular Device, Inc, Sunnyvale, CA) and digitized with Array-Pro image analysis software (Media Cybernetics, Silver Spring, MD). Data were analyzed by first subtracting the background and then normalizing the signals using a LOWESS filter (Locally-weighted Regression) to compensate for the intensity difference between Cy3 and Cy5. The ratio of the two sets of detected signals (log2 transformed, balanced) and P-values of the t-test were calculated. Significantly different signal intensities were those with less than 0.01 P-values.

Functional inference of miRNA

The target genes of differentially expressed miRNAs (as determined by microarray analysis) among control, AB, and LM sperm populations were extracted using the MAMI server and database. Targets were predicted for up- and down-regulated miRNAs from either AB or LM compared to the controls. A MAMI sensitivity of 0.46 and a specificity of 1.0 were used to minimize Type I Errors. DAVID was used to annotate MAMI-predicted target genes of differentially expressed miRNAs and to identify significant

functional enrichment in the miRNA targets relative to the whole genome background. Entrez gene IDs were uploaded to the functional annotation tool and enriched biological themes were analyzed using a modified Fisher's exact test (EASE score; $P < 0.05$), medium classification stringency, minimum enrichment score of 1.3, and a minimum fold enrichment of 1.5. Functional annotation charts and clustering were analyzed to determine enriched molecular functions, biological processes, cellular components, biochemical pathways, and other gene annotations.

Results

Microarray

Of the 1097 miRNAs probed, microarray results showed that 62 miRNAs were differentially expressed between C and AB (5.7%), with 38 up-regulated in AB and 24 down-regulated. There were 66 miRNAs differentially expressed between C and LM (6.0%), with 33 up-regulated and 33 down-regulated in LM. Forty-eight miRNAs were differentially expressed in both the AB and LM samples, with 2 miRNAs (miRs-25 and -92a) up-regulated in AB but down-regulated in LM. Twenty-four miRNAs were up-regulated in both AB and LM groups compared to controls and 19 were down-regulated in both groups.

In contrast to the results described in Chapter III and other published reports (Ach et al., 2008; Chen et al., 2009; Mattie et al., 2006), the qRT-PCR data reported in Chapter IV did not support the microarray results. Unexpectedly, results were inversely related (Table B.2).

Table B.1. Number of miRNAs differentially expressed among normal (C), abnormal (AB), and low motility (LM) sperm samples by array

	C vs. AB	C vs. LM	Total
Differentially expressed (%)	62 (5.7)	66 (6.0)	80 (7.3)
Up-regulated (%)	38 (3.5)	33 (3.0)	47 (4.3)
Down-regulated (%)	24 (2.2)	33 (3.0)	39 (3.6)

Table B.1 shows the number of miRNAs that were differentially expressed between groups. The numbers in parenthesis are the percent of all miRNAs probed that were differentially expressed. The Total column does not equal the sum of C vs. AB and C vs. LM because some differentially expressed miRNAs were shared between groups.

Table B.2. Comparison of microarray and qRT-PCR results in AB and LM sperm

	Abnormal		Low Motility	
miRNA	**microarray**	**qRT-PCR**	**microarray**	**qRT-PCR**
let-7a	↓	↑	↓	nsd
let-7d	↓	↑	↓	nsd
let-7e	↓	↑	↓	↑
let-7i	↓	nsd	↓	↑
miR-15b	nsd	↓	↓	nsd
miR-182	↓	nsd	nsd	nsd
miR-22	nsd	↑	nsd	nsd
miR-24	nsd	nsd	nsd	nsd
miR-92	nsd	nsd	nsd	nsd

Table B.2 shows the microarray and real time qRT-PCR results in both AB and LM groups compared to Controls. A down-pointing arrow (↓) indicates a significant decrease ($P < 0.05$) in expression compared to Controls, whereas an upward-pointing arrow (↑) indicates a significant increase in expression compared to Controls. Nsd= no significant difference.

Functional inference of miRNA

The bioinformatic analyses not only provided putative gene targets for the miRNAs of interest, but also allowed inferences to be deduced by grouping the genes into functional categories. When the differentially expressed miRNAs between the C and AB groups (as determined by microarray) were subjected to MAMI analysis, targets were identified for 24 miRNAs (38.7%) with a total of 537 gene-miRNA interactions. Ninety of these genes were predicted to be targeted by multiple miRNAs and resulted in 307 unique predicted targets. The mean number of gene targets per miRNA was 26.6 with a minimum of 15 and a maximum of 49. The MAMI results for the differentially expressed miRNAs between the C and LM identified targets for 27 (40.9%) miRNAs with a total of 557 gene-miRNA interactions. One hundred eighteen of the genes were predicted to be targeted by two or more miRNAs, and again, coincidentally, resulted in 307 unique predicted targets. The mean number of gene targets per miRNA was 21.4 with a minimum of two and a maximum of 55. A combined analysis showed that a total of 33 miRNAs were predicted to target 392 unique genes.

