

PHYSIOLOGY AND REPRODUCTION

Comprehensive analysis of miRNAs, lncRNAs, and mRNAs reveals potential players of sexually dimorphic and left-right asymmetry in chicken gonad during gonadal differentiation

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ABSTRACT Despite thousands of sex-biased genes being found in chickens, the genetic control of sexually dimorphic and left-right asymmetry during gonadal differentiation is not yet completely understood. This study aimed to identify microRNAs (**miRNAs**), long noncoding RNAs (**lncRNAs**), messenger RNAs (**mRNAs**), and signaling pathways during gonadal differentiation in chick embryos (day 6/stage 29). The left and right gonads were collected for RNA sequencing. Sex-biased, side-biased miRNAs, lncRNAs, mRNAs, and shared differentially expressed miRNAs (**DEmiRNA**)–differentially expressed mRNAs (**DEmRNA**)–differentially expressed lncRNAs (**DElncRNA**) interaction networks were performed. A total of 8 DEmiRNAs, 183 DElncRNAs, and 123 DEmRNAs were identified for the sex-biased genes, and 7 DEmiRNAs, 189 DElncRNAs, and 183 DEmRNAs for the side-biased

genes. The results of quantitative real-time PCR were generally consistent with the RNA-sequencing results. The study suggested that miRNAs and lncRNAs regulation were novel gene-specific dosage compensation mechanism and they could contribute to left-right asymmetry of chicken, but sex-biased and side-biased miRNAs, lncRNAs, and mRNAs were independent of each other. The competing endogenous RNA (**ceRNA**) networks showed that 17 target pairs including miR-7b (*CYP19A1*, *FSHR*, *GREB1*, *STK31*, *CORIN*, and *TDRD9*), miR-211 (*FSHR*, *GREB1*, *STK31*, *CORIN*, and *TDRD9*), miR-204 (*FSHR*, *GREB1*, *CORIN*, and *TDRD9*), and miR-302b-5p (*CYP19A1* and *TDRD9*) may play crucial roles in ovarian development. These analyses provide new clues to uncover molecular mechanisms and signaling networks of ovarian development.

Key words: chicken, miRNAs, lncRNAs, mRNAs, gonadal differentiation

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INTRODUCTION

In mammals, females are XX and males XY, but in birds, females are ZW and males ZZ. The Z and W sex chromosomes of birds evolved independently of the eutherian X and Y (Fridolfsson et al., 1998; Nanda et al., 1999; Ross et al., 2005). Sexual dimorphism in chicken which has a Z/W sex chromosome system is thought to arise largely as a consequence of sex-biased

gene expression, including thousands of protein-coding genes and several noncoding RNAs (**ncRNAs**) (Ayers et al., 2013; Grath and Parsch, 2016; Mank, 2017; Warnefors et al., 2017). ncRNAs Have no ability of coding proteins while they can act as functional RNAs. miRNAs Are a class of small ncRNAs (≈ 22 nts) which play multiple roles in various biological processes by regulating expression of their target genes and long noncoding RNAs (**lncRNAs**) (Dragomir et al., 2018). lncRNAs Is a type of ncRNA with a length of longer than 200 nts which play critical and complicated roles in the regulation of various biological processes, including chromatin modification, transcription, and posttranscriptional processing (Wei et al., 2017). Several lncRNAs are able to regulate the expression of other RNAs, sharing responsive elements for the same miRNA (**MREs**), thus acting as competing endogenous RNA

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(**ceRNA**) (Salmena et al., 2011). Currently, growing evidences indicated that there are interactions between lncRNAs and miRNAs, the downstream target genes of which have been closely related to animal reproduction (Hawkins and Matzuk, 2010; Yerushalmi et al., 2014; Li et al., 2015; Kimura et al., 2017; Zhang et al., 2019). But the relationship between sex-biased mRNAs and miRNAs in chickens was unclear. In addition, less attention has been given to the potential impact of sex-biased lncRNAs. And few know what the interactions between lncRNAs and miRNAs, and their downstream target genes contribute to sexual dimorphism during the gonadal differentiation in chicken.

In chicken, sex-specific differentiation of the gonads becomes apparent from embryonic day 6–6.5 (E6–6.5/stage 29–30). After gonadal differentiation, male birds develop bilateral testes, whereas female birds develop only a left ovary. In female birds, an asymmetry between left and right gonads becomes very evident after E8 (stage 34). The aforementioned studies in chicken focused on protein-coding genes, and many genes were related to the asymmetrical development and the right ovary degeneration, such as *Bmp7*, *Pitx2*, *GOS2*, TDRD members, and so on (Andrews et al., 1997; Hoshino et al., 2005; Guioli and Lovell-Badge, 2007; Rodriguez-Leon et al., 2008; Wan et al., 2017). However, there was little known about the contributions of miRNAs and lncRNAs in left-right asymmetry in chicken. Several miRNAs were reported to play key role in testicular development and the proliferation of primordial germ cells, such as miR-202, miR-107 and miR-302b (Bannister et al., 2011; Miao et al., 2016; Lazar et al., 2018). Currently, growing evidences indicated that lncRNAs might have contributed to ovarian development (Bouckenheimer et al., 2018; Li et al., 2018; Liu et al., 2018). These suggested a role for miRNAs and lncRNAs in left-right asymmetry in chicken.

Taken together, sexual dimorphism depends on sex-biased gene expression, but the contributions of lncRNAs have not been assessed, and there was little known about the contributions of miRNA and lncRNA in left-right asymmetry. In addition, whether the sex-biased gene has an impact on left-right asymmetry or not. We therefore produced a deep RNA sequencing data to identify potential players involved in sexually dimorphic and left-right asymmetry by comprehensive analysis of miRNA, lncRNA, and mRNA expression during gonadal differentiation in chicken gonad (day 6/stage 29).

MATERIALS AND METHODS

Fertilized Eggs and Animal Care

Fertilized eggs of Huiyang Bearded chickens were obtained by artificial insemination. All chick embryos used in this study were taken care as outlined in the Guide for the Care and Use of Experimental Animals (Institutional Animal Care and Use Committees of Guangdong

Academy of Agricultural Sciences) and as specifically approved for this study (GAAS-IAS-P-01-2018-03).

