Epididymal mRNA and miRNA transcriptome analyses reveal important genes and miRNAs related to sperm motility in roosters

Kai Xing,* Yu Chen,[†] Liang Wang,[†] Xueze Lv,[†] Zheng Li,[‡] Xiaolong Qi,* Xiangguo Wang,* Longfei Xiao,* Hemin Ni,* Yong Guo,* and Xihui Sheng ⓘ*,[1]

*Animal Science and Technology College, Beijing University of Agriculture, Beijing 102206, China; [†]Beijing General Station of Animal Husbandry, Beijing 100107, China; and [‡]Beijing Institute of Feed Control, Beijing 100107, China

ABSTRACT Sperm motility is a crucial trait in chicken production, and the epididymis is an essential organ in the reproductive system. Currently, the molecular mechanisms underlying sperm motility in the epididymis are unclear. In this study, 8 cDNA libraries and eight miRNA libraries were constructed from roosters (4 chickens per group) with diverse sperm motility. After a comparative analysis of epididymal transcriptomes, we detected 84 differentially expressed genes (**DEG**s) using the edgeR package. Integrated interpretation of DEGs indicated that *MMP9, SLN, WT1, PLIN1*, and *LRRIQ1* are the most promising candidate genes affecting sperm motility in the epididymis of roosters. MiR-146a, mir-135b, and mir-205 could play important regulatory roles in sperm maturation, capacitation, and motility. Additionally, a comprehensive analysis of the mRNA and miRNAs transcriptomes in silico identified a promising gene-miRNA pair miR-135b-HPS5, which may be a vital regulator of sperm motility in the epididymis. Our findings provide novel integrated information of miRNAs and genes that shed light on the regulatory mechanisms of fertility in roosters.

Key words: transcriptome, miRNA, epididymis, rooster, sperm motility

INTRODUCTION

The reproductive potential of a rooster depends on semen quality, which includes semen volume, sperm motility, and sperm density, along with other parameters. The epididymis is an important organ in the reproductive system of male animals, since its function is not only to store spermatozoa but also to confer motility and fertilization competency (Cornwall and Hann, 1995). Recent studies suggested that avian sperm acquire necessary capabilities for fertilization in this organ and that sperm motility was greatly increased during epididymal transit (Ahammad et al., 2011; Nixon et al., 2014). However, the molecular mechanisms underlying sperm maturation in the epididymis and its effect on sperm quality and male fertility in roosters remain unclear.

MicroRNAs (**miRNAs**) are a group of small (approximately 22 nucleotides in lengths) non-coding RNAs that play crucial roles in diverse biological processes (Valencia-Sanchez et al., 2006). More than 200 miRNAs have been detected in mammalian and human epididymis (Belleannée et al., 2012). Although the expression profiles of miRNAs in epididymis are essential in promoting sperm maturation (Belleannee et al., 2012; Belleannée et al., 2013; Anderson et al., 2015; Tian et al., 2020), miRNAs in chicken epididymis and their exact correlation with epididymis development and semen quality are still unknown. Paired gene and miRNA expression profiling is an efficient method to investigate key miRNAs and their target genes (Huang et al., 2007; Xing et al., 2019).

In this study, we comprehensively compared epididymis mRNA and miRNAs transcriptomes between roosters with highly divergent semen quality using high-throughput sequencing data. An integrated analysis of differentially expressed genes (**DEGs**) and miRNAs was performed to identify critical genes, miRNAs, pathways, and miRNA−gene pairs associated with rooster reproductive traits in the epididymis.

MATERIALS AND METHODS

Ethics Statement

Animal welfare practices and experimental procedures were performed in accordance with the Guide for the

Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006). All procedures were approved by the Animal Ethics Committee of Beijing University of Agriculture.

Experimental Animals and Sample Preparation

In our study, 232 Hy-Line Brown laying hen roosters were housed at the Wei farm in Beijing, China, under identical environmental and nutritional conditions from 36-wk-old to 45-wk-old. Semen samples were collected 10 times from all roosters once a week using the dorsoabdominal massage technique, and semen motility was tested using a computer-assisted sperm analyser system (WLJX-9000 Weili Color Sperm Analysis System, Weili New Century Science & Tech Dev., Beijing, China). Five randomly selected microscopic fields were analyzed for each semen sample, and the mean value represented the sperm motility of samples. Based on the averaged semen motility, 8 roosters were selected from 232 Hy-Line Brown laying hen roosters. Four roosters had higher sperm motility (86.20 ± 1.81%, HSM group) whereas the other 4 roosters had significantly lower sperm motility (28.85 ± 2.25%, $P < 0.01$, LSM group). After slaughter, epididymis samples were dissected for sequencing and stored in liquid nitrogen.

RNA Extraction, Library Preparation, and Sequencing

Total RNA was extracted from each epididymis sample using TRIzol reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions. The purity and concentration of RNA were evaluated using the ARNA 6000 Nano LabChip Kit for Bioanalyzer 2100 (Agilent, CA). RNA with an integrity number >7 and a 28S/18S ratio >0.7 was used for further high-throughput sequencing.

Eight cDNA libraries were manufactured by reverse-transcription under the protocol for the mRNA-Seq Sample Preparation Kit (Illumina, San Diego, CA). HiSeq 2500 was used for paired-end sequencing according to the manufacturer's instructions (Illumina).

Eight small RNA libraries were also prepared using TruSeq Small RNA Sample Prep Kits and sequenced on an Illumina HiSeq 2500 platform by single-end sequencing based on the manufacturer's instructions (Illumina).

Data Analysis of Gene Expression

Raw sequencing data were collected using Illumina Pipeline v1.5 software. The data were filtered to removed reads with more than 10% unknown sequences, "N", and low-quality sequences (quality score <20). The quality of the remaining reads was assessed using FastQC software (Andrews, 2010). High-quality reads were mapped to the chicken reference genome sequence (version: *Galgal* 6.0: ftp://ftp.ensembl.org/pub/release-101/fasta/gallus_gallus/dna/) and alignments were guided by a gene transfer format file (http://asia.ensembl.org/Gallus_gallus/Info/Index) using TopHat v2.0.1 (Kim et al., 2013). Cufflinks was used to assemble reads (Trapnell et al., 2012). The expression level of each gene was quantified using HT-seq software (Anders et al., 2015). DEGs were detected using the edgeR package between the HSM and the LSM group using false positive rate (**FDR**) ≤ 0.05 and |Log$_2$ FC| ≥ 1 (Robinson et al., 2010).

Data Analysis of miRNA Expression

Eight small RNA libraries were sequenced on a HiSeq 2500 platform (Illumina), and single-end reads were obtained. Before analyzing the pipeline, the Fastx-tool kit was used for quality control to remove adapters and low-quality reads (Gordon, 2010). The CAP-miRSeq pipeline was used for miRNA analysis (Sun et al., 2014). Chicken precursor and mature miRNA information was available from the miRbase database (Kozomara and Griffiths-Jones, 2013). The known miRNAs were quantitated by MirDeep2 (Friedländer et al., 2011). Differentially expressed miRNAs (DEMs) (FDR ≤0.05, and |Log2 FC| ≥1) between the 2 groups were identified using the edgeR package (Robinson et al., 2010).

Verification of Genes and miRNAs Expression Level Using Quantitative Real-Time PCR

Quantitative real-time PCR (**qPCR**) were used to confirm genes and miRNAs expression level in this study. Six DEGs and five DEMs were selected for validation. As the manufacturer's protocol, total RNA was isolated from eight epididymis samples, and then reversed transcription into cDNA using Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc, Waltham, MA). All primers used in this study are shown in Table S1, including the reference genes U6 and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) for miRNAs and genes, respectively. Then, triplicate qPCR in each sample was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) in an ABI 7500 Real-Time PCR System (Applied Biosystems). The ΔCt values were used for calculating the expression of genes in $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The qPCR was accomplished by the Beijing SinoGene Scientific Co., Ltd., China.

Functional Analysis of the DEGs

Kyoto Encyclopedia of Genes and Genomes (**KEGG**) pathway enrichment analysis (Kanehisa and Goto, 2000) and Gene Ontology (**GO**) functional classification including biological process (**BP**) terms, molecular function (**MF**) terms, and cell component (**CC**) term (Ashburner et al., 2000) were performed for the screened DEGs using the R package ClusterProfiler
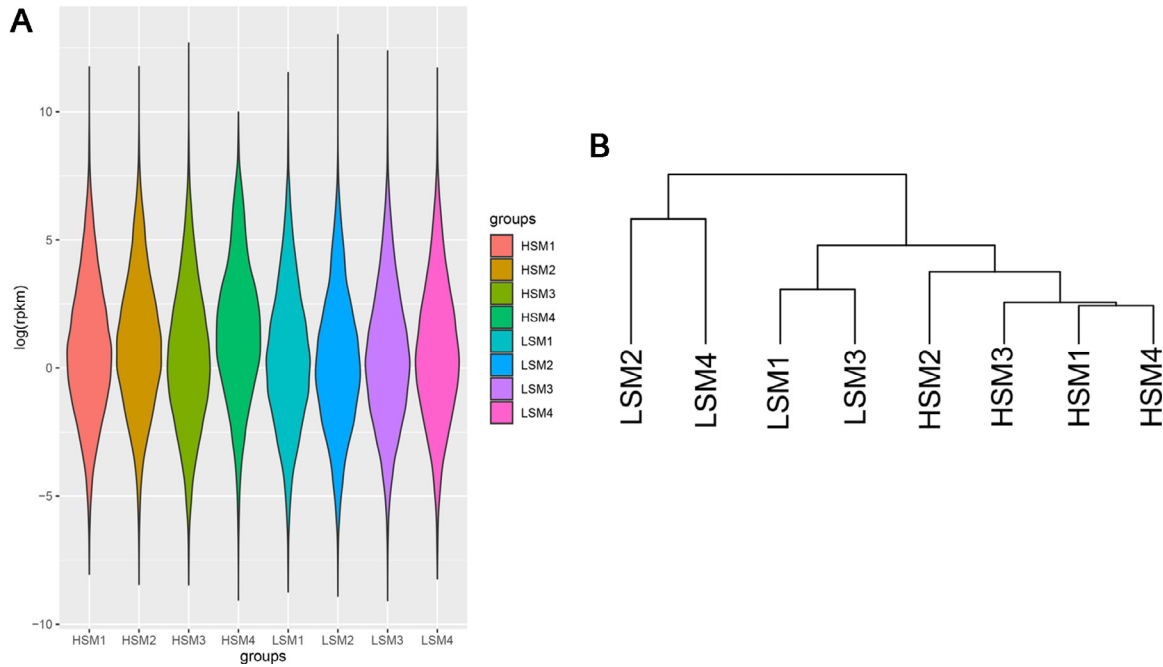
Figure 1. Gene expression and hierarchical clustering of all samples. (A) Gene expression level across all samples. (B) Hierarchical clustering of all samples.

(v3.16.1). GO terms and KEGG pathways with P.adjust < 0.05 were considered significant. Functional protein interaction networks were predicted using the STRING v10 database (Szklarczyk et al., 2014). The Gallus gallus database was used as the reference (Gene Ontology database doi: 10.5281/zenodo.3954044).

Prediction and Functional Analysis of Target Genes of miRNAs

The potential targets of all DEMs were predicted using the multiMiR R package, including nearly 50 million records from 14 different databases (Ru et al., 2014). The predicted genes were maintained and functionally analyzed using ClusterProfiler in more than 1 database. The threshold for significant GO terms and KEGG pathways was set at P.adjust < 0.05. The potential regulatory relationship of predicted miRNA-gene pairs was demonstrated using Cytoscape software (Shannon et al., 2003). Pearson's correlation analysis of each pair was calculated using R software.

RESULTS

Overview of Sequencing Data

In this study, 8 cDNA libraries of epididymis from four HSM roosters and 4 LSM roosters were sequenced. The deep sequencing data was submitted to the National Center for Biotechnology Information (**NCBI**) Sequence Read Archive (**SRA**) under accession no. SUB6851711, Bioproject: PRJNA660289. An average of 31.79 M clean raw data (150 bp) was obtained, and 79.63% were mapped to the reference genome (Table S2). The high mapping ratio and quality scores of Q20 (98.6%) and Q30 (95.3%) demonstrated that the reliability and quality of the reads were adequate for further analysis.

After annotation in the reference genome *Galgal* 6.0, 20656 ensemble genes were detected. Pearson's correlation between the expression of biological replicate samples showed a highly positive correlation (r = 0.9896), and the majority (91.8%) of the epididymis genes is conserved between 2 groups. In addition, hierarchical clustering confirmed 2 distinct clusters with different semen quality (LSM/HSM, Figure 1).

DEGs in the Epididymis Tissue Between the HSM and LSM Groups

A total of 84 DEGs were identified between the HSM and LSM groups, based on the criteria of that FDR was below 0.05 and log2 fold change was above 1 (Table S3). Among them, 51 genes were upregulated in HSM compared with LSM (Figure 2).