DAVID analysis of genes predicted to be targeted by miRNAs differentially expressed in AB sperm by microarray showed enriched gene ontology (GO) categories for regulation of developmental processes, apoptosis, cellular development, cell projection morphogenesis, nucleotide binding, GTP binding, and cellular component enrichment for membrane-bound organelles. Targets of miRNAs differentially expressed in LM sperm were overrepresented for GO categories such as regulation of metabolic, cellular, and

biological processes, protein binding, protein kinase activity, metal/ ion binding, and transcription factor activity, with a cellular component enrichment of the nucleus.

Discussion

Surprisingly, the PCR data showed a reverse trend when compared to the microarray results. The same RNA samples used in the microarray were included in the PCR analyses and also clashed with microarray data. These results conflicted with literature reports that qRT-PCR results are supportive of microarray results. Although the cause for this discord is unknown, it seems an unlikely coincidence that the results were nearly opposite and are likely due to technician error, such as mislabeling of samples. Chen et al. (2009) found low correlation between qRT-PCR (Taqman) and microarrays (LC Sciences) when using different aliquots of the same RNA and noted that higher variation was observed in miRNAs with low expression; however, in this study, the same aliquots of RNA were used for microarray analysis and subsequently for qRT-PCR. Ach et al. (2008) found high correlation of 88.3% of miRNAs compared using Taqman qRT-PCR and Agilent microarrays. A study by Mattie et al. (2006) reported high correlation between microarray and PCR data, with the exception of let-7, which was found to be up-regulated in microarray data but not significantly different in Taqman qRT-PCR results. It is possible that, in the microarray, multiple members of the let-7 family hybridized to the same probes, thereby inflating the signal. These contradictory data reinforce the recommendation to verify miRNA expression levels via PCR.

The miRNAs chosen for qRT-PCR analysis were chosen, in part, because of the difference in expression determined by the microarray. Regardless of discrepancies

between the microarray data and the qRT-PCR results, in general, the miRNAs that were deemed significantly different via microarray were still significantly different by qRT-PCR and those that showed no significant differences remained so across platforms.

Appendix C

Differentially expressed miRNAs among control, AB, and LM sperm

Differentially Expressed miRNA between Control sperm and Morphologically Abnormal or Low Motility Sperm as Determined by Microarray

Upregulated in AB vs. Controls

hsa-miR-1182
hsa-miR-1207-5p
ptr-miR-1224-5p
hsa-miR-1224-5p
hsa-miR-1228*
hsa-miR-1268
hsa-miR-1275
hsa-miR-1300
ptr-miR-1300b
hsa-miR-1469
hsa-miR-149*
hsa-miR-150*
hsa-miR-188-5p
hsa-miR-1915
ggo-miR-198
ggo-miR-25
ptr-miR-25
ggo-miR-30a-5p
hsa-miR-30c-1*
ggo-miR-30d
rno-miR-327
rno-miR-345-3p
hsa-miR-371-5p
hsa-miR-375
hsa-miR-483-5p
hsa-miR-574-3p
hsa-miR-601
hsa-miR-638
hsa-miR-659
hsa-miR-663
hsa-miR-671-5p
hsa-miR-675
hsa-miR-760
hsa-miR-765
ggo-miR-92
rno-miR-92a
hsa-miR-92b
hsa-miR-939

Downregulated in AB vs. Controls

hsa-let-7a
hsa-let-7b
ssc-let-7c
hsa-let-7d
hsa-let-7e
ssc-let-7f
hsa-let-7g
hsa-let-7i
ssc-miR-103
ssc-miR-125b
hsa-miR-151-5p
ssc-miR-16
hsa-miR-182
ppy-miR-182
hsa-miR-1826
hsa-miR-191
hsa-miR-20a
ssc-miR-26a
ssc-miR-30b
ggo-miR-30b
ssc-miR-30c
hsa-miR-342-3p
hsa-miR-720
hsa-miR-923