Sample Preparation

Huiyang Bearded eggs were incubated in a Rcom PRO 50 egg incubator (Rutoex Co., Ltd.) until stage 29 (embryonic day 6, E6) at 38°C (Hamburger and Hamilton, 1951). Gonads were dissected from the ventral surface of the mesonephric kidney of chicken embryos at E6 in Petri dishes (BD Falcon) containing Dulbecco's phosphate-buffered saline (DPBS; Invitrogen Inc., Carlsbad, CA) under a microscope. The left and right gonad were removed and immediately frozen in liquid nitrogen and stored at –80°C, respectively. The gonad was stored in the central position of the thermal container with dry ice between withdrawal and extraction. Embryonic body was collected and stored at –20°C prior to DNA extraction (Magen Biotech Co., Ltd.). Embryos were sexed by PCR using the primers 5'-GTTACTGATTCGTCTACGAGA-3' and 5'-ATTGAAATGATCCAGTGCTTG-3'. PCR reactions were 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 57°C for 45 s, 72°C for 30 s, and a final extension step of 72°C for 5 min. PCR products were analyzed by using 1% agarose gel. More than 700 embryos were collected, and gonadal tissues were pooled according to sex. After sexing, 100 left or right gonads were pooled with the TRIzolreagent (Invitrogen, Inc., Carlsbad, CA) for each replicate (3 replicates were collected for each tissue), respectively.

RNA Extraction and Library Preparation

Total RNA was extracted using the Trizol reagent (Invitrogen Inc., Carlsbad, CA) according to the manufacturer's protocol. The integrity of RNA was checked by using 2% agarose gel. The RNA quality was evaluated using the K5500Plus Spectrophotometer (Beijing Kaiiao Technology Development Co., Ltd., Beijing, China) and Agilent 2,100 Bioanalyzer (Agilent Technologies Inc., La Jolla, CA). Purified RNA was stored at –80°C until required. RNA with amount >6 µg, concentration ≥200 ng/ml, 1.8 < OD260/280 < 2.2, and RIN >8.5 was used to prepare cDNA library construction.

Small RNA Library Construction, Sequencing, and Data Processing

After extraction and purification, about 2 µg total RNA per sample was used to construct the small RNA library using TruSeq small RNA sample preparation kit (Illumina Inc., San Diego, CA) according to the manufacturer's instruction. Adapters were ligated to the 3' end of the RNA, followed by the ligation of the 5' adapter. Subsequently, the RNA was reverse transcribed to create single-stranded cDNA, followed by single-end sequencing (50 base pairs in length) on an Illumina HiSeq 2,500 platform (Illumina Inc., San Diego, CA).

Raw data (raw reads) were processed with in-house scripts consisting of adapter trimming, read alignment, and read counting. By using Bowtie1 (<http://bowtie-bio.sourceforge.net/index.shtml>), the clean reads were mapped to the chicken reference genome, GRCg6a (GCA_000002315.5). Then, the expression of miRNA was quantified by using miRDeep2 (<https://www.mdcbertlin.de/8551903/en/>). Mapping reads to mature miRNA and hairpin which is recorded in miRbase to identify known miRNA. After excluding the reads mapped to known miRNA/ncRNA/repeat region/mRNA region, the remaining reads were used to predict novel miRNA, the key of prediction is hairpin structure and stability. Moreover, the differentially expressed miRNAs (**DEmiRNAs**) between samples were identified using DESeq package in R. miRNA With P value < 0.01 and $|\log_2(\text{Fold_change})| > 2$ were used as the criteria of significance.

lncRNA + mRNA High-throughput Sequencing and Data Processing

A total of 3- μ g RNA per sample was used for the RNA sample preparations. We constructed the library for high throughput sequencing of lncRNA and mRNA as follows: 1) Remove the ribosomal RNA from total RNA by Ribo-Zero Magnetic gold kit (EpiCentre, Madison, WI); 2). Construct the library with NEB Next Ultra Directional RNA Library Prep Kit (NEB Inc., Ipswich, MA). RNA was purified and fragmented into 200~500 base pairs; the first cDNA strand was synthesized by RNA fragments primed with random hexamer primers; the second cDNA strand was synthesized with dUTP instead of dTTP; after purification by Qiaquick PCR purification kit, end repair 3'end adenylation and adapter ligation were performed; the second cDNA strand was digested by using UNG enzyme (Illumina, Inc., San Diego, CA) and PCR was performed to construct library for high throughput sequencing of lncRNA and mRNA; 3) All libraries for high-throughput sequencing of lncRNA and mRNA were amplified through PCR for 15 cycles; Then, purification was performed by Certified Low Range Ultra Agarose (Bio-Rad), and quantification was performed by Picogreen (Molecular probes) on TBS380 (Turner Biosystems); Bridge PCR was performed on cBot; 4) The libraries were sequenced on an Illumina HiSeq X platform (Illumina Inc., San Diego, CA), and 125-bp paired-end reads were generated.

By using base calling, all the raw data obtained from high-throughput RNA-sequencing were translated into raw FASTQ sequence data. Then raw reads of FASTQ format were processed with Perl scripts to ensure the quality of data used in the following analysis. To obtain the clean reads from RNA-sequencing results for lncRNA and mRNA, sequence with low quality including adaptor sequences, sequences with quality score < 20 , and sequences with N base rate of raw reads $> 10\%$ were removed by using cutadapt (<http://cufflinks.cbc.umd.edu/>). After sequence with low

quality including adaptor sequences, sequences with quality score < 20 , sequences with N base, and sequence less than 18 bp were removed by using Fastx-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). After filtering the raw reads, we obtained the clean reads. And statistics were performed on its quantity and quality, including Q30 (the proportion of nucleotides with quality values > 30) (Ewing and Green, 1998) statistics, data quantity statistics, base content statics, and so forth.

The clean reads were mapped to the chicken reference genome, GRCg6a (GCA_000002315.5), using HISAT2. Then read count for each gene in each pool was counted by HTSeq, and FPKM (Fragments Per Kilobase of exon model per million mapped reads) were then calculated to represent the expression level of genes in the sample.

The DErnAs and DELncRNAs between samples were identified using DESeq (<http://www.bioconductor.org/packages/release/bioc/html/DESeq.html>). The P value < 0.01 and $|\log_2(\text{Fold_change})| > 1$ were used as the criteria of significance.