To further elucidate the potential roles of the 84 differentially expressed genes, we performed GO and KEGG pathway enrichment analysis using the ClusterProfiler package. We found three significantly enriched KEGG pathways (P.adjust < 0.05), which were cytokine-cytokine receptor interaction, PPAR signaling pathway, and toll-like receptor signaling pathway (Table 1). Sixteen GO terms were significantly associated with DEGs (Figure 3), including 5 BP terms, 10 MF terms, and 1 CC term (P.adjust < 0.05). The details of the GO term analysis are shown in Table S4.
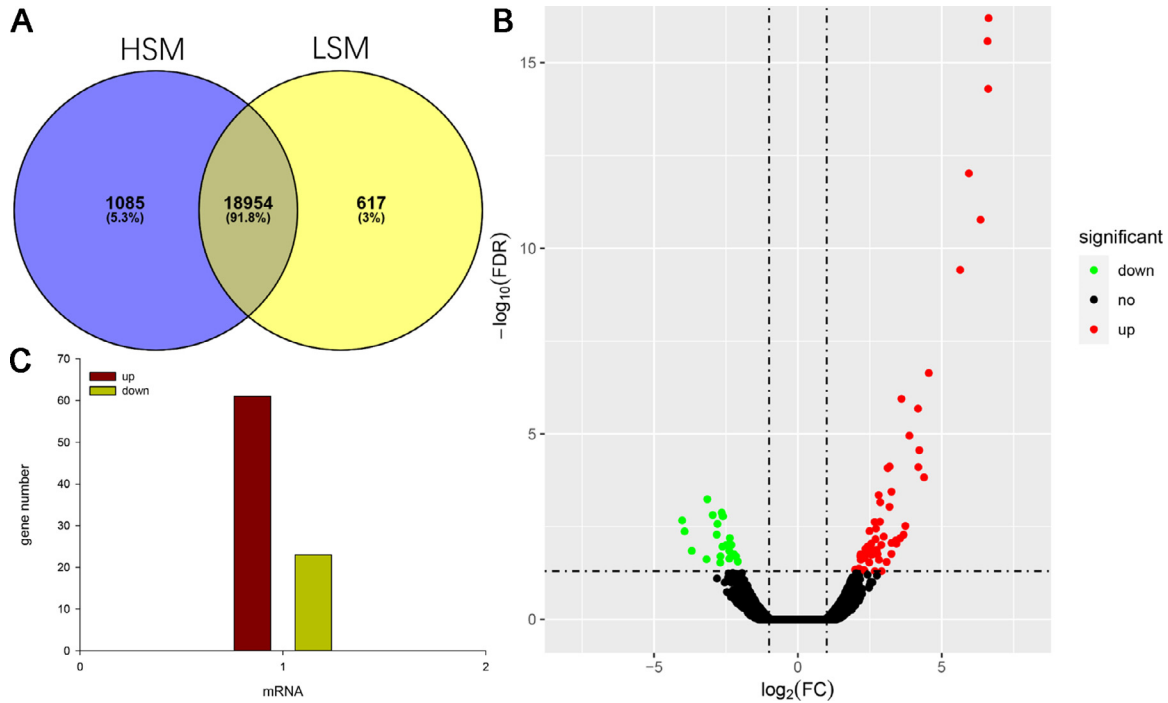
Figure 2. Gene expression in the epididymis of roosters with high sperm motility (HSM) and low sperm motility (LSM). (A) Venn diagrams showing the total number of shared and unique expressed genes in each group. (B) Volcano plot of differentially expressed genes; significantly upregulated genes are represented as red dots and significantly downregulated genes are represented as green dots. (C) Number of significantly upregulated genes and downregulated genes.

Table 1. Pathways (P.adjust < 0.05) enriched in genes differentially expressed between epididymal transcriptomes of high sperm motility and low sperm motility groups.

Pathway ID	Description	P.adjust	Genes
gga04060	Cytokine-cytokine receptor interaction	0.000171	CXCL13, LOC100857191, CCL17, CSF3R, CXCL13L2, CCL4, CXCL13L3
gga03320	PPAR signaling pathway	0.004186	HMGCS2, PLIN1, APOC3
gga04620	Toll-like receptor signaling pathway	0.009336	SPP1, CCL4, CCL17

DEMs in Epididymis Tissue Between the HSM and LSM Groups

Eight small RNA libraries were sequenced from the epididymis in HSM and LSM roosters. The miRNA deep sequencing data was submitted to NCBI SRA under accession no. SUB8071823, Bioproject: PRJNA660426. An average of 20.16 M clean reads were obtained from the chicken epididymis tissue for further analysis. Approximately 72% of clean reads were mapped to the reference genome. After sequencing analysis, a total of 463 miRNAs were found in all samples. Among these miRNAs, 6 DEMs were identified between the two groups using edgeR (Figure 4). The details of DEMs were shown in Table 2.

Expression Level of Genes and miRNAs Verified Using qPCR

Six DEGs (SLN, WT1, PLIN1, LRRIQ1, HSPA5, and DMA) and 6 DEMs (gga-mir-146a, gga-miR-215, gga-miR-194, gga-miR-135b, gga-mir-205b, and gga-mir-214) were chosen to validate the expression trend in the

high throughput sequencing data using the qPCR. The fold change of selected genes or miRNAs were shown the same trend in both RNA-seq or miRNA-seq data and qPCR (Figure 5). Those results indicated that the DEGs and DEMs identified in NGS data were reliable and efficient.

Functional Analysis of Differentially Expressed miRNAs

To understand the function of these six DEMs, we first identified a total of 4,169 putative target genes presented in more than one database in the multiMiR R package. In addition, KEGG and GO pathway analysis enabled us to identify 17 significantly enriched KEGG pathways and 123 GO terms (q-value < 0.05). The detailed information is shown in Table S5.

miRNA-Gene Interaction Related to Semen Quality

As evidenced by the integrated analysis of differentially expressed genes and miRNAs expression profiles, 7 DEGs
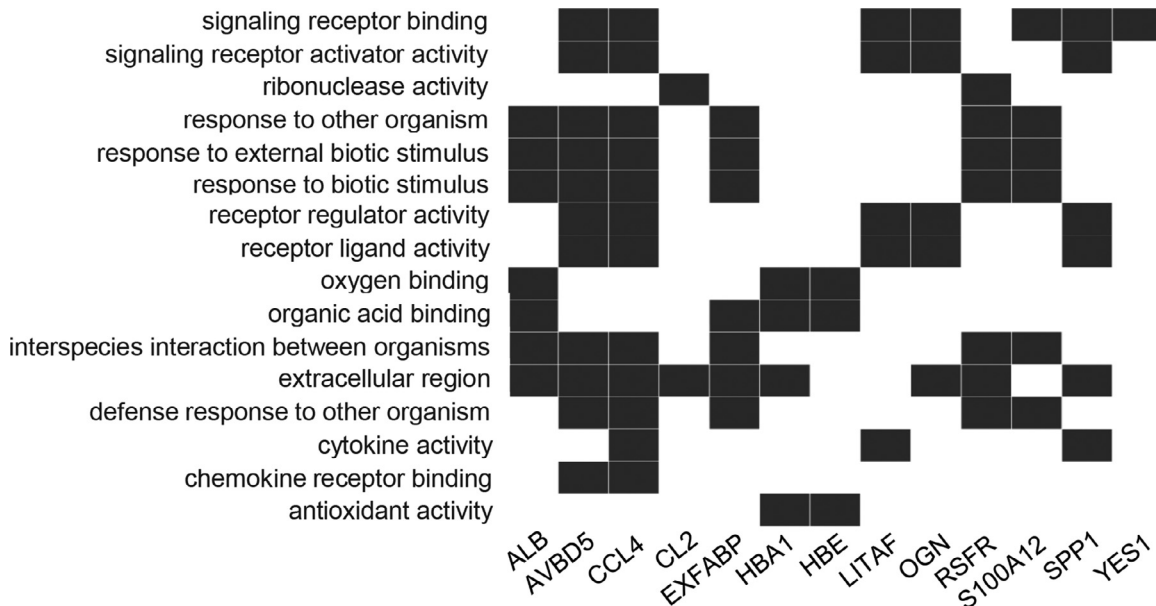
Figure 3. Heatmap of DEGs and their enriched GO terms. Abbreviations: DEGs, differentially expressed genes; GO, Gene Ontology.
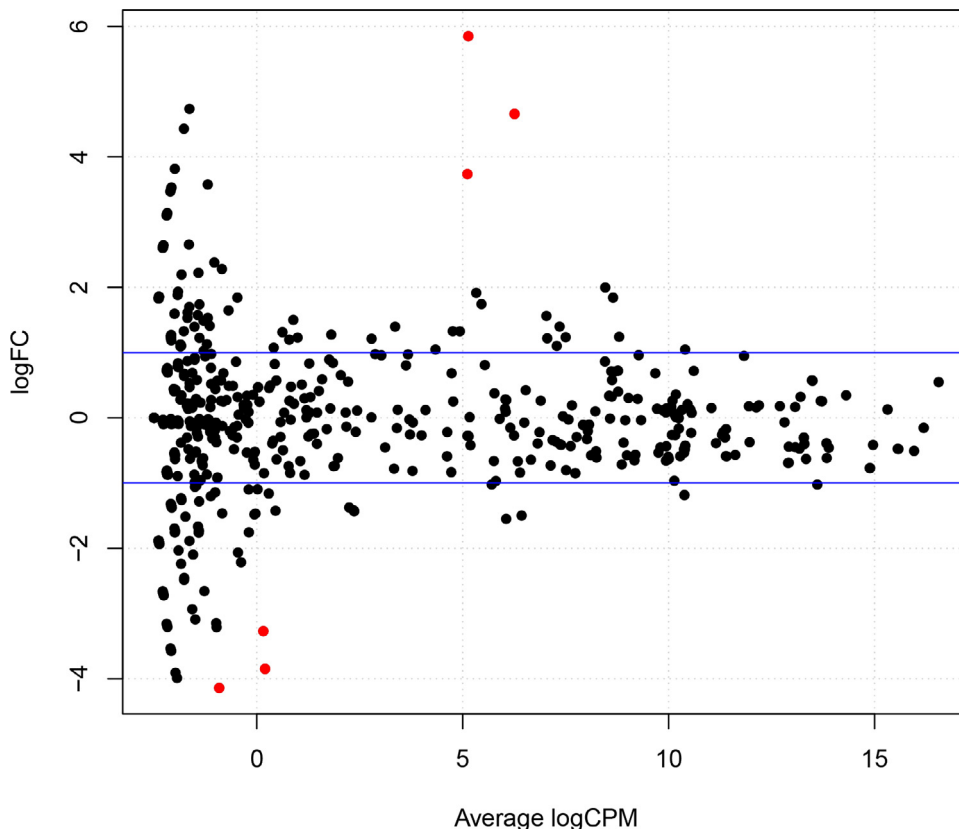

Figure 4. Expression scatter plot of the DEMs between HSM and LSM groups. Red dot represents differently expressed miRNAs with fold change ≥ 2 and P-value ≤ 0.05. X-axis values are average $\log_2$ (counts per million) and y-axis values are $\log_2$ (fold change). Abbreviations: DEMs, Differentially expressed miRNAs; HSM, high sperm motility; LSM, low sperm motility.

(*SRGN, F13A1, YES1, HSP5, EMS1, CENPO,* and *ALB*) were found among the 4,169 predicted target genes of differentially expressed miRNAs, could be regulated by DEM (Figure 6A). A total of 8 miRNA-gene pairs were differentially expressed between the HSM and LSM groups. Five of these pairs of genes and miRNAs showed opposite expression trends when comparing the 2 groups. However, only 2 pairs (miR-135b-HPS5 and miR-205b-*CENPO*) showed significantly negative relationships (Pearson's correlation value ≤-0.8; P ≤ 0.05). The correlation coefficients between miRNAs and their potential targeted genes for each pair are shown in Figure 6B.

Table 2. The six differentially expressed miRNAs detected in roosters with high and low sperm motility.

miRNA	MIMAT ID	Mature sequence	Log$_2$ (FC)	FDR
gga-mir-146a	MIMAT0001163	5' UGAGAACUGAAUUCCAUGGGUU	5.85	6.95E-09
	MIMAT0026529	3' ACCCAUGGGGCUCAGUUCUUCAG		
gga-miR-215	MIMAT0001134	5' AUGACCUAUGAAUUGACAGAC	4.66	1.41E-06
		3' CCUGUCAUUUCUAUAGGCCAAUA		
gga-miR-194	MIMAT0001133	5' UGUAACAGCAACUCCAUGUGGA	3.74	2.88E-04
gga-miR-135b	MIMAT0007536	5' UAUGGCUUUUUAUUCCUUUU	−3.85	8.22E-04
gga-mir-205b	MIMAT0001164	5' CCCUUCAUUCCACCGGAAUCUG	−3.27	6.33E-03
gga-mir-214	MIMAT0050062	5' UGCCUGUCUGUGCCUGCUGUAC	−4.14	1.51E-02
	MIMAT0050063	3' CACAGCAAGUGUAGACAGGCA		
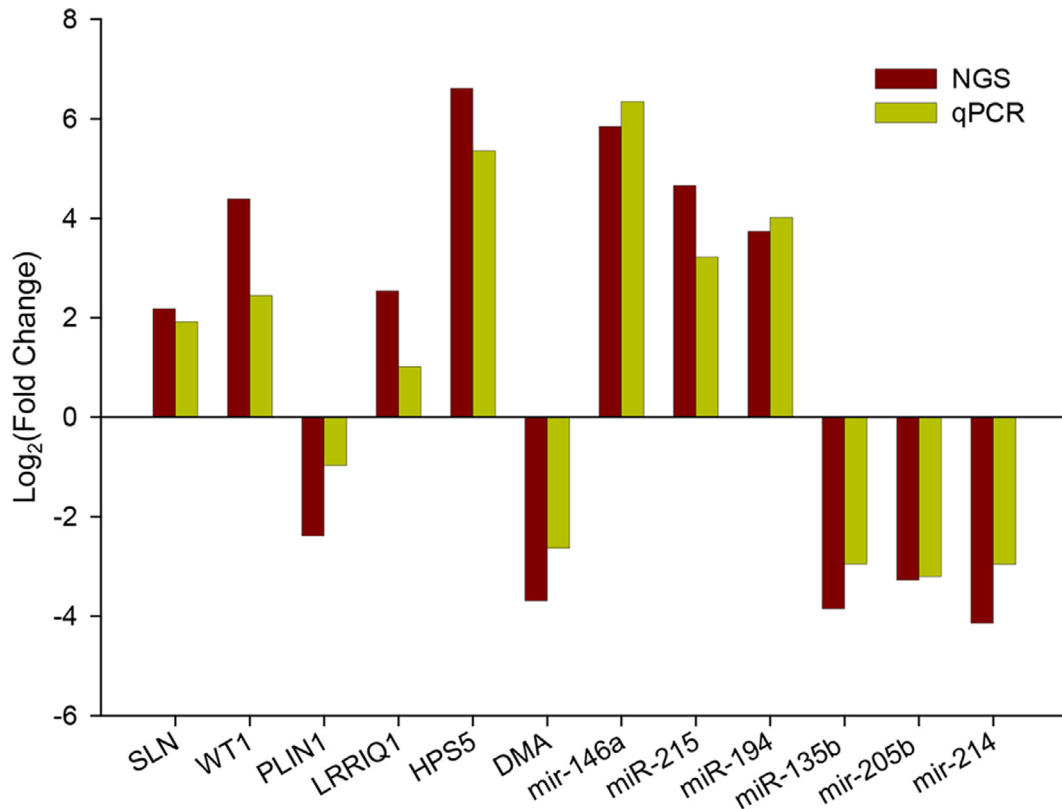
Figure 5. Validation of DEGs and DEMs expression level using next generation sequencing (NGS) and qPCR. Abbreviations: DEGs, differentially expressed genes; DEMs, Differentially expressed miRNAs.

DISSCUSSION

Sperm motility in roosters is an important reproductive trait for chicken husbandry (Reddy and Sadjadi, 1990). The epididymis is necessary for sperm maturation, capacitation, and development of progressive motility in bird (Jones and Lin, 1993). In our study, we focused on identifying key genes and miRNAs affecting sperm motility in the epididymis of roosters using an integrated analysis of miRNA and mRNA transcriptomes. This successful approach helps us better understand the molecular mechanisms of rooster sperm motility and provides a basis for future research.

Measuring the expression of epididymis genes in chickens with divergent sperm motility can provide information on how genes are regulated and reveal details of epididymal biology (Lowe et al., 2017). After removal of low-quality reads, there were average 79.63% reads mapped to the reference genome. This was similar to that of other studies on chicken transcriptome, such as 73.1% (Liu et al., 2018) and 80.2% (Xing et al., 2020) in testis, 82% in sperm storage tubules (Yang et al., 2020), and 75.73% in intraepithelial lymphocyte natural killer cells (Boo et al., 2020). In our previous studies, screening candidate genes associated with sperm motility in testis of chickens with divergent sperm motility by RNA-seq was performed (Xing et al., 2020), and 3 DEGs ([matrix metalloproteinase-9] *MMP9*, [AHNAK nucleoprotein 2] *AHNAK2*, and [sarcolipin] *SLN*) were identified. In the present study, the expression of these 3 genes differed more than 4-fold in the epididymis of chickens with different sperm motility. This implied that these 3 genes might play an important role in the regulation of sperm motility. MMP9 is a member of the proteolytic enzyme family (Fowlkes and Winkler, 2002), which is located on the sperm flagella membrane and involved in the regulation of spermatogenesis in epididymis, and considered a biomarker for semen quality (Warinrak et al., 2015). A
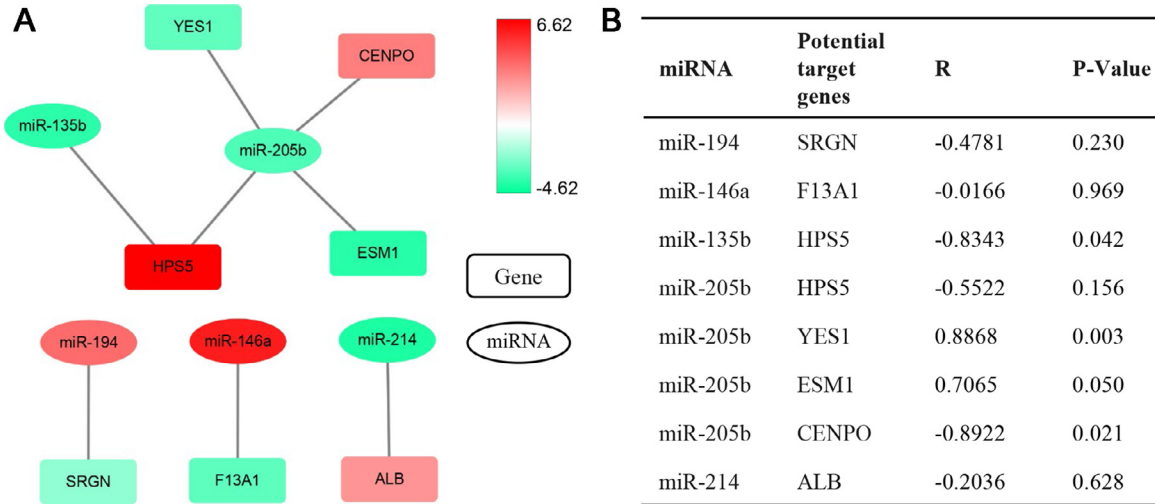
A

B

miRNA	Potential target genes	R	P-Value
miR-194	SRGN	-0.4781	0.230
miR-146a	F13A1	-0.0166	0.969
miR-135b	HPS5	-0.8343	0.042
miR-205b	HPS5	-0.5522	0.156
miR-205b	YES1	0.8868	0.003
miR-205b	ESM1	0.7065	0.050
miR-205b	CENPO	-0.8922	0.021
miR-214	ALB	-0.2036	0.628

Figure 6. Putative regulatory relationships between differentially expressed genes (DEGs) and miRNAs (DEMs). (A) Potential network of genes and miRNAs differentially expressed between HSM and LSM roosters. Squares represent miRNAs, and ellipses represent DEGs, respectively. Upregulated genes or miRNAs are shown in red, and downregulated genes or miRNAs are shown in green to LSM. The network diagram was generated using Cytoscape. (B) The correlation coefficient and *P*-value of each miRNA-gene pair. R means pearson correlation value. Abbreviations: HSM, high sperm motility; LSM, low sperm motility.

small amount of fat is deposited in the epididymis (Guo et al., 2019). During sperm maturation in the epididymis, lipid contents in the sperm membrane change, which facilitates sperm motility (Bjorkgren et al., 2015). When fed a high-fat diet, a significantly greater number of epididymal adipocytes were found in SLN$^{-/-}$ mice than in wild-type mice. This could be why differential expression of SLN in epididymis alters sperm motility. However, the physiological function of AHNAK2 in male reproductive traits remains unclear.

The complex interaction between the epididymis and the immune system has been demonstrated in many reports (Voisin et al., 2019; Wijayarathna and Hedger, 2019). In our study, 8 genes (i.e., *CXCL13, LOC100857191, CCL17, CSF3R, CXCL13L2, CCL4, CXCL13L3*, and *SPP1*) were significantly enriched in two immune-related pathways (cytokine-cytokine receptor interaction and toll-like receptor signaling pathway). It has been documented in male semen that various cytokines act as growth and differentiation factors during physiological processes and pathological conditions (Paulesu et al., 2010). The presence of these molecules in semen plays an important role in both implantation and development of embryos (Robertson et al., 2001). Our results may explain the relationship between reproductive performance and immunobiology in the epididymis of roosters.

Previous studies have indicated that Wilms tumor (**WT1**), perilipin 1 (**PLIN1**), and leucine-rich repeats and IQ motif containing 1 (**LRRIQ1**), which are DEGs in the epididymal tissue of roosters with diverging semen quality, are critical for spermatogenesis and semen quality. In mice, the WT1 gene is a specific biomarker for Sertoli cells, and Sertoli cell-specific factors are vital for differentiation of spermatogonia (Zheng et al., 2014). In addition, WT1 is necessary for spermatogenesis in mice since it regulates Sertoli cell polarity (Wang et al., 2013). It has also been reported that WT1 mutations are associated with male infertility in populations of

European ancestry (Seabra et al., 2015). PLIN1 was reported as a crucial regulator of fat deposition in human adipocytes (Grahn et al., 2013) and porcine intramuscular fat content (Li et al., 2018). PLIN1 is involved in spermatogenesis at the meiotic stage and maturation through deficiency affecting spermatogenesis gene expression in mice (Chen et al., 2014). LRRIQ1 is highly expressed in testis tissue and has been shown to be significantly associated with sperm concentration and testis size in humans. It was also proved that LRRIQ1 may affect male fertility by responding to inhibin B levels (Sato et al., 2020). In our study, the expression of WT1, PLIN1 and LRRIQ1 in the epididymis of high sperm motility chickens was more than 4 times higher than that of low sperm motility chickens, which provided more evidence that these genes affect male reproductive traits.

Previous studies have indicated that miRNAs in the epididymis are implicated in gametogenesis and sperm maturation in various species (Belleannee et al., 2013; Browne et al., 2018; Twenter et al., 2017; Nixon et al., 2015a). However, there are no reports of chicken epididymosomes to our knowledge. We detected a total of 463 miRNAs and 6 differentially expressed miRNAs in epididymis tissue from roosters with diverging sperm motility. In these DEMs, some miRNAs that are involved in male fertility have been verified. For instance, miR-146a, a well-known biomarker of male etiologic factors, contributes to inflammation and cell apoptosis processes (Jacobo et al., 2011). In a previous study, mir-146a was highly expressed in the testis of chickens with higher sperm motility (Liu et al., 2018) and seminal plasma of males with asthenozoospermia (Wang et al., 2011). It has also been reported that miR-135b contributes to spermatogonial stem cell maintenance by regulating forehead box protein O1 activity (Moritoki et al., 2014). In line with our results, miR-135b was more abundant in chickens with low sperm motility as well (Liu et al., 2018). Mir-205, a marker of

the origin of azoospermia in humans (Barcelo et al., 2018), is related to proliferation inhibition in adult epididymal epithelia (Ma et al., 2012). Three other DEMs (miR-194, mir-214, and miR-215) were detected in the epididymis tissue of various species, such as humans (Belleannee et al., 2012), mice (Nixon et al., 2015b), rats (Chu et al., 2015), and equine species (Twenter et al., 2020). The conservation of miRNAs in the epididymis implies that they may control sperm maturation, capacitation, or development of progressive motility. Nevertheless, further studies are required to confirm the functions of these DEMs.

As we know, the regulative way of animal miRNAs is inhibiting translation and/or by inducing degradation of target messenger RNAs (Bartel, 2009). In addition, researches showed that miRNAs also can downregulate mRNA levels (Lim et al., 2005). To delineate the molecular mechanisms between genes and miRNAs that regulate sperm motility, potential regulatory relationships were constructed using a comprehensive profile of miRNA and mRNA transcriptomes. In our study, only 2 pairs (miR-135b-HPS5 and miR-205b and *CENPO*) showed significantly negative relationships. HSP5 is a member of the heat shock protein 70 chaperon family, which protects cells from chemical and heat shock (Kamaruddin et al., 2004). HSP5 was detected in mouse sperm membranes (Choi et al., 2015) and on the surface of human sperm (Naaby-Hansen and Herr, 2010) and was found to be more abundant in caput epididymal sperm than in cauda epididymal sperm in bovine species (Frenette et al., 2006). Besides, HSP5 is localized to the acrosome and principal piece in caput epididymal hamster sperm during epididymal maturation (Kameshwari et al., 2010). Some evidence suggests that HSP5 contributes to sperm capacitation by forming a chaperon complex with ADAM7 (a disintegrin and metallopeptidase domain 7) (Ijiri et al., 2011; Choi et al., 2015). In addition, a previous study has reported that HSP5 could be targeted by miR-205 in gastric cancer (Pavithra et al., 2018). These findings indicate that HSP5 is a novel candidate that affects sperm motility and may be downregulated by miR-205 in rooster epididymis. Lastly, centromere protein O (**CENPO**) is a constitutive centromeric protein involved in cell cycle, chromosome segregation, meiosis, and DNA repair regulation in the human testis (Smith et al., 2014). It was also reported that *CENPO* is down-regulated in the testis of varicocele rats. However, the function of these miRNAs and genes on protein level and their roles in sperm maturation, capacitation, and motility need further studies.

CONCLUSIONS

In this study, we first described the expression patterns of genes and miRNAs in epididymis from HSM and LSM groups using high-throughput sequencing data. A total of 84 genes and 6 miRNAs were identified to be differentially expressed between the 2 groups.

Integrated interpretation of DEGs indicated that *MMP9, SLN, WT1, PLIN1,* and *LRRIQ1* were the most promising candidate genes affecting sperm motility in the epididymis of roosters. MiR-146a, mir-135b, and mir-205 could play important regulatory roles in sperm maturation, capacitation, and motility. Additionally, a comprehensive in silico analysis of the mRNA and miRNAs transcriptomes identified an important correlation in a gene-miRNA pair, miR-135b-HPS5, which may be a key regulator of sperm motility in the epididymis. Our findings provide novel information on the molecular mechanisms underlying reproductive traits in roosters.

DISCLOSURES

The authors confirm they have no conflicts of interest to declare.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2021.101558.

REFERENCES

Ahammad, M. U., C. Nishino, H. Tatemoto, N. Okura, Y. Kawamoto, S. Okamoto, and T. Nakada. 2011. Maturational changes in motility, acrosomal proteolytic activity, and penetrability of the inner perivitelline layer of fowl sperm, during their passage through the male genital tract. Theriogenology. 76:1100–1109.

Anders, S., P. T. Pyl, and W. Huber. 2015. HTSeq—a Python framework to work with high-throughput sequencing data. Bioinformatics. 31:166–169.

Anderson, A. L., S. J. Stanger, B. P. Mihalas, S. Tyagi, J. E. Holt, E. A. McLaughlin, and B. Nixon. 2015. Assessment of microRNA expression in mouse epididymal epithelial cells and spermatozoa by next generation sequencing. Genomics Data 6:208–211.

Andrews, S. 2010. Babraham Bioinformatics − FastQC a quality control tool for high throughput sequence data. Accessed Nov. 2021. http://www.bioinformatics.babraham.ac.uk/projects/fastqc/.

Ashburner, M., C. A. Ball, J. A. Blake, D. Botstein, H. Butler, J. M. Cherry, A. P. Davis, K. Dolinski, S. S. Dwight, and J. T. Eppig. 2000. Gene Ontology: tool for the unification of biology. Nat. Genet. 25:25.

Barcelo, M., A. Mata, L. Bassas, and S. Larriba. 2018. Exosomal microRNAs in seminal plasma are markers of the origin of azoospermia and can predict the presence of sperm in testicular tissue. Hum. Reprod. 33:1087–1098.

Bartel, D. P. 2009. MicroRNAs: target recognition and regulatory functions. Cell. 136:215–233.

Belleannee, C., E. Calvo, J. Caballero, and R. Sullivan. 2013. Epididymosomes convey different repertoires of microRNAs throughout the bovine epididymis. Biol. Reprod. 89:30.

Belleannée, C., É. Calvo, J. Caballero, and R. Sullivan. 2013. Epididymosomes convey different repertoires of microRNAs throughout the bovine epididymis. Biol. Reprod. 89 30, 31-11.

Belleannee, C., E. Calvo, V. Thimon, D. G. Cyr, C. Legare, L. Garneau, and R. Sullivan. 2012. Role of microRNAs in

controlling gene expression in different segments of the human epididymis. PLoS One. 7:e34996.

Belleannée, C., V. Thimon, and R. Sullivan. 2012. Region-specific gene expression in the epididymis. Cell Tissue Res. 349:717–731.

Boo, S. Y., S. W. Tan, N. B. Alitheen, I. Ho, C. L. Ho, A. R. Omar, and S. K. Yeap. 2020. Transcriptome analysis of chicken intraepithelial lymphocyte natural killer cells infected with very virulent infectious bursal disease virus. Sci. Rep. 10:18348.

Bjorkgren, I., H. Gylling, H. Turunen, I. Huhtaniemi, L. Strauss, M. Poutanen, and P. Sipila. 2015. Imbalanced lipid homeostasis in the conditional Dicer1 knockout mouse epididymis causes instability of the sperm membrane. FASEB J. 29:433–442.

Browne, J. A., S. H. Leir, S. E. Eggener, and A. Harris. 2018. Region-specific microRNA signatures in the human epididymis. Asian J. Androl. 20:539–544.

Chen, M., H. Wang, X. Li, N. Li, G. Xu, and Q. Meng. 2014. PLIN1 deficiency affects testicular gene expression at the meiotic stage in the first wave of spermatogenesis. Gene. 543:212–219.

Choi, H., C. Han, S. Jin, J. T. Kwon, J. Kim, J. Jeong, J. Kim, S. Ham, S. Jeon, Y. J. Yoo, and C. Cho. 2015. Reduced fertility and altered epididymal and sperm integrity in mice lacking ADAM7. Biol. Reprod. 93:70.

Chu, C., G. Zheng, S. Hu, J. Zhang, S. Xie, W. Ma, M. Ni, C. Tang, L. Zhou, Y. Zhou, M. Liu, Y. Li, and Y. Zhang. 2015. Epididymal region-specific miRNA expression and DNA methylation and their roles in controlling gene expression in Rats. PLoS One. 10:e0124450.

Cornwall, G. A., and S. R. Hann. 1995. Specialized gene expression in the epididymis. J. Androl. 16:379–383.

Fowlkes, J. L., and M. K. Winkler. 2002. Exploring the interface between metallo-proteinase activity and growth factor and cytokine bioavailability. Cytokine Growth Factor Rev. 13:277–287.

Frenette, G., J. Girouard, and R. Sullivan. 2006. Comparison between epididymosomes collected in the intraluminal compartment of the bovine caput and cauda epididymidis. Biol. Reprod. 75:885–890.

Friedländer, M. R., S. D. Mackowiak, N. Li, W. Chen, and N. Rajewsky. 2011. miRDeep2 accurately identifies known and hundreds of novel microRNA genes in seven animal clades. Nucleic Acids Res. 40:37–52.

Gordon, A., and G. Hannon. 2010. Fastx-toolkit. Accessed Nov. 2021. http://hannonlab.cshl.edu/fastx_toolkit.5.

Grahn, T. H., Y. Zhang, M. J. Lee, A. G. Sommer, G. Mostoslavsky, S. K. Fried, A. S. Greenberg, and V. Puri. 2013. FSP27 and PLIN1 interaction promotes the formation of large lipid droplets in human adipocytes. Biochem. Biophys. Res. Commun. 432:296–301.

Guo, R. M., R. Z. Zhao, J. Zhang, F. Yang, H. Q. Wen, J. Wang, Y. Zhang, and Q. L. Li. 2019. Quantification of fat deposition in the testis and epididymis using mDIXON Quant sequence: correlation with age and ejaculation. Abdom. Radiol. 44:1528–1534.

Huang, J. C., T. Babak, T. W. Corson, G. Chua, S. Khan, B. L. Gallie, T. R. Hughes, B. J. Blencowe, B. J. Frey, and Q. D. Morris. 2007. Using expression profiling data to identify human microRNA targets. Nat. Methods. 4:1045–1049.

Ijiri, T. W., T. Merdiushev, W. Cao, and G. L. Gerton. 2011. Identification and validation of mouse sperm proteins correlated with epididymal maturation. Proteomics. 11:4047–4062.

Jacobo, P., V. A. Guazzone, and M. S. Theas. 2011. Testicular autoimmunity. Autoimmun. Rev. 10:201–204.

Jones, R. C., and M. Lin. 1993. Spermatogenesis in birds. Oxf. Rev. Reprod. Biol. 15:233–264.

Kamaruddin, M., T. Kroetsch, P. K. Basrur, P. J. Hansen, and W. A. King. 2004. Immunolocalization of heat shock protein 70 in bovine spermatozoa. Andrologia. 36:327–334.

Kameshwari, D. B., S. Bhande, C. S. Sundaram, V. Kota, A. B. Siva, and S. Shivaji. 2010. Glucose-regulated protein precursor (GRP78) and tumor rejection antigen (GP96) are unique to hamster caput epididymal spermatozoa. Asian J. Androl. 12:344–355.

Kanehisa, M., and S. Goto. 2000. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res. 28:27–30.

Kim, D., G. Pertea, C. Trapnell, H. Pimentel, R. Kelley, and S. L. Salzberg. 2013. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol. 14:R36.

Kozomara, A., and S. Griffiths-Jones. 2013. miRBase: annotating high confidence microRNAs using deep sequencing data. Nucleic Acids Res. 42:D68–D73.

Li, B., Q. Weng, C. Dong, Z. Zhang, R. Li, J. Liu, A. Jiang, Q. Li, C. Jia, W. Wu, and H. Liu. 2018. A key gene, PLIN1, can affect porcine intramuscular fat content based on transcriptome analysis. Genes. 9:194.

Lim, L. P., N. C. Lau, P. Garrett-Engele, A. Grimson, J. M. Schelter, J. Castle, D. P. Bartel, P. S. Linsley, and J. M. Johnson. 2005. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature. 433:769–773.

Liu, Y., Y. Sun, Y. Li, H. Bai, S. Xu, H. Xu, A. Ni, N. Yang, and J. Chen. 2018. Identification and differential expression of microRNAs in the testis of chicken with high and low sperm motility. Theriogenology. 122:94–101.

Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)). Method. Methods. 25:402–408.

Lowe, R., N. Shirley, M. Bleackley, S. Dolan, and T. Shafee. 2017. Transcriptomics technologies. PLoS Comput. Biol. 13:e1005457.

Ma, W., S. Xie, M. Ni, X. Huang, S. Hu, Q. Liu, A. Liu, J. Zhang, and Y. Zhang. 2012. MicroRNA-29a inhibited epididymal epithelial cell proliferation by targeting nuclear autoantigenic sperm protein (NASP). J. Biol. Chem. 287:10189–10199.

Moritoki, Y., Y. Hayashi, K. Mizuno, H. Kamisawa, H. Nishio, S. Kurokawa, S. Ugawa, Y. Kojima, and K. Kohri. 2014. Expression profiling of microRNA in cryptorchid testes: miR-135a contributes to the maintenance of spermatogonial stem cells by regulating FoxO1. J. Urol. 191:1174–1180.

Naaby-Hansen, S., and J. C. Herr. 2010. Heat shock proteins on the human sperm surface. J. Reprod. Immunol. 84:32–40.

Nixon, B., K. A. Ewen, K. M. Krivanek, J. Clulow, G. Kidd, H. Ecroyd, and R. C. Jones. 2014. Post-testicular sperm maturation and identification of an epididymal protein in the Japanese quail (Coturnix coturnix japonica). Reproduction. 147:265–277.

Nixon, B., S. J. Stanger, B. P. Mihalas, J. N. Reilly, A. L. Anderson, M. D. Dun, S. Tyagi, J. E. Holt, and E. A. McLaughlin. 2015a. Next generation sequencing analysis reveals segmental patterns of microRNA expression in mouse epididymal epithelial cells. PLoS One. 10:e0135605.

Nixon, B., S. J. Stanger, B. P. Mihalas, J. N. Reilly, A. L. Anderson, S. Tyagi, J. E. Holt, and E. A. McLaughlin. 2015b. The microRNA signature of mouse spermatozoa is substantially modified during epididymal maturation. Biol. Reprod. 93:91.

Paulesu, L., S. Jantra, F. Ietta, R. Brizzi, and E. J. H. C. Bigliardi. 2010. Biology. 2010. Cytokines in vertebrate reproduction. Herpetol. Conservat. Biol. 5:335–340.

Pavithra, D., K. Sabitha, and T. Rajkumar. 2018. Identification of small molecule inhibitors for differentially expressed miRNAs in gastric cancer. Comput. Biol. Chem. 77:442–454.

Reddy, R. P., and M. Sadjadi. 1990. Selection for growth and semen traits in the poultry industry: what can we expect in the future. Colloq. l'INRA 54:47–60.

Robertson, S. A., C. Sjoblom, M. J. Jasper, R. J. Norman, and R. F. Seamark. 2001. Granulocyte-macrophage colony-stimulating factor promotes glucose transport and blastomere viability in murine preimplantation embryos. Biol. Reprod. 64:1206–1215.

Robinson, M. D., D. J. McCarthy, and G. K. Smyth. 2010. edgeR: a bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 26:139–140.

Ru, Y., K. J. Kechris, B. Tabakoff, P. Hoffman, R. A. Radcliffe, R. Bowler, S. Mahaffey, S. Rossi, G. A. Calin, L. Bemis, and D. Theodorescu. 2014. The multiMiR R package and database: integration of microRNA-target interactions along with their disease and drug associations. Nucleic Acids Res. 42:e133.

Sato, Y., A. Tajima, M. Kiguchi, S. Kogusuri, A. Fujii, T. Sato, S. Nozawa, M. Yoshiike, T. Mieno, K. Kojo, M. Uchida, H. Tsuchiya, K. Yamasaki, I. Imoto, and T. Iwamoto. 2020. Genome-wide association study of semen volume, sperm concentration, testis size, and plasma inhibin B levels. J. Hum. Genet. 65:683–691.

Seabra, C. M., S. Quental, A. C. Lima, F. Carvalho, J. Goncalves, S. Fernandes, I. Pereira, J. Silva, P. I. Marques, M. Sousa, A. Barros, S. Seixas, A. Amorim, and A. M. Lopes. 2015. The mutational spectrum of WT1 in male infertility. J. Urol. 193:1709–1715.

Shannon, P., A. Markiel, O. Ozier, N. S. Baliga, J. T. Wang, D. Ramage, N. Amin, B. Schwikowski, and T. Ideker. 2003.

Cytoscape: a software environment for integrated models of bio-molecular interaction networks. Genome Res. 13:2498–2504.

Smith, J. F., P. Yango, E. Altman, S. Choudhry, A. Poelzl, A. M. Zamah, M. Rosen, P. C. Klatsky, and N. D. Tran. 2014. Testicular niche required for human spermatogonial stem cell expansion. Stem Cells Transl. Med. 3:1043–1054.

Sun, Z., J. Evans, A. Bhagwate, S. Middha, M. Bockol, H. Yan, and J.-P. Kocher. 2014. CAP-miRSeq: a comprehensive analysis pipeline for microRNA sequencing data. BMC Genomics. 15:423.

Szklarczyk, D., A. Franceschini, S. Wyder, K. Forslund, D. Heller, J. Huerta-Cepas, M. Simonovic, A. Roth, A. Santos, and K. P. Tsafou. 2014. STRING v10: protein−protein interaction networks, integrated over the tree of life. Nucleic Acids Res. 43:D447–D452.

Tian, F., J. Wang, Y. Li, C. Yang, R. Zhang, X. Wang, Z. Ju, Q. Jiang, J. Huang, and C. Wang. 2020. Integrated analysis of mRNA and miRNA in testis and cauda epididymidis reveals candidate molecular markers associated with reproduction in Dezhou donkey. Livest. Sci. 234:103885.

Trapnell, C., A. Roberts, L. Goff, G. Pertea, D. Kim, D. R. Kelley, H. Pimentel, S. L. Salzberg, J. L. Rinn, and L. Pachter. 2012. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat. Protoc. 7:562–578.

Twenter, H., K. Klohonatz, K. Davis, L. Bass, S. J. Coleman, G. J. Bouma, and J. E. Bruemmer. 2020. Transfer of MicroRNAs from epididymal epithelium to equine spermatozoa. J. Equine Vet. Sci. 87:102841.

Twenter, H. M., A. D. Belk, K. M. Klohonatz, L. K. Bass, G. J. Bouma, and J. E. Bruemmer. 2017. An investigation into miRNAs in the equine epididymis as potential regulators of spermatozoal maturation. J. Equine Vet. Sci. 48:61–68.

Valencia-Sanchez, M. A., J. Liu, G. J. Hannon, and R. Parker. 2006. Control of translation and mRNA degradation by miRNAs and siRNAs. Genes Dev. 20:515–524.

Voisin, A., F. Saez, J. R. Drevet, and R. Guiton. 2019. The epididymal immune balance: a key to preserving male fertility. Asian J. Androl. 21:531–539.

Wang, C., C. Yang, X. Chen, B. Yao, C. Yang, C. Zhu, L. Li, J. Wang, X. Li, Y. Shao, Y. Liu, J. Ji, J. Zhang, K. Zen, C. Y. Zhang, and C. Zhang. 2011. Altered profile of seminal plasma microRNAs in the molecular diagnosis of male infertility. Clin. Chem. 57:1722–1731.

Wang, X. N., Z. S. Li, Y. Ren, T. Jiang, Y. Q. Wang, M. Chen, J. Zhang, J. X. Hao, Y. B. Wang, R. N. Sha, Y. Huang, X. Liu, J. C. Hu, G. Q. Sun, H. G. Li, C. L. Xiong, J. Xie, Z. M. Jiang, Z. M. Cai, J. Wang, J. Wang, V. Huff, Y. T. Gui, and F. Gao. 2013. The Wilms tumor gene, Wt1, is critical for mouse spermatogenesis via regulation of sertoli cell polarity and is associated with non-obstructive azoospermia in humans. PLos Genet. 9: e1003645.

Warinrak, C., J. T. Wu, W. L. Hsu, J. W. Liao, S. C. Chang, and F. P. Cheng. 2015. Expression of matrix metalloproteinases (MMP-2, MMP-9) and their inhibitors (TIMP-1, TIMP-2) in canine testis, epididymis and semen. Reprod. Domest. Anim. 50:48–57.

Wijayarathna, R., and M. P. Hedger. 2019. Activins, follistatin and immunoregulation in the epididymis. Andrology. 7:703–711.

Xing, K., M. Gao, X. Li, Y. Feng, Y. Ge, X. Qi, X. Wang, H. Ni, Y. Guo, and X. Sheng. 2020. An integrated analysis of testis miRNA and mRNA transcriptome reveals important functional miRNA-targets in reproduction traits of roosters. Reprod. Biol. 20:433–440.

Xing, K., X. Zhao, H. Ao, S. Chen, T. Yang, Z. Tan, Y. Wang, F. Zhang, Y. Liu, H. Ni, Y. Guo, Z. Hou, and C. Wang. 2019. Transcriptome analysis of miRNA and mRNA in the livers of pigs with highly diverged backfat thickness. Sci. Rep. 9:16740.

Yang, L., X. Zheng, C. Mo, S. Li, Z. Liu, G. Yang, Q. Zhao, and S. Li. 2020. Transcriptome analysis and identification of genes associated with chicken sperm storage duration. Mou. Poult. Sci. 99:1199–1208.

Zheng, Q. S., X. N. Wang, Q. Wen, Y. Zhang, S. R. Chen, J. Zhang, X. X. Li, R. N. Sha, Z. Y. Hu, F. Gao, and Y. X. Liu. 2014. Wt1 deficiency causes undifferentiated spermatogonia accumulation and meiotic progression disruption in neonatal mice. Reproduction. 147:45–52.