Upregulated in LM vs. Controls	Downregulated in LM vs. Controls
hsa-miR-1182	hsa-let-7a
hsa-miR-1207-5p	hsa-let-7b
hsa-miR-1224-5p	ssc-let-7c
ptr-miR-1224-5p	hsa-let-7d
hsa-miR-1228*	hsa-let-7e
hsa-miR-1268	ssc-let-7f
hsa-miR-1275	hsa-let-7g
hsa-miR-1281	hsa-let-7i
hsa-miR-134	ssc-let-7i
hsa-miR-1469	hsa-miR-10b
hsa-miR-149*	ssc-miR-125b
hsa-miR-150*	hsa-miR-128
hsa-miR-188-5p	hsa-miR-1280
ggo-miR-198	hsa-miR-1308
hsa-miR-198	hsa-miR-151-5p
ggo-miR-200c	ssc-miR-15b
hsa-miR-200c	ssc-miR-16
rno-miR-200c	hsa-miR-1826
hsa-miR-30c-1*	hsa-miR-191
hsa-miR-320a	ggo-miR-25
rno-miR-327	ssc-miR-26a
rno-miR-345-3p	ggo-miR-30b
hsa-miR-371-5p	ssc-miR-30b
hsa-miR-483-5p	ssc-miR-30c
hsa-miR-574-3p	ggo-miR-30d
hsa-miR-663	rno-miR-329
hsa-miR-671-5p	hsa-miR-34b
hsa-miR-675	hsa-miR-425
hsa-miR-760	hsa-miR-720
hsa-miR-765	ggo-miR-92
hsa-miR-874	hsa-miR-923
hsa-miR-936	rno-miR-92a
hsa-miR-939	hsa-miR-92b

Appendix D

Detection of miRNA in other porcine tissues

In addition to porcine gametes and embryos, endpoint RT-PCR was also performed on RNA isolated from additional porcine tissues to examine tissue-specific expression. Other tissues included: cumulus-oophorus complexes (COCs) aspirated from abattoir gilt ovaries; lung, heart, kidney, liver, uterus, corpus luteum (CL), oviduct, and ovary collected from gilts at time of slaughter; piglet testes collected at castration (7 days of age), and; boar testes opportunistically harvested at euthanasia.

Total RNA enriched for small RNAs was isolated from tissue samples using the *mir*Vana™ miRNA Isolation Kit (Ambion Inc., Austin, TX) as per manufacturer's protocol. Sample concentration and quality were determined by spectrophotometry using the Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Endpoint RT-PCR was conducted using the *mir*Vana™ qRT-PCR miRNA Detection Kit (Ambion, Austin, TX) and was used to determine the presence of 22 specific miRNAs: let-7a, -7d, -7e, -7f, -7i, miR-9, -15b, -16, -21, -22, -24, -27a, -31, -92, -124a, -132, -134, -150, -181a, -182, -212, and-345. PCR reactions, gel electrophoresis, and sequence analysis were conducted as described in Chapter III.

Several miRNAs exhibited tissue-specific expression (Table D.1). It is interesting to note that COCs and sperm showed the highest degree of variation in miRNA expression. It is possible that the miRNAs absent in those tissues are expressed by endothelial cells of blood vessels and, since COCs and sperm are the only two tissues examined that do not

contain vasculature, were not present in those tissues. MiR-134 was not present in boar testis or sperm cells, but was detected in every other tissue examined, including piglet testis. MiR-124a expression appeared to be restricted to testis and sperm, indicating a possible role in spermatogenesis.

Table D.1. Identification of miRNAs in porcine tissues by RT-PCR

	Tissue											
miRNA	lung	heart	kidney	liver	uterus	corpus luteum	oviduct	COC	ovary	sperm	boar testis	piglet testis
hsa-let-7a	√	√	√†	√	√	√	√	√	√	√†	√	√
hsa-let-7d		√	√	√	√	√	√	√	√	√†	√	√
hsa-let-7e	√	√	√†	√	√	√	√	√	√	√†	√	√
hsa-let-7f	√	√	√†	√	√	√	√	√	√	√†	√	√
hsa-let-7i		√	√	√	√	√	√	√	√	√†	√	√
hsa-miR-124a	-	-	-	-	-	-	-	-	-	√†	√	√
hsa-miR-132	√	√	√†	√	√	√	√	√	√	√†	√	√
hsa-miR-134	√	√	√	√	√	√	√	√	√	-	-	√
hsa-miR-150	√	√	√†	√	√	√	√	√	√	-	√	√
hsa-miR-15b	√	√	√†	√	√	√	√	√	√	√†	√	√
hsa-miR-16	√	√	√†	√	√	√	√	√	√	√†	√	√
hsa-miR-181a	√	√	√†	√	√	√	√	-	√	√†	√	√
hsa-miR-182	√	√	√	√	√	√	√	-	√	√†	√	√
hsa-miR-21	√	√	√†	√	√	√	√	√	√	√†	√	√
hsa-miR-212	√	√	√†	√	√	√	√	√	√	√†	√	√
hsa-miR-22	√	√	√†	√	√	√	√	√	√	√†	√	√
hsa-miR-24	√	√	√†	√	√	√	√	√	√	√†	√	√
hsa-miR-27a	√	√	√†	√	√	√	√	√	√	√†	√	√
hsa-miR-31	√	-	√†	-	√	√	√	√	√	-	√	√
hsa-miR-345	√	√	√	√	√	√	√	√	√	√†	√	√
hsa-miR-9	√	√	√†	√	√	√	√	-	√	√†	√	√
hsa-miR-92	√	√	√†	√	√	√	√	√	√	√†	√	√

√= detected; √†= detected and sequenced; - = not detected.

Appendix E

Estrous grading scale for gilts

Background

Achievement of effective estrous detection in gilts can be difficult due to various factors such as housing, heat stress, genetics (Rydhmer et al., 1994), and experience of handlers. Cronin et al. (1982) found that 70% of gilts culled for not cycling had actually ovulated, indicating either subtle estrous behaviors and/or poor estrous detection. In order to facilitate estrous detection and eliminate subjective biases among multiple observers, including previously untrained students participating in a Clemson University Creative Inquiry project, an objective estrous scoring system was developed.

Methods

In order to optimize gilt estrous detection for artificial insemination and embryo collection purposes, a numerical estrous scoring system was developed (Table E.1). A maximum of twenty-two gilts were housed in individual pens (approx. 3 ft x 6 ft) in two rows in the Isolation room at the Clemson University Swine Farm. Minimal contact between gilts in adjacent pens was possible, as was visualization of gilts in pens on the opposite aisle. Estrous checks were performed between 6am- 8am and again between 4pm and 6pm, every day. Students were instructed to observe each gilt and assign and record estrous scores on gilts' individual records at each check. A boar, housed in the same room, was permitted to walk in front of the gilts to elicit behaviors indicative of estrus. Nose-to-nose contact was encouraged between the boar and every gilt.

Table E.1. Estrous grading scale

Score	Behavioral and physical indicators of estrus
0	No signs
1	Interest in boar
2	Pink/ red vulva OR vulvar swelling
3	Pink/ red vulva, vulvar swelling/ mucous discharge, vocalization, ear-perking
4	Scores 1-3 and lordosis response; standing estrus

Heat score descriptions

At each heat check, all gilts were assigned a score from zero to four. A zero indicated that the gilt exhibited no signs of estrus and usually referred to the gilt ignoring the boar. Students were advised that every gilt must be encouraged to stand because a pig in strong estrus may 'lock up' while lying, so that a score of four could be easily be mistaken for a zero. A score of one was appointed when gilts showed interest in the boar, either standing up or walking to the front of the pen as he approached.

Swelling and/ or redness of the vulva can occur two to six days prior to estrus and it was observed that some gilts exhibited redness prior to swelling while others showed swelling first. If either of these were seen, a score of two of was assigned. Swelling and/ or redness sometimes dissipated immediately (~12 hr) prior to standing estrus. As circulating estrogen levels rise due to the presence of dominant follicles, a vulvar mucous discharge may occur. Another behavior associated with estrus is ear perking (also called

ear pricking). Gilts hold their ears erect and pointed caudally, with intermittent twitching in the forward direction. Little information as to the physiology behind or the adaptive advantage of ear perking can be found in the literature, although it is likely a result of elevated estrogen and may provide a visual cue to the boar.

A score of four was assigned only when a gilt was observed to be in standing estrus, or 'locking up'. Locking up describes the posture of a female in estrus accepting the application of pressure to her lower back, such that it mimics a boar attempting to mount her. Her ears stand erect and the gilt braces her legs and sometimes pushes back against the pressure. Sows in estrus may also mount other sows, but since the gilts in this study were housed individually, such behavior was not observed; however, some gilts close to estrus were observed attempting to climb the sides of their enclosure.

Results and Discussion

The scoring system provided effective communication among observers and allowed students to track gilts throughout their cycles. Knowing the date of an animal's last estrus was beneficial in predicting the next. Those that were not cycling (no signs of estrus for > 5 weeks or constant estrous behavior due to ovarian cysts) were culled from the study, saving both labor and expense.

To the author's knowledge, this is the first porcine estrous scoring system developed. A logical follow-up study would compare the pregnancy and litter sizes resulting from either the described estrous scoring technique or traditional heat check methods. Another interesting study would utilize ultrasonography to determine the time of ovulation relative to estrous behaviors.

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