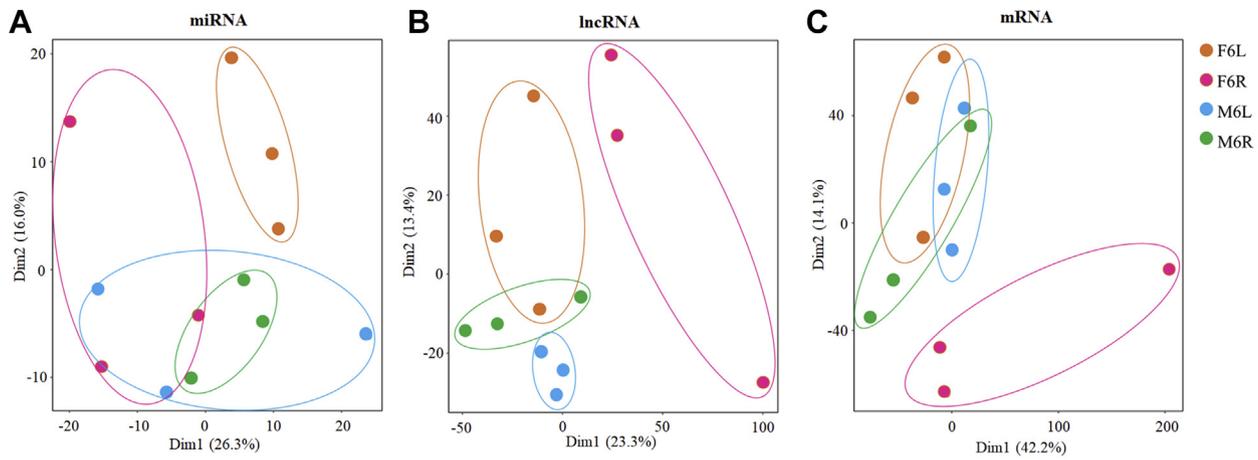
Schema for Integrative Analysis of DEmiRNAs, DELncRNA, and DEMRNA

Systematic bioinformatic analysis was developed based on possible functional relationships between DEmiRNAs, DELncRNA, and DEMRNA. First, the putative targeted DELncRNAs of DEmiRNAs and DEMRNAs of DEmiRNAs were predicted by 3 bioinformatic algorithms including miRanda (MicroRNA Target Prediction Database), PITA (Probability of Interaction by Target Accessibility), and Targetscan (Prediction of microRNA targets), respectively. Second, we selected the mRNAs with high Spearman correlation coefficient ($\rho \geq 0.9$) as the trans-targets, and the mRNAs within the 50-kb upstream and downstream regions of each DELncRNAs were cis-targets. According to the functional relationships between these molecules, the miRNA target gene regulatory network, miRNA-lncRNA target regulatory network, and lncRNA-mRNA coexpression network were established, respectively. Then, we constructed the ceRNA network. The ceRNA network was reconstructed by aggregating all coexpression competing triplets identified previously and was visualized using Cytoscape software at the same time. All node degrees of the ceRNA network were calculated simultaneously.

Gene Ontology and Kyoto Encyclopedia of Genes and Genomes Pathway Enrichment Analysis

We performed gene ontology (**GO**) enrichment and Kyoto Encyclopedia of Genes and Genomes (**KEGG**) pathway analyses on these DEMRNAs and predicted target genes of DEmiRNAs and DELncRNAs. GO term and KEGG pathway analyses of coding genes were performed by using an online-based software program

Principal component analyses of miRNA, lncRNA, and mRNA expression profiles



Unsupervised clustering analysis showing expression profiles of miRNAs, mRNAs, and lncRNAs

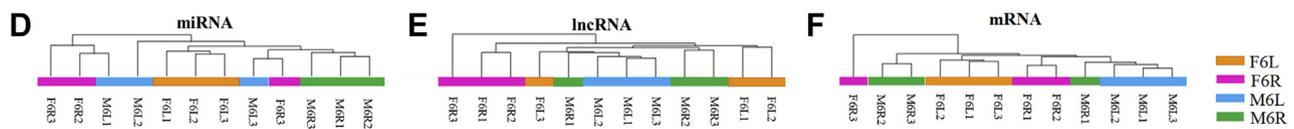


Figure 1. RNA-seq reveals distinct expression pattern of miRNAs, lncRNAs, and mRNAs at E6. (A–C) Principal component analyses of miRNA, lncRNA, and mRNA expression profiles. (D–F) Unsupervised clustering analysis showing expression profiles of miRNAs, mRNAs, and lncRNAs among the 4 groups. Abbreviations: lncRNAs, long noncoding RNAs; miRNAs, microRNAs; mRNAs, messenger RNAs.

GeneCoDis3 (<http://genecodis.cnb.csic.es/analysis>). Both GO terms and KEGG pathways with corrected P values < 0.05 were considered to be significantly enriched.

Real-Time PCR

Dissected left or right gonads from embryonic stage 29 were pooled according to sex, including 3 biological replicates. Approximately 50 gonads were used for each pool. Total RNA was isolated by using the Trizol reagent (Invitrogen Inc., Carlsbad, CA) according to manufacturer's protocol. The levels of miRNAs were measured by quantitative real-time PCR (qRT-PCR) using miDETECT A Track miRNA qRT-PCR Kit (RiboBio, Guangzhou, China) according to the manufacturer's instructions. The primers for gga-miR-2954, gga-miR-202-5p, gga-miR-302b-3p, gga-miR-302b-5p, gga-miR-302d, and U6 small nuclear RNA were obtained from RiboBio Company (Guangzhou, China). The miRNA expression levels were normalized to the expression of the internal control U6 using the $2^{-\Delta\Delta CT}$. The mRNA and lncRNA template were reversely transcribed into cDNA using a FastQuant RT Kit according to the manufacturer's protocol. qRT-PCR was carried out using iTaq Universal SYBR Green Supermix kit (Bio-RAD, Berkeley, CA) in Bio-RAD CFX96 Real-Time Detection system. Samples were normalized against *HPRT* using the comparative C_T method ($\Delta\Delta C_T$) as previously described (Smith et al., 2008;

Ayers et al., 2013). The primers sequences of mRNAs and lncRNAs used in this study are listed in Supplementary Table 1.

RESULTS

Analysis of Transcriptome Sequencing

Deep whole-transcriptome sequencing was used to profile coding and ncRNA expression of 12 pools from 3 pools of female gonads on the left, 3 pools of female gonads on the right, 3 pools of male gonads on the left, and 3 pools of male gonads on the right during the sexual differentiation in chick embryos (E6). Counts of clean reads, Q30, and mapped ratio of sequencing results were displayed in Supplementary Table 2. All coding and ncRNAs that identified in at least 3 of 6 gonad replicates (left and right) were kept for further analysis. Deep RNA-seq analysis detected expression of 1,557 miRNA, 16,540 lncRNA, and 16,910 mRNA, including novel 445 miRNA, 4,284 lncRNA, and 666 mRNA precursors. Principal component analyses revealed that miRNA, lncRNA, and mRNA expression profiles distinguished from the 4 groups: female gonads on the left or right (F6L and F6R) and male gonads on the left or right (M6L and M6R) (Figures 1A–1C). Unsupervised hierarchical clustering of the miRNAs, lncRNAs, and mRNAs showed each group clustered together too, despite inter-individual variation (Figures 1D–1F).

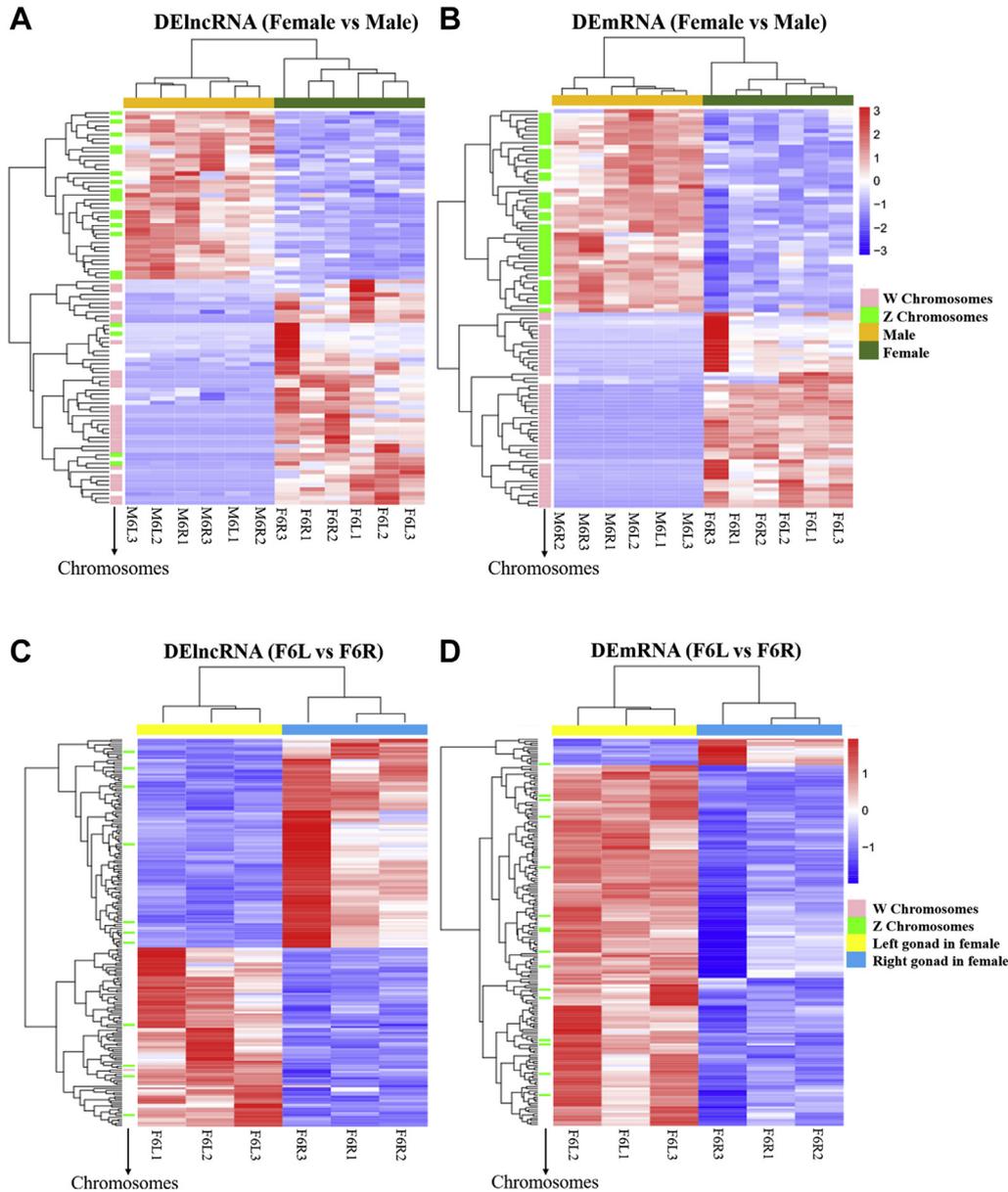


Figure 2. RNA-seq reveals distinct expression pattern of DE miRNAs, DE lncRNAs, and DEMRNAs at E6. (A–B) Unsupervised clustering analysis showing expression profiles of DE miRNAs, DEMRNAs, and DE lncRNAs between the sexes at E6. (C–D) Unsupervised clustering analysis showing expression profiles of DE miRNAs, DEMRNAs, and DE lncRNAs between the left and right gonads in female. Abbreviations: Chr, chromosomes; DE lncRNAs, differentially expressed long noncoding RNAs; DE miRNAs, differentially expressed microRNAs; DEMRNAs; differentially expressed messenger RNAs.

Deep RNA-seq Revealed Characteristic of Active Phase of Sexual Differentiation in Chicken

To identify the sex-biased genes, we compared the gene expression profiles between the sexes at E6. Unsupervised hierarchical clustering of the DE miRNAs, DE lncRNA, and DEMRNA revealed a distinct expression signature of all 3 RNA species in female and male gonads (Figures 2A, 2B). The majority of these DE genes reside on the sex chromosomes (W or Z chromosome). The most sex-biased miRNAs, lncRNAs, and mRNAs exhibiting significant upregulation and downregulation were listed in Table 1. Of the 8 miRNAs, 2 DE miRNAs

were reported, such as gga-miR-7b and gga-miR-2954 (Ayers et al., 2013; Warnefors et al., 2017). Similarly, over 40 (total 123) genes were reported to be associated with sex-biased in chicken gonads, such as 27 W-linked genes, *CYP19A1/Aromatase*, and so on (Ayers et al., 2013; Bellott et al., 2017). Functional annotation revealed that DEMRNAs were mainly involved in forty-four GO terms, DNA integration (FDR = 2.612E-10), viral genome integration into host DNA (FDR = 9.70E-080), viral nucleocapsid (FDR = 2.25E-05), and RNA-DNA hybrid ribonuclease activity (2.67E-12) were significantly enriched GO terms (Table 2). There were no significantly enriched pathways between female and male gonads at E6.

Table 1. The most sex-biased known miRNAs, lncRNAs and mRNAs per gonadal pool.

miRNAs			
Gene symbol	Chr ²	Log ₂ FC	P _{adj}
miR-1416-5p	Z	-1.30	0.0243
miR-204	10	-1.23	0.0031
miR-211	28	-1.23	0.0031
miR-2954	Z	-2.96	4.88E-16
miR-3538	1	1.28	0.0448
miR-6606-5p	Un ³	6.41	1.08E-09
miR-7b	W	7.01	2.82E-47
lncRNAs			
Ensembl Gene ID	Chr ²	Log ₂ FC	P _{adj}
ENSGALG00000051419	Z	6.64	3.87E-06
ENSGALG00000053643	Z	-1.85	0.0024
ENSGALG00000052444	Z	-2.22	0.0452
ENSGALG00000053311	7	-2.75	0.0222
ENSGALG00000033566	5	-2.99	0.0183
ENSGALG00000050966	3	-4.48	0.0229
ENSGALG00000048380	10	-4.92	0.0103
ENSGALG00000050161	Z	-5.64	1.34E-10
mRNAs ¹			
Gene name	Chr ²	Log ₂ FC	P _{adj}
<i>GPBP1W</i>	W	8.72	1.06E-08
<i>SMAD2W</i>	W	8.32	9.18E-06
<i>MIER3W</i>	W	8.04	3.98E-06
<i>ZFRW</i>	W	7.96	5.70E-18
<i>SPIN1W</i>	W	7.79	7.73E-11
<i>NIPBL</i>	W	7.67	3.16E-20
<i>VCPW</i>	W	7.63	3.81E-17
<i>UBE2R2W</i>	W	7.55	1.75E-13
<i>Wpkci-7</i>	W	7.41	3.36E-32
<i>NEDD4LW</i>	W	7.40	0.0002
<i>FGD5</i>	12	-1.27	0.0001
<i>CHD1Z</i>	Z	-1.28	0.0270
<i>NSA2</i>	Z	-1.29	0.0007
<i>FRMD3</i>	Z	-1.29	8.42E-05
<i>CLTA</i>	Z	-1.41	7.15E-06
<i>SMC2</i>	Z	-1.42	0.0262
<i>C1H21ORF91</i>	1	-1.55	7.55E-07
<i>CETN3</i>	Z	-1.67	2.15E-07
<i>PRLR</i>	Z	-2.37	1.47E-14
<i>FAM240 B</i>	Z	-2.62	5.08E-08

Abbreviations: lncRNAs, long noncoding RNAs; miRNAs, microRNAs; mRNAs, messenger RNAs.

¹Top 10 upregulated and downregulated mRNAs per gonadal pool, and the FPKM > 1.

²Chromosomes.

³Nonrandom.

In addition, we compared the gene expression profiles between the left and right gonads to identify the side-specific genes in female. Unsupervised hierarchical clustering of the expression profiles of DE miRNAs, DE lncRNAs, and DE mRNAs revealed that all 3 RNA species expression profiles can significantly distinguish the female left and right gonad (Figures 2C, 2D). The majority of these DE genes between left and right gonads reside on the autosomal chromosomes. The most significant miRNAs, lncRNAs, and mRNAs exhibiting significant upregulation and downregulation were listed in Table 3. Of the 183 DE mRNAs, over 10 were reported to be associated with left-right asymmetry in female chicken gonads, such as *PITX2*, *TDRD15*, *SLC1A3*, *DAZL*, *TDRD9*, and so on (Wan et al., 2017).

Meanwhile, we compared the most side-biased known miRNAs, lncRNAs, and mRNAs in female with those in males (Supplementary Table 3) and found that there were 6 shared DE miRNAs in both of them: miR-302 cluster and miR-34a. Functional annotation revealed that DE mRNAs in female were mainly involved in forty-six GO terms (Table 4). As showed in Table 4, piRNA metabolic process (FDR = 2.84208E-05), intracellular ribonucleoprotein complex (FDR = 1.28E-06), ribonucleoprotein complex (FDR = 1.35E-06), and RNA binding (FDR = 0.000911) were significantly enriched GO terms. Based on the KEGG enrichment analysis, ribosome was a significantly enriched pathway (Supplementary Figure 2).

Sex-Related ceRNA Network in the Female and Male Gonads

The miRNA-mRNA target prediction analyses identified 179 miRNA-mRNA target pairs and only 5 significant miRNAs, miRNA-7b (degree = 37), miR-211 (degree = 16), miR-6606-5p (degree = 6), miR-2954 (degree = 4), and miR-204 (degree = 4) (Figure 3A). Thirty-eight possible miRNA-lncRNA target pairs were found, and only one significant miRNA, miR-6606-5p (degree = 6), while the lncRNAs were all novel precursors (Supplementary Figure 1A). We annotated the location relationship between each lncRNA and its trans and cis target genes, and 35 significant lncRNAs and their target genes pairs were obtained. However, the lncRNAs were all novel precursors, and more than 90% were reside on the sex chromosomes (28 on W chromosomes and 5 on Z chromosomes) (Supplementary Figure 1B). According to the target pairs of miRNA-mRNA, miRNA-lncRNA, and lncRNA-mRNA, we constructed a ceRNA network, and *GREB1*, *NIPBL*, *CBWD1*, *SLITRK6*, *ZSWIM6W*, and *VCPW* were hub DE mRNAs of this network (Figure 3B).

Left-Right Asymmetry-Related ceRNA Network in the Female Gonads

Totally, 58 possible significant miRNA-mRNA interaction pairs were obtained, and gga-miR-302b-5p (degree = 19) had the most target genes in female left-right asymmetry (Figure 3C). The miRNA-lncRNA target prediction analyses identified 78 miRNA-lncRNA interaction pairs and only 4 significant miRNAs, miR-302b-3p (degree = 3), miR-302d (degree = 1), miR-34a-5p (degree = 1), miR-302b-5p (degree = 1) (Supplementary Figure 1C). We annotated the location relationship between each lncRNA and its trans and cis target genes and obtained 6 significant lncRNAs and their target gene pairs in total, and the lncRNAs all reside on the autosome chromosomes (Supplementary Figure 1D). Totally, 4 lncRNAs, 4 miRNAs, and 4 mRNAs were involved in the ceRNA network (Figure 3D). *TEX14*, *MYT1L*, *RNF17*, and *GFRA1* were hub DE mRNAs of this network.

Table 2. The top 10 and some more GO terms between the female and male.

GO ID	GO name	Observed gene count	False discovery rate
Biological process			
GO:0015074	DNA integration	14	2.61E-10
GO:0044826	viral genome integration into host DNA	11	9.70E-08
GO:0019043	establishment of viral latency	11	9.70E-08
GO:0075713	establishment of integrated pro-viral latency	11	9.70E-08
GO:0046718	viral entry into host cell	11	1.00E-07
GO:0044409	entry into host	11	1.00E-07
GO:0051806	entry into cell of other organism involved in symbiotic interaction	11	1.00E-07
GO:0051828	entry into other organism involved in symbiotic interaction	11	1.00E-07
GO:0030260	entry into host cell	11	1.00E-07
GO:0006310	DNA recombination	14	6.23E-07
Cellular component			
GO:0019013	viral nucleocapsid	6	2.25E-05
GO:0044423	virion part	6	0.000142
Molecular function			
GO:0004523	RNA-DNA hybrid ribonuclease activity	15	2.67E-12
GO:0016891	endoribonuclease activity, producing 5'-phosphomonoesters	15	8.29E-12
GO:0016893	endonuclease activity, active with either ribo- or deoxyribonucleic acids and producing 5'-phosphomonoesters	15	1.08E-11
GO:0004540	ribonuclease activity	16	1.14E-11
GO:0004521	endoribonuclease activity	15	1.78E-11
GO:0004518	nuclease activity	17	9.86E-11
GO:0003964	RNA-directed DNA polymerase activity	17	1.54E-10
GO:0004519	endonuclease activity	15	3.17E-10
GO:0003887	DNA-directed DNA polymerase activity	13	3.17E-10
GO:0034061	DNA polymerase activity	17	3.52E-10

Abbreviation: GO, gene ontology.

Confirmation by qRT-PCR

We performed the confirmation of 5 DE miRNAs (gga-miR-2954, gga-miR-202-5p, gga-miR-302b-3p, gga-miR-302b-5p, and gga-miR-302d), 2 DE lncRNAs (ENSGALG00000048380 and ENSGALG00000048071), and 5 DE mRNAs (*CYP19A1*, *FSHR*, *STK31*, *DDX4*, and *TDRD9*) by qRT-PCR. As shown in Figure 4, expression of these 5 miRNAs, 2 lncRNAs, and 5 genes was consistent with that in RNA-seq results of this study.

DISCUSSION

This study used next-generation sequencing to provide a quantitative and comprehensive analysis of the coding and noncoding transcriptome in sexually dimorphic and left-right asymmetry of chicken at first. These analyses revealed significant differences in the patterns of miRNA, lncRNA, and mRNA expression in sexually dimorphic and left-right asymmetry of chicken, as well as provided insight into the earliest steps of gonad sex differentiation and identified novel candidate genes for ovarian and testis development.

Based on the RNA sequence data, we identified 7 miRNAs, 91 lncRNAs, and 123 mRNAs were differentially expressed between the sexes at E6, and the majority of these (57.14% of DE miRNAs, 54.95% of DE lncRNAs and 87.00% of DE mRNAs) reside on the sex chromosomes [W, Z or suspected W chromosome (un-random)] (Figures 2A, 2B and Table 1). Seven DE miRNAs, 189

DE lncRNAs, and 183 DE mRNAs reside between left and right gonad in female, but only a few of these (0% of DE miRNAs, 6.35% of DE lncRNAs and 8.15% of DE mRNAs) reside on the sex chromosomes (Figures 2C, 2D and Table 3). The previous studies reported that the sex-biased mRNAs were primarily derived from the sex rather than the autosome chromosome, the side-biased mRNAs had the opposite (Ayers et al., 2013, 2015; Wan et al., 2017; Warnefors et al., 2017). Furthermore, the present study showed that not only the mRNAs, but also the miRNAs and lncRNAs had the same. Finally, most of the female-biased miRNAs, lncRNAs, and mRNAs reside on the W chromosomes, and the male-biased were on the Z chromosomes. These results may be supported and explained by Warnefors et al., who suggested that there is no global dosage compensation mechanism that restores expression in ZW females after almost all genes on the W chromosome decayed (Warnefors et al., 2017). Overall, our findings suggest that miRNAs and lncRNAs regulation are novel gene-specific dosage compensation mechanism and they could contribute to left-right asymmetry of chicken, but sex-biased and side-biased miRNAs, lncRNAs, and mRNAs are independent of each other.

Several studies indicate that sex-biased miRNAs in chicken were likely to have important roles in sexual development. MiR-2954 showed conserved preference for dosage-sensitive genes on the Z Chromosome and acted to equalize male-to-female expression ratios of its targets (Zhao et al., 2010; Warnefors et al., 2017). The W-linked

Table 3. The most side-biased known miRNAs, lncRNAs, and mRNAs in female.

miRNAs			
Gene symbol	Chr ²	Log ₂ FC	P _{adj}
miR-302c-5p	4	2.27	0.0011
miR-302c-3p	4	2.20	9.00E-06
miR-302b-5p	4	2.17	1.22E-06
miR-34a-5p	21	1.83	0.0029
miR-302a	4	1.76	0.0014
miR-302b-3p	4	1.63	0.0088
miR-302d	4	1.63	0.0008
lncRNAs			
Ensembl Gene ID	Chr ²	Log ₂ FC	P _{adj}
ENSGALG00000046774	5	6.53	7.82E-07
ENSGALG00000052590	4	3.40	2.13E-09
ENSGALG00000048772	12	3.33	0.0102
ENSGALG00000001935	21	2.82	0.0457
ENSGALG00000048071	4	2.44	0.0432
ENSGALG00000050584	3	2.24	0.0353
ENSGALG00000048597	7	2.11	0.0320
ENSGALG00000047853	9	1.71	0.0230
ENSGALG00000046481	Z	1.47	0.0292
ENSGALG00000048136	1	-2.12	0.0376
mRNAs ¹			
Gene name	Chr ²	Log ₂ FC	P _{adj}
<i>GRIA1</i>	13	3.70	4.25E-13
<i>ASZ1</i>	1	3.51	1.24E-09
<i>TDRD15</i>	3	3.48	3.09E-09
<i>STK31</i>	2	3.25	4.25E-13
<i>TTC39 A</i>	8	3.18	4.25E-14
<i>DAZL</i>	2	3.03	1.83E-12
<i>DDX4</i>	1	3.03	1.76E-09
<i>ESRP2</i>	11	2.99	1.45E-10
<i>HKDC1</i>	6	2.81	6.74E-10
<i>RYR3</i>	5	2.73	2.05E-09
<i>APP</i>	5	-1.01	0.0013
<i>ANKRD50</i>	4	-1.09	0.0029
<i>MBNL1</i>	9	-1.15	0.0213
<i>WNK1</i>	1	-1.18	0.0485
<i>ADAM12</i>	6	-1.19	0.0035
<i>DAB2</i>	17	-1.26	1.36E-06
<i>GNB1</i>	21	-1.27	4.43E-06
<i>TRPC4</i>	1	-1.29	0.0011
<i>UNC5C</i>	4	-1.35	0.0001
<i>PCDHGA2</i>	13	-1.86	1.67E-10

Abbreviations: lncRNAs, long noncoding RNAs; miRNAs, microRNAs; mRNAs, messenger RNAs.

¹Top 10 up- and down-regulated mRNAs between left and right gonad in female, and the FPKM > 1.

²Chromosomes.

miR-7b, which was first identified in chicken embryonic gonads at day 4.5 (Ayers et al., 2013), was the most sex-biased miRNAs in chicken brain, heart, and liver (Warnefors et al., 2017). Our work revealed that miR-2954 and miR-7b were also the most sex-biased miRNAs in chicken gonad. In our sex-related ceRNA network (Figures 3A, 3B), we also found miR-7b had most target mRNAs, suggested that miR-7b may play an important role in sexual development and the sex-biased gene-related pathways. As expected, we found several novel candidate miRNAs for sexually dimorphic, such as miR-1416-5p, miR-204, miR-211, miR-3538, and miR-6606-5p. Many reports showed that miR-204 and miR-211 might play an important role in differentiation and

migration of cells (Avendano-Felix et al., 2019; Song et al., 2019). However, there was little known about the miR-1416-5p, miR-3538, and miR-6606-5p. In chicken primordial germ cells (PGCs), miRNAs might have contribution on the regulation of PGCs proliferation. MiR-302 b and miR-17-5p regulated Glucose Phosphate Isomerase (GPI) in chicken PGCs proliferation (Rengaraj et al., 2013). MiR-181-5p, miR-2127, and members of the miR-302/367 cluster had a dominant role in the regulation of PGCs proliferation (Lee et al., 2011; Lazar et al., 2018). The number of PGCs and germ cells proliferated rapidly, and in the left gonad, it was greater than that in the right gonad during gonadal differentiation (Vallisneri et al., 1990). Interestingly, we found that miR-302 cluster (miR-302c-5p, miR-302c-3p, miR-302b-5p, miR-302a, miR-302b-3p, and miR-302d) were not only the left side-biased miRNAs in female (Table 3) but also in male (Supplementary Table 3), suggesting that miR-302 cluster might perform multiple roles in sexual development of chicken. MiR-363, miR-363*, miR-1788-5p, and miR-12222-5p were identified in chicken gonadal sex differentiation (Huang et al., 2010; Warnefors et al., 2017). Unfortunately, these miRNAs were not significantly different in sexually dimorphic or left-right asymmetry of chicken in our study. The main reason was that we collected the gonad on the other time to sequence and used the most recent chicken genome release (GRCg6a).

There were 27 W-linked genes expression in our study, and they were associated with sex-biased in chicken gonads, including the embryo-specific expression gene *FAF* (data not shown). Interesting, *BTF3W* and *GPBP1W* genes were detected in high expression in adult chicken ovary and very low or even no expression in other tissue (Bellott et al., 2017). However, they were not expressed in our study by comparing with the report that the adult chicken W chromosome was thought to host only 28 protein-coding genes (Bellott et al., 2017), suggesting that they might be associated with the development of ovary. Of the 28 genes on the W chromosome with homologous sequences on the Z-chromosome, 7 genes, *SPIN1Z*, *C18ORF25*, *HNRNPK*, *TXNL1*, *MIER3*, *NIPBL*, and *UBAP2*, were upregulated in male (data not shown). Out of the 52 Z-linked genes that were associated with sex-biased in our study, 2 high-expression genes (FPKM > 100) may have contribution to the development of testis (Table 1). *FAM240 B* gene was highly expressed in mouse testicle but none in ovary (Yue et al., 2014). The present study showed that *FAM240 B* gene expression was 6-fold higher in male than in female, suggesting that *FAM240 B* gene may be associated with the development of testis in chicken. The prolactin receptor (PRLR) was an important factor in female reproductive physiology. It also presented in male reproductive organs and may have a possible role during spermatogenesis (Raut et al., 2019). However, its function in male reproduction still remains majorly unexplored. *PRLR* gene expression was 5-fold higher in male than in female in our study, demonstrating that it may play a greater role in the development of testis than ovary

Table 4. The top 10 significantly enriched GO terms between the left and right gonads in female.

GO ID	GO name	Observed gene count	False discovery rate
Biological process			
GO:0034587	piRNA metabolic process	6	2.84E-05
GO:0007140	male meiotic nuclear division	7	5.47E-05
GO:0000280	nuclear division	7	7.98E-05
GO:0140013	meiotic nuclear division	7	7.98E-05
GO:0043046	DNA methylation involved in gamete generation	5	0.0002
GO:0048285	organelle fission	7	0.0016
GO:0022412	cellular process involved in reproduction in multicellular organism	11	0.0051
GO:0007141	male meiosis I	4	0.0054
GO:0010529	negative regulation of transposition	3	0.0063
GO:0010528	regulation of transposition	3	0.0063
Cellular component			
GO:0030529	intracellular ribonucleoprotein complex	27	1.28E-06
GO:1990904	ribonucleoprotein complex	27	1.35E-06
GO:0043186	P granule	6	1.35E-06
GO:0071546	pi-body	4	4.67E-05
GO:0022625	cytosolic large ribosomal subunit	7	4.85E-05
GO:0033391	chromatoid body	4	0.0002
GO:0044391	ribosomal subunit	10	0.0004
GO:0015934	large ribosomal subunit	7	0.0049
GO:0044445	cytosolic part	9	0.006
GO:0044424	intracellular part	152	0.0090
Molecular function			
GO:0003723	RNA binding	33	0.0009
GO:0003735	structural constituent of ribosome	9	0.0064

Abbreviation: GO, gene ontology.

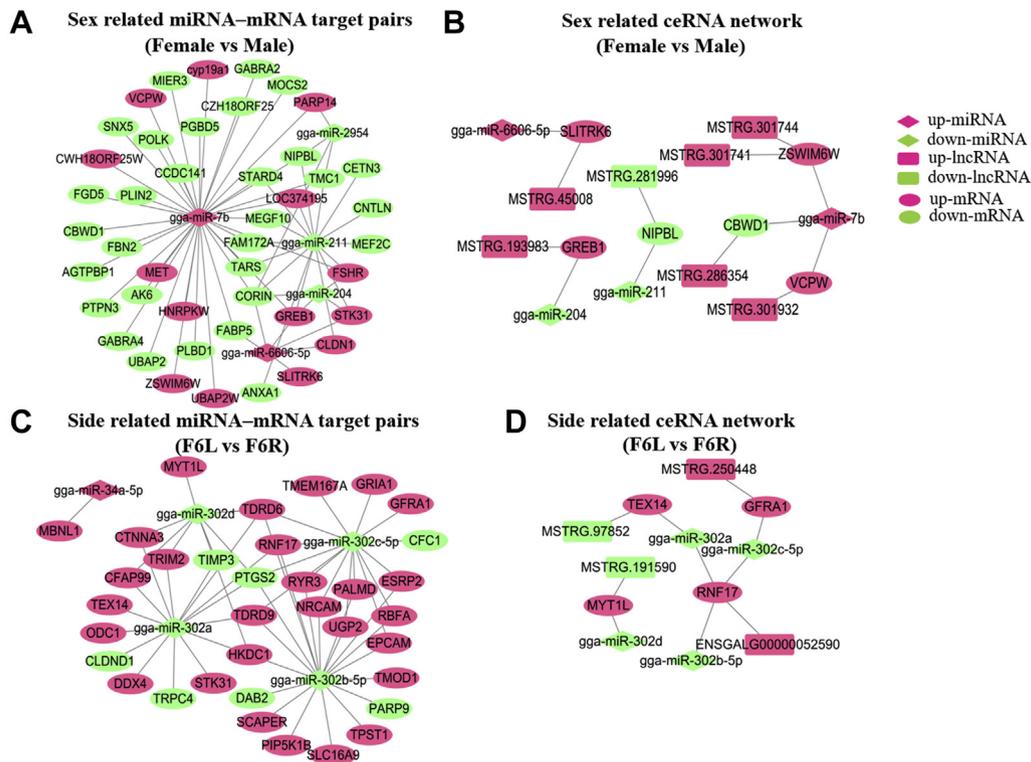


Figure 3. The ceRNA network. (A) Sex-related ceRNA networks of DEmiRNAs-DEmRNAs. This network consists of 5 DEmiRNAs and 44 DEmRNAs. (B) Global view of the sex-related ceRNA network. This network consists of 4 DEmiRNAs, 7 DELncRNAs, and 6 DEmRNAs. (C) Side-related ceRNA networks of DEmiRNAs-DEmRNAs. This network consists of 5 DEmiRNAs and 35 DEmRNAs. (D) Global view of the side-related ceRNA network. This network consists of 4 DEmiRNAs, 4 DELncRNAs, and 4 DEmRNAs. Red and green color represented upregulation and downregulation in the 2 groups. Abbreviations: ceRNA, competing endogenous RNA; DEmiRNAs, differentially expressed microRNAs; DEmRNAs, differentially expressed messenger RNAs.

The expression of miRNAs, lncRNAs and genes was validated using qRT-PCR and compared with that from RNA-seq

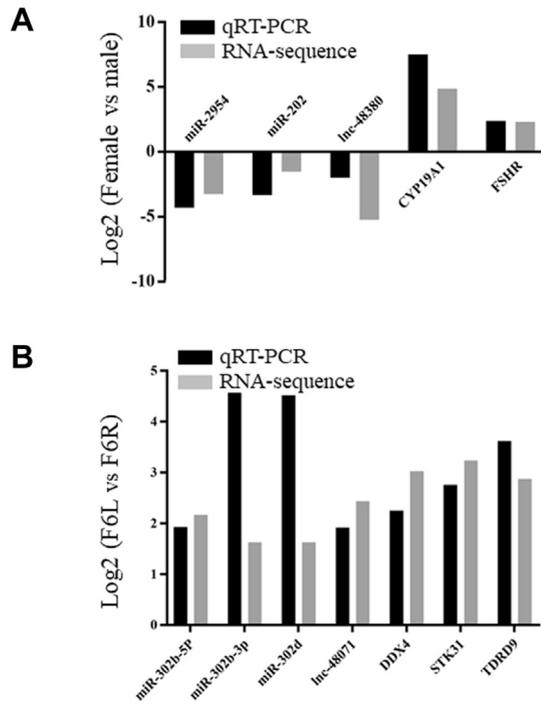


Figure 4. Verification of differentially expressed miRNAs, lncRNAs and genes by qRT-PCR. (A) The expression of 2 miRNAs, one lncRNAs, and 2 genes was validated using qRT-PCR and compared with the expression levels obtained from RNA-seq. Expression data are presented as expression values of genes in female gonads relative to that in male sample. (B) The expression of 3 miRNAs, one lncRNAs, and 3 genes was validated using qRT-PCR and compared with the expression levels obtained from RNA-seq. Expression data are presented as expression values of genes in right gonads relative to that in left sample. The qRT-PCR of miRNAs values were normalized relative to the expression levels of U6 in the same cDNA sample, and lncRNAs and genes were relative to the expression levels of *HPRT*. Abbreviations: lncRNAs, long noncoding RNAs; miRNAs, microRNAs; qRT-PCR, quantitative real-time PCR.

in chicken. Moreover, the functional enrichment of 123 DE mRNAs between the sexes showed that mainly enriched GO terms were viral related, such as viral genome integration into host DNA, establishment of viral latency, viral nucleocapsid, and so on (Table 2). This unexpected result may be supported and explained by several studies, which reported that the function and development of immune system had emerged from avian embryo (Seto, 1981; Dieterlen-Lievre, 1989; Hansell et al., 2007). Hence, the E6 may be an important moment of the immune system development in chicken reproductive organs. In addition, the related to ovarian development genes *CYP19A1*, *FSHR*, *FOXL2*, and *