Transcriptome analysis of the testes of male chickens with high and low sperm motility

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ABSTRACT The reproductive performance of chicken breeders has significant economic importance in the poultry industry, and sperm motility is an indicator of reproductive performance. This study performed RNA-seq of the testes of Gushi chicken roosters with high and low sperm motility and identified differentially expressed RNAs involved in sperm motility. RNA-seq analysis showed that 73 and 67 differentially expressed mRNAs were up- and downregulated, and 47 and 56 differentially expressed long non-coding RNAs were up- and downregulated, respectively. The genes related to sperm motility and spermatogenesis included KIFC1, KCNK2, and RECS. Functional enrichment analysis revealed that the pathways related to sperm motility included oxidative phosphorylation and glycine, serine, and threonine metabolism. In addition, the MSTGG.15920.1-RECS-MSTGG.11860.2-VWC2 pathway may regulate sperm motility. This study helped elucidate the molecular genetic mechanism of sperm motility in chicken.

Key words: Gushi chicken, RNA-seq, testis, sperm motility

INTRODUCTION

The breeding male occupies a pivotal position in the breeding system. Semen quality is an index of the breeding ability of roosters and affects the egg fertilization rate and even the production performance of the offspring. Low sperm motility is the main manifestation of low fertility in poultry production, and 80% of low fertility breeding males have low sperm motility (Hu et al., 2013).

Sperm motility is an indicator of semen quality (Bonidarenko et al., 2019) and is measured by the percentage of sperm cells that move in a straight line during ejaculation. In mammals, spermatozoa have weak or no motility after exiting the varicocele; motility is acquired in the epididymis but remains quiescent (Vyklicka et al., 2020). Spermatozoa undergo several physiological and biochemical modifications in the male reproductive tract to gain fertilization competence (Zalazar et al., 2020). Poultry sperm viability is low, intermediate, and high in the testis, epididymis, and vas deferens, respectively (Ahammad et al., 2011). Chicken spermatozoa are immotile at ≥40°C and resume motility at 30°C. Motility is determined by Ga2+ homeostasis and the regulation of multiple enzymes, including myosin light chain kinase, protein kinase A, and phosphatases (Ashizawa et al., 2010; Nguyen, 2019).

Transcriptional gene regulation plays a key role in multiple physiological processes, including sperm motility (Song et al., 2019). For instance, DNAH1 mutations in the testis of mice led to defects in sperm flagella, resulting in reduced sperm motility (Coutton et al., 2019). In Atlantic salmon, the inhibitor HC-056456 bound to CatSper channel and blocked Ca2+ influx, inhibiting sperm activation and fertilization (Lissabet et al., 2020). While the genetic molecular mechanisms underlying sperm motility have been studied in terrestrial mammals and marine animals, most studies on sperm motility in chickens focused on the effect of external factors and trace elements. For instance, the addition of trace elements such as zinc and selenium and organic matter such as flaxseed oil to the diet can improved sperm motility and sperm quality in poultry (Huang et al., 2019; Abbaspour et al., 2020). Sperm motility and viability were higher in geese maintained under artificial white light than in birds kept under blue and red light (Chang et al., 2016). However, improvements in poultry husbandry reduced the effects of external factors on sperm motility. Proteomic analysis has
shown that seminal plasma proteins adhesion G protein-coupled receptor and serine peptidase inhibitor Kazal-type 2 have a key role in maintaining sperm motility in chickens (Li et al., 2020).

This study performed RNA-Seq of the testes of sexually mature Gushi roosters with high and low sperm motility to identify differentially expressed (DE) RNAs involved in sperm motility. These results helped identify the factors affecting the reproductive performance of chickens.

**MATERIALS AND METHODS**

All animal experiments were performed in accordance with the regulations of the Chinese National Research Council (1994) and were approved by the Animal Care and Use Committee of Henan Agricultural University (Permit Number:11-0085).

**Sample Collection**

From 238 roosters, 79 healthy 28-wk-old Gushi roosters of similar weight were selected and these animals were kept in the same environment. Semen quality was assessed twice a week for 4 wk using a light microscope, and pH was measured using a pH test paper. At 32 wk of age, 3 animals with high sperm motility (H1, H2, and H3) and 3 with low sperm motility (L1, L2, and L3) were anesthetized with an intravenous injection of sodium pentobarbital (30 mg/kg) and euthanized by cervical dislocation. The right testis of each animal was excised, transferred to an RNase-free centrifuge tube containing tissue RNA preservation solution, and stored at −80°C for analysis.

**RNA Library Preparation and Sequencing**

Total RNA was extracted from the testes using TRIzol (Vazyme, Zhengzhou, China) and resuspended in RNase-free ddH2O. RNA degradation and contamination were detected using 1% agarose gels, and RNA integrity was assessed using an Agilent 2100 bioanalyzer (Agilent Technologies, CA). Total RNA (3 μg per sample) was used to construct each sequencing library. In this project, the mRNA with polyA structure in the total RNA was enriched by Oligo(dT) magnetic beads, and the RNA was broken to a fragment of about 300 bp in length by ion interruption. The first strand of cDNA was synthesized with 6-base random primers and reverse transcriptase using the RNA as template, and the second strand of cDNA was synthesized using the first strand of cDNA as template. After library construction, PCR amplification was used for library fragment enrichment, followed by library selection based on fragment size, which was 450 bp. Then, the libraries were quality checked by Agilent 2100 Bioanalyzer, and the total library concentration and effective library concentration were tested. The libraries containing different Index sequences (each sample with a different Index, and finally the downstream data of each sample according to the Index) were mixed proportionally according to the effective concentration of the library and the amount of data required for the library. The mixed libraries are uniformly diluted to 2 nM, and single-stranded libraries are formed by alkali denaturation. After RNA extraction, purification, and library construction, these libraries were sequenced using Next-Generation Sequencing (NGS) based on the Illumina sequencing platform with Paired-end (PE) sequencing.

**Read Alignment and Assembly**

Adapter sequences, primers, and reads with quality scores below 20 were removed using Cutadapt software, and paired-end reads were mapped to the chicken genome (GCF_000002315.6_GRCg6a_genomic.fna) using HISAT2 software. The number of aligned reads was counted using the HTSeq package for Python, and transcripts were classified as coding sequences, introns, intergenic regions, and UTR. Contigs were assembled into transcripts using Cufflinks version 2.2.1. Transcript levels were expressed as fragments per kilobase of exon per million fragments mapped (FPKM), and transcripts with FPKM >1 were considered differentially expressed.

**Identification of IncRNAs**

The eukaryotic transcriptome non-strand-specific library was used to obtain IncRNAs, and mapped reads were assembled using the Stringtie/Hisat2 pipeline. Transcripts with indeterminate strand orientation were removed, and IncRNAs were identified. The coding potential of predicted IncRNAs can determine whether new transcripts encode proteins.

**Analysis of Differential Expression**

The differential expression of mRNAs and IncRNAs was quantified with DESeq using log2 fold-change>|1.5| and false-discovery rate <0.05 as cut-off criteria. Samples were hierarchically clustered based on RNA expression levels using the hclust package in R. DE IncRNAs between roosters with high and low sperm motility were identified by two-way hierarchical clustering analysis using complete linkage Euclidean distances.

**Prediction of Target Genes**

Cis-regulatory elements located within 10 kbp upstream and downstream of IncRNAs were predicted using jPREdictor (http://bibiserv.techfak.uni-bielefeld.de/jpredictor), as described previously. Pearson correlation coefficients between DE IncRNAs and mRNAs were calculated to identify co-expressed IncRNA-mRNA pairs. The threshold was set to >0.5, and the FDR was set to <0.01.
Functional Enrichment Analysis

Gene Ontology (GO) enrichment analysis was performed using topGO, assuming that the expected number of target genes assigned to each GO term followed a hypergeometric distribution. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed using g:Profiler (https://biit.cs.ut.ee/gprofiler/gost/). P-values of less than 0.05 were considered statistically significant.

Real-Time Quantitative Polymerase Chain Reaction

RNA was reverse transcribed into cDNA using the HiScript III RT SuperMix kit (Vazyme). Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) was performed using SYBR Green I on a LightCycler96 system (Roche Diagnostics, Zhengzhou, China). The transcription level of each target gene was normalized to the housekeeping gene GAPDH using the 2^(-ΔΔCt) method. The reaction mixture contained 5 μL of SYBR Green Mix, 3 μL of RNA-free water, 0.5 μL of each primer, and 1 μL of cDNA. Amplification conditions consisted of an initial denaturation step at 95°C for 5 min followed by 40 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The experiments included 6 biological replicates performed in triplicate. Primers are shown in Table 1.

Table 1. Gene primer sequences.

| Genes   | Primer sequences | Login Number | Product length |
|---------|------------------|--------------|---------------|
| GAPDH   | F: GACATCATCCACCAAGGTCAC  | NM_204305    | 132           |
|         | R: CACCAAGCTGCAAGTCACAC  |             |               |
| KCNK2   | F: ATCCCAATCGGCCACCTCAG  | XM_004935320 | 229           |
|         | R: AGGTTCATGAGGCTGCTCC   | NM_204114    | 195           |
| DIO2    | F: GCTTCTGACTTTCGCGGTCCT | NM_001277379 | 243           |
|         | R: TCTCTCCAACTGATGGCCCGC |             |               |
| HABP2   | F: ATCCTCAAAAGACATAGCCCG | XM_04053731  | 208           |
|         | R: AGGAACCTTGCCATGTCGAG  |             |               |
| AN08    | F: GGGAGGTGTCGTCAAGAGGTG  | XM_004938942 | 222           |
|         | R: TAGACCCAGCGTGCCTCAATGG |             |               |
| OMP     | F: GACGACACATCAGGTTGATG   | XM_01527919  | 207           |
|         | R: TTTGCTTCCGCTCCCTGTTA  | NM_04047085  | 204           |
| TPUPP3  | F: GCACCCCGGCGGCGGGCGA    |             |               |
|         | R: CACAGCTGCTGCTACGTAT    | NM_001081698 | 152           |
| KCNIP1  | F: CACTTCTTCGATCCGCAGGTC  |             |               |
|         | R: GTCTCTTGAGTTCTCCATGA   | NM_205176    | 162           |
| CRYAB   | F: ACCTCCTTCGCCGGTCTCTTC  |             |               |
|         | R: CAGCACTGAGGGCTGCTGCT  | NM_205489    | 217           |
| KIFC1   | F: ACAATCGCTGTGGTGTGATG   |             |               |
|         | R: GTCTTCCATCGTCTCCATAGA | NM_205489    | 217           |
| MSTRG.15920.1 | F: TGAAGAGGTGCGGGTGCTTTC   |             | 157           |
|         | R: GGGGGTCCCAAGACCTTTT   |             |               |
| HBBA    | F: CTATTCCCAAGCGGAGAAGAAGA |             |               |
|         | R: AGGTTTCCCAAGAGCGCTGAA  |             |               |
| MSTRG.33244.1 | F: CATIGGAACCTTCGCGAGACT  |             | 197           |
|         | R: ATGGTCTCATCCCCGACGAC   |             |               |
| MSTRG.9747.1 | F: GGCAAGCTGGGAACCCCTTAT   |             | 168           |
|         | R: AGGCCATTACCTGAGCCCCGCC |             |               |
| REC8    | F: TTTGCTTGATCTGTGCTGCC  | XM_040655012 | 166           |
|         | R: GGTCCCCAAAAAGAGCGCGCG |             |               |
| MSTRG.11860.2 | F: GAGAAAGGAGTGGAGAGGACACCC |             | 157           |
|         | R: CAGATGAGGGCCACAGCCTGAT |             |               |
| VWC2    | F: TTTGCGGCGGAGAACCTCTCC  | XM_040378591 | 228           |
|         | R: GTCAGCTCCACCTCTCGGCG   |             |               |

Statistical Analyses

Data were analyzed by one-way analysis of variance followed by Duncan’s multiple range test using SPSS version 23.0. P-values of less than 0.05 were considered statistically significant. Data are means ± standard errors and were plotted using GraphPad Prism version 7.0.

RESULTS

Assessment of Semen Quality

The ejaculate volume of 79 roosters was 0.37 ± 0.12 mL, pH was 7.70 ± 0.20, sperm motility was 0.51 ± 0.12, sperm viability was 0.76 ± 0.05, sperm density was 20.00 ± 460 million/mL, and sperm malformation rate was 0.13 ± 0.03. The median sperm motility was 0.52, and the lower and upper quartile was 0.42 and 0.61 (Figure 1A). The frequency distribution of sperm motility was normal (Figure 1B). Based on previous results, sperm motility below 0.42 and above 0.61 was considered low and high, respectively. Three animals with high sperm motility (0.74 ± 0.03) and 3 with low motility (0.24 ± 0.02) were selected for analysis of differential expression.

Quality of Transcriptome Data

To screen genes related to testicular development and sperm viability, 6 libraries (L1, L2, L3, H1, H2, H3)
were paired-end sequenced on an Illumina platform. The number of high-quality reads in these libraries was 49673570, 45968990, 43805848, 41691346, 46183652, and 39461786, respectively. The percentage of mapped and unique reads was >92% and >96%.

Analysis of DE mRNAs in Testicular Tissue

DE mRNAs from both groups were counted. A total of 16,837 mRNAs were identified, of which 191 and 195 were specifically expressed in the low and high sperm motility groups, respectively (Figure 2A). A total of 140 mRNAs were DE, of which 73 were upregulated and 67 were downregulated (Figure 2B). Several genes were associated with spermatogenesis and sperm motility, including KIFC1, REC8 and DIO2 (Table 2). DE mRNAs were clustered into 2 major groups based on fold-change (Figure 2C).

GO and KEGG Enrichment Analyses

Functional enrichment analysis was performed to identify pathways associated with sperm viability. mRNAs were enriched in genes encoding G protein-coupled receptors (GABBR2), heat-shock proteins (CRYAB and HSPB2), and alpha-B crystallin (CRYAB) (Table 3). KEGG analysis showed that genes associated with oxidative phosphorylation (NDUFB1, COX5A [downregulated] and MAOB [upregulated]) and glycine, serine, and threonine metabolism could be involved in the regulation of sperm motility (Table 4; Huang et al., 2018; Nguyen et al., 2019).
Validation of RNA-Seq results

RNA-Seq data were validated by RT-qPCR. Nine DE genes—KCNK2, DIO2, HABP2, ANO8, CRYAB, KIFC1, OMP, TPPP3, and KCNIP1—were selected at random (Table 5). PCR results showed that KCNK2, DIO2, HABP2, ANO8, and CRYAB were upregulated, whereas KIFC1, OMP, TPPP3, and KCNIP1 were downregulated in the group with high sperm motility, consistent with RNA-Seq results (Figure 3; Li et al., 2019).

Identification of lncRNAs

LncRNAs were identified using PLEK, CNCI, and PfamScan (Kong et al., 2007), and 7,830 lncRNAs were selected for subsequent analysis. To assess the effect of mRNA levels on sperm viability, DE lncRNAs between the 2 study groups were integrated. mRNA sequences were longer than lncRNAs (Figure 4A). The number of transcripts (Figure 4B). Most lncRNAs contained 2 or 3 exons, whereas most mRNAs had more than 10 exons (Figure 4C), in line with previous results (Li et al., 2020). A total of 7,400 lncRNAs were co-expressed in the high and low sperm viability groups; of these, 227 and 202 were specifically expressed in the low sperm viability and high sperm viability groups, respectively (Figure 4D). A total of 103 lncRNAs were DE: 56 were downregulated (31 in the low sperm viability group), and 47 were upregulated (25 in the high sperm viability group; Figure 4E). LncRNAs were clustered into 2 major groups based on fold-change (Figure 4F).

Analysis of DE lncRNAs

To further investigate how lncRNAs and their target genes regulate sperm motility, functional enrichment

Table 2. Differential genes related to spermatogenesis and sperm motility.

| Gene     | Expression status | Function                                                                 |
|----------|-------------------|--------------------------------------------------------------------------|
| KIFC1    | down              | Participates in mitosis of spermatogonia, meiosis of spermatocytes and    |
|          |                   | acrosome formation during spermatogenesis (Hao et al., 2019; Gao et al., |
|          |                   | 2019).                                                                   |
| REC8     | up                | Meiosis affecting spermatogenesis (Xu et al., 2005; Lin et al., 2020).    |
| KCNK2    | up                | K(2P) channels regulate processes such as germ cell maturation, fertiliz- |
|          |                   | ation and development (Hur et al., 2009).                                |
| UXT      | up                | Essential for spermatogenesis and fertility in germ cells (Schäfer et al., |
|          |                   | 2018; Thomas et al., 2020).                                             |
| SLC26A7  | up                | Regulation of testicular anions, thus regulating sperm motility (Kujala et |
|          |                   | al., 2007).                                                              |
| DIO2     | up                | Acts in testicular cells, thereby affecting steroidogenesis, sperm pro-   |
|          |                   | duction and male fertility (Hernandez 2018).                             |
| C1QTNF3  | down              | Increasing C1q/TNF-related protein 3 improves sperm count and sperm       |
|          |                   | motility (Mu et al., 2018).                                              |

Table 3. The most highly enriched GO terms.

| Category | GO ID       | Term                                   | Total DEG | P-value  | Up_Gene | Down_Gene |
|----------|-------------|----------------------------------------|-----------|----------|---------|-----------|
| CC       | GO:0038039  | G protein-coupled receptor het-         | 1         | 1        |         |           |
|          |             | erodimeric complex                     |           |          | GABBR2  |           |
| CC       | GO:0005581  | collagen trimer                        | 31        | 2        | C1QL1   |           |
| CC       | GO:0005576  | extracellular region                   | 774       | 10       | BMP3, C1QL1, FGF13, LOC10058942, OVALX |           |
| CC       | GO:0031838  | haptoglobin-hemoglobin complex         | 5         | 1        |         | C1QTNF3  |
| CC       | GO:0038037  | G protein-coupled receptor dimeric      | 5         | 1        |         | C1QTNF3  |
| CC       | GO:0097648  | G protein-coupled receptor complex      | 5         | 1        |         | C1QTNF3  |
| MF       | GO:0005212  | structural constituent of eye lens      | 14        | 1        | CRYAB, HSPB2 |           |
| MF       | GO:0000036  | acyl carrier activity                  | 1         | 1        |         | NDUFAB1  |
| MF       | GO:0030492  | hemoglobin binding                     | 1         | 1        |         | HBBBA    |
| MF       | GO:0041620  | ACP phosphopantetheine attachment site  | 1         | 1        |         | NDUFAB1  |
| MF       | GO:0051192  | prosthetic group binding               | 1         | 1        |         | NDUFAB1  |
| MF       | GO:0070579  | methylocytosine dioxygenase activity    | 1         | 1        |         | TET2     |
| BP       | GO:0006211  | 5-methylocytosine catabolic process     | 1         | 1        |         | TET2     |
| BP       | GO:0019857  | 5-methylocytosine metabolic process    | 1         | 1        |         | TET2     |
| BP       | GO:0021577  | hindbrain structural organization       | 1         | 1        |         | DAB1     |
| BP       | GO:0021589  | cerebellum structural organization      | 1         | 1        |         | DAB1     |
| BP       | GO:0021942  | radial glia guided migration of Purkinje cell |         | 1        |         | DAB1     |
| BP       | GO:1905907  | negative regulation of amyloid fibril   | 1         | 1        | CRYAB    |           |

CC stands for "cellular component", MF stands for "molecular function" and BP stands for "biological process".
analysis was performed on 15,895 genes regulated in cis and 3,000 genes regulated in trans. DE genes were implicated in growth and development, reproduction, immunity, and metabolism (Figure 5A) and were significantly enriched in mTOR signaling, VEGF signaling, phosphatidylinositol signaling, RNA transport, and aminoacyl-tRNA biosynthesis (Figure 5B).

### LncRNA-mRNA Interaction Network

The interactions between lncRNAs and mRNAs were analyzed using the igraph package in R (Figure 6). Seven cis-regulated genes interacted with 7 lncRNAs. The genes HBBA, TET2, and SH3TC1 were downregulated, and C1QL1, LOC112530336, LOC100858942, and CNDP1 were upregulated.

### Table 4.
The top 20 KEGG pathways significantly enriched to differential genes.

| Pathway ID | Pathway                              | total_number | DEG_number | P-value  | Up_gene | Down_gene |
|------------|--------------------------------------|--------------|------------|----------|---------|-----------|
| gga00340   | Histidine metabolism                 | 20           | 2          | 0.0075   | MAOB    | CNDP1     |
| gga04060   | Cytokine-cytokine receptor interaction| 202          | 5          | 0.011    | IL1R2, TNFRSF8, IL18R1, BMP3 | IL1RL2 |
| gga04672   | Intestinal immune network for IgA production | 38          | 2          | 0.026    | BLB2    | MADCAM1   |
| gga00330   | Arginine and proline metabolism      | 42           | 2          | 0.031    | MAOB    | CNDP1     |
| gga05114   | Cell adhesion molecules (CAMs)       | 125          | 3          | 0.049    | CD2, BLB2 | MADCAM1   |
| gga00360   | Phenylalanine metabolism             | 13           | 1          | 0.082    | MAOB    | -         |
| gga00770   | Pantetheine and CoA biosynthesis     | 14           | 1          | 0.088    | VNN1    | -         |
| gga00532   | Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate | 19          | 1          | 0.12     | -       | CHST12    |

### Table 5.
Information on the expression amount, location, and length of some of the differential genes in the sequenced samples.

| ID          | baseMean_A | baseMean_B | foldChange(B/A) | P-value | Chromosome | Start site | End site | Length |
|-------------|------------|------------|-----------------|---------|------------|------------|----------|--------|
| C1QTNF3     | 80.44      | 40.42      | 0.50            | 0.0040  | NC_006127.5 | 10370845  | 10387499 | 2927   |
| OMP         | 23.62      | 5.75       | 0.24            | 0.0052  | NC_006088.5 | 1.95E+08  | 1.95E+08 | 5634   |
| GABBR2      | 127.19     | 81.34      | 0.64            | 0.019   | NC_006089.5 | 45450067  | 45474587 | 5383   |
| HBBA        | 38.12      | 18.18      | 0.48            | 0.026   | NC_006088.5 | 1.97E-08  | 1.97E-08 | 618    |
| COX5A       | 138.19     | 58.48      | 0.42            | 0.043   | NC_006097.5 | 2847964   | 2852187  | 639    |
| KCNIP1      | 95.01      | 32.91      | 0.35            | 1.14E-05| NC_006100.5 | 4271439   | 4564776  | 3017   |
| TTPP3       | 2,375.80   | 1,177.92   | 0.55            | 2.64E-05| NC_006098.5 | 1221961   | 1224452  | 1188   |
| ZNF692      | 398.77     | 223.88     | 0.56            | 4.74E-05| NC_006103.5 | 2469991   | 2453265  | 1686   |
| ATP13A4     | 356.24     | 116.87     | 0.33            | 0.00029 | NC_006096.5 | 13237777  | 13270344 | 4810   |
| KIFC1       | 1,068.47   | 293.20     | 0.29            | 0.00044 | NC_006103.5 | 2439128   | 2443841  | 2181   |
| SH3TC1      | 158.32     | 25.78      | 0.16            | 0.00045 | NC_006091.5 | 80795858  | 80854199 | 4861   |
| NDUFAB1     | 508.75     | 312.58     | 0.61            | 0.029   | NC_006101.5 | 471439    | 4564776  | 3017   |
| DAB1        | 30.53      | 13.79      | 0.45            | 0.044   | NC_006095.5 | 2659068   | 26522972 | 6174   |
| AN08        | 125.74     | 282.97     | 2.25            | 5.69E-08| NC_006115.5 | 3514382   | 35235366 | 3767   |
| REC8        | 258.06     | 499.61     | 1.94            | 1.25E-07| NW_02110164.1 | 1747896   | 1751916  | 1417   |
| CRYAB       | 63.29      | 190.53     | 3.01            | 5.61E-06| NC_006111.5 | 6242317   | 6246235  | 3219   |
| SLCEA11     | 132.42     | 240.89     | 1.82            | 9.06E-05| NC_006099.5 | 4455919   | 4556066  | 5563   |
| VWC2        | 135.21     | 220.34     | 1.63            | 0.0019  | NC_006089.5 | 80439066  | 80551992 | 1889   |
| MAOB        | 80.73      | 135.18     | 1.67            | 0.0057  | NC_006088.5 | 7277376   | 7270310  | 6090   |
| BMP3        | 47.97      | 118.42     | 2.47            | 0.0079  | NC_006091.5 | 45450607  | 45474587 | 5383   |
| MROHTL4     | 74.68      | 122.93     | 1.65            | 0.0096  | NC_006127.5 | 67440358  | 67443254 | 1070   |
| SLC26A7     | 109.64     | 167.81     | 1.53            | 0.013   | NC_006089.5 | 4752222   | 47601212 | 6090   |
| PKP3        | 79.43      | 158.77     | 2.00            | 0.018   | NC_006092.5 | 1782404   | 1792449  | 3150   |
| UXT         | 58.32      | 119.15     | 0.50            | 0.024   | NC_028739.2 | 337454    | 345057   | 763    |
| DIO2        | 12.67      | 30.35      | 2.40            | 0.027   | NC_006092.5 | 4057222   | 4076922  | 6900   |
| HABP2       | 8.183      | 21.14      | 2.58            | 0.050   | NC_006093.5 | 2847745   | 28407872 | 2212   |
and LOC112531346 were upregulated. Thirteen trans-regulated genes interacted with 12 lncRNAs. The genes LRRC74A, BORCS6, TPPP3, TEF, LOC112530178, CHST12, and ZNF692 were downregulated, and LOC101749307, LOC107054355, MROH7L4, CRYAB, REC8, and VWC2 were upregulated. REC8 inhibited MSTRG.15920.1 and MSTRG.11860.2, whereas VWC2 inhibited MSTRG.11860.2. REC8 affects sperm meiosis, and VWC2 is a bone morphogenetic protein antagonist (Almehmadi et al., 2018). BMP4 promotes the formation of primordial germ cells in chickens (Zuo et al., 2019).

**DISCUSSION**

Chicken is an economically important livestock product. The reproductive performance of breeding males has a significant economic impact on the poultry industry, and semen quality is an indicator of the reproductive performance of males (Bhave et al., 2020; Tesfay et al., 2020). Nonetheless, international standards for evaluating chicken semen quality are currently unavailable, and the present study intended to fill this gap. Sperm viability below 0.42 and above 0.61 was considered low and high, respectively. These data can serve as a basis for assessing the semen quality of breeding hens.
Avian spermatogenesis is a complex process regulated by the endocrine hypothalamic-pituitary-testicular axis (Xu et al., 2017; Wang et al., 2019). RNA-Seq of testes from animals with high and low sperm viability was performed to identify RNAs affecting spermatogenesis and sperm motility. Several genes were DE between these groups, including \textit{KCNK2} and \textit{KIFC1}. \textit{KIFC1}, a member of the kinesin 14 family, was expressed in human spermatocytes, and the knockdown of this gene inhibited testicular cancer cell division (Xiao et al., 2017; Teng et al., 2019). \textit{KIFC1} may be involved in mitosis in spermatogonia, meiosis in spermatocytes, and acrosome formation in spermatogenesis in Japanese shrimp, and the inhibition of \textit{KIFC1} expression causes apoptosis in spermatocytes (Hao et al., 2019). \textit{KIFC1} is essential for spermatogenesis in Phascolosoma esculenta, red swamp crayfish (\textit{Procambarus clarkii}), Chinese mitten crab (\textit{Eriocheir sinensis}), and Portunus trituberculatus (Ma et al., 2017; Gao et al., 2019; Wei et al., 2019). \textit{KCNK2} is a member of the K2P channel family and is expressed in bovine ovaries, testes, oocytes, and spermatozoa (Hur et al., 2009). Potassium channels regulate various physiological processes in mammalian germ cells, including sperm motility (Winston et al., 2004; Yang et al., 2004).

The molecular mechanisms and signal transduction pathways controlling sperm motility include intracellular...
calcium levels, lipid transfer in the sperm plasma membrane, phospholipid remodeling, and protein phosphorylation (Ashizawa et al., 1994; Han et al., 2007). Moreover, protein kinase A (PKA) and phosphatases control sperm motility (Priyadarshana et al., 2020). Mitochondria are the energy-producing units of the testis that support the energy expenditure of sperm to obtain power, and if they are damaged sperm viability must be affected. Functional enrichment analysis showed that pathways associated with oxidative phosphorylation and glycine, serine, and threonine metabolism affected sperm viability, and NDUFA1 (Hou et al., 2019), COX5A (Xiyang et al., 2020), and MAOB (Raghavan et al., 2020) affect mitochondrial function. Our analysis showed that DE genes were enriched in processes related to reproduction and immunity. Immune cells protect germ cells against pathogens, and testicular macrophages promote spermatogenesis (Bhushan et al., 2020). The mTOR signaling pathway has an important role in spermatogonial stem cell proliferation and differentiation, blood-testis barrier remodeling, and spermatogenesis of Sertoli cells (Moreira et al., 2019).

Our results showed that IncRNAs regulated several genes, including REC8, CRYAB, and TPPP3. MSTRG.15920.1 upregulated the gene adhesion element REC8, which upregulated the IncRNA MSTRG.11860.2 and the mRNA VWC2. REC8 is expressed in mouse spermatocytes and oocytes (Lee et al., 2002; 2003b; Rong et al., 2016) and has a key role in spermatogenesis and oogenesis (Xu et al., 2005; Lin et al., 2020). The cysteine junction protein VWC2 increases bone formation through an antagonistic effect on BMP signaling (Almehmadi et al., 2018). The IncRNA MSTRG.22136.1 regulated the alpha-B crystallin gene CRYAB. CRYAB is involved in eye and heart diseases and cancers (Tang et al., 2018; Zhang et al., 2019; Ruan et al., 2020). In addition, CRYAB regulates apoptosis, inflammation, and oxidative stress.

Mounting evidence suggests that CRYAB was significantly upregulated in the testis of animals with high sperm motility, suggesting that this gene regulates sperm viability. MSTRG.9747.1 upregulated the tubulin polymerization-promoting protein family member 3 gene (TPPP3). TPPP3 inhibition induces apoptosis in human endometrial stromal cells and reduces mitochondrial membrane potential, resulting in embryonic metaphase (Shukla et al., 2019). Moreover, the knockdown of TPPP3 inhibits the proliferation, migration, and invasion ability of human rectal cancer cells and induces apoptosis in vitro (Ye et al., 2017). The interaction of these target genes may affect sperm motility.
CONCLUSIONS

RNA-Seq analysis identified differentially expressed mRNAs and lncRNAs in the testicular tissue of Gushi roosters. Several genes were involved in sperm motility, including KIFC1 and REC8. The MSTRG.15920.1-REC8-MSTRG.11860.2-VWC2 pathway and the lncRNA MSTRG.15920.1 affected sperm motility by regulating REC8. This study helped elucidate the molecular mechanisms of sperm motility in chicken.

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DISCLOSURES

The manuscript has been read and approved by all the named authors and there is no conflict of interest between us. We have given due consideration to the protection of intellectual property rights in relation to this work and there are no impediments to publication, including timing of publication, etc. We further confirm that any aspect of the experimental animal work involved in this manuscript was conducted with the ethical approval of all relevant institutions. We understand that the corresponding author is the sole point of contact for the editorial process (including the editorial manager and direct communication with the office). He/she is responsible for communicating progress with other authors, revision submissions and final approval proof-reading.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2022.102183.

REFERENCES

Abbaspour, B., S. D. Sharifi, S. Ghazanfari, A. Mohammad-Sangcheshme, and S. Hoonabakhsh. 2020. Effect of dietary supplementation of whole flaxseed on sperm traits and sperm fatty acid profile in aged broiler breeder roosters. Reprod. Domest. Anim. 55:594–603.

Ahmad, M. U., C. Nishino, H. Tatamoto, N. Okura, K. 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.

Almehmadi, A., Y. Ohya, M. Kaku, A. Alamoudi, D. Husein, M. Katafuchi, Y. Mishina, and Y. Mochida. 2018. VWC2 increases bone formation through inhibiting activin signaling. Calcif. Tissue Int. 103:663–674.

Ashizawa, K., N. Kawaji, S. Nakamura, D. Nagase, H. Tatamoto, S. Katayama, K. Narumi, and Y. Tsuchi. 2010. Temperature-dependent regulation of sperm motility of Iijima’s copper pheasants (Symaticus semmerringii iiijimae), one of ‘near threatened’ species. Anim. Reprod. Sci. 121:181–187.

Ashizawa, K., H. Tomonaga, and Y. Tsuchi. 1994. Regulation of flagellar motility of fowl spermatozoa: evidence for the involvement of intracellular free Ca2+ and calmodulin. J. Reprod. Fertil. 101:265–272.

Bhavik, K., T. P. J. Koilpillai, V. Ragothaman, S. Jothi, G. Joshi, and V. Ducrocq. 2020. Semen production and semen quality of indigenous buffalo breeds under hot semiarid climatic conditions in India. Trop. Anim. Health Prod. 52:2529–2539.

Bhushan, S., M. S. Thiru, V. A. Guazzzone, P. Jacobo, M. Wang, M. Fijak, A. Meinhardt, and L. Lustig. 2020. Immune cell subtypes and their function in the testis. Front. Immunol. 11:58304.

Bondarenko, V., and J. Cosson. 2019. Structure and beating behavior of the sperm motility apparatus in aquatic animals. Theriogenology 135:152–163.

Chang, S. C., Z. X. Zhuang, M. J. Lin, C. Y. Cheng, T. Y. Lin, Y. S. Jea, and S. Y. Huang. 2016. Effects of monochromatic light sources on sex hormone levels in serum and on semen quality of ganders. Anim. Reprod. Sci. 167:96–102.

Coutton, C., G. Martinez, Z. E. Kherraf, A. Amiri-Yekta, M. Boguenet, A. Saut, X. He, F. Zhang, M. Cristen-Kent, J. Escoffier, M. Bildat, V. Satre, B. Conne, S. Fourati Ben Mustapha, L. El Sanoufi, M. Marrakchi, M. Makni, H. Latrous, M. Kharouf, K. Perret-Gallay, M. Bonhivers, S. Hennebicq, N. Rives, E. Dulouast, A. Toure, H. Gourabi, Y. Cao, R. Zouari, S. H. Rosseini, S. Nej, N. Thierry-Mieg, C. Arnoult, and P. F. Ray. 2019. Bi-allelic mutations in ARMC2 lead to severe astheno-teratozoospermia due to sperm flagellum malformations in humans and mice. Am. J. Hum. Genet. 104:331–340.

Gao, X. M., D. L. Mu, C. C. Hou, J. Q. Zhu, S. Jin, and C. L. Wang. 2019. Expression and putative functions of KIFC1 for nuclear reshaping and midpiece formation during spermiogenesis of Phaseolosoma esculentum. Gene 683:169–183.

Han, Y., C. J. Haines, and H. L. Feng. 2007. Role(s) of the serine/threonine protein phosphatase I on mammalian sperm motility. Arch. Androl. 53:169–177.

Hao, S. L., and W. X. Yang. 2019. KIFC1 is essential for normal spermatogenesis and its deletion results in early germ cell apoptosis in the Kuruma shrimp. Prenaes (Marsupenaeus) japonicus. Aging (Albany NY) 11:12773–12792.

Hernandez, A. 2018. Thyroid hormone role and economy in the developing testis. Vitam. Horm. 106:473–500.

Hou, T., R. Zhang, C. Jian, W. Ding, Y. Wang, S. Ling, Q. Ma, X. Hu, H. Cheng, and X. Wang. 2019. NDUFAB1 confers cardio-protection by enhancing mitochondrial bioenergetics through coordination of respiratory complex and supercomplex assembly. Cell Res. 29:754–766.

Hu, J. L., L. Chen, J. Wen, G. P. Zhao, M. Q. Zheng, R. R. Liu, W. P. Liu, L. H. Zhao, G. F. Liu, and Z. W. Wang. 2013. Estimation of the genetic parameters of semen quality in Beijing-You chickens. Poult. Sci. 92:2606–2612.

Huang, L. X., L. Li, W. Wang, L. Yang, and Y. Zhu. 2019. The role of zinc in poultry breeder and hen nutrition: an update. Biol. Trace Elem. Res. 192:308–318.

Huang, S., S. Cao, T. Zhou, L. Kong, and G. Liang. 2018. 4-tert-octylphenol injures motility and viability of human sperm by affecting cAMP-PKA/PKC-tyrosine phosphorylation signals. Environ. Toxicol. Pharmacol. 62:234–243.

Hur, C. G., C. Choe, G. T. Kim, S. K. Cho, J. Y. Park, S. G. Hong, C. L. Wang. 2019. Expression and putative functions of KIFC1 for nuclear reshaping and midpiece formation during spermiogenesis of Phaeocolosoma esculenta. Gene 683:169–183.

Kong, L., Y. Zhang, Z. Q. Ye, X. Q. Liu, S. Q. Zhao, L. Wei, and G. Gao. 2007. CPC: assess the protein-coding potential of transcripts using sequence features and support vector machine. Nucleic. Acids. Res. 35:W345–W349.

Kujawa, M., S. Hihana, T. Tanaka, K. Kaunisto, J. Hästbacka, C. L. Holmberg, J. Kere, and P. Höglund. 2007. Expression of ion transport-associated proteins in human efferent and epididymal ducts. Reproduction 133:775–784.

Lee, J., T. Iwai, T. Yokota, and M. Yamashita. 2003. Temporally and spatially selective loss of Rec8 protein from meiotic chromosomes during mammalian meiosis. J. Cell Sci. 116:2781–2790.

Lee, J., T. Yokota, and M. Yamashita. 2002. Analyses of mRNA expression patterns of cohesin subunits Rad21 and Rec8 in mice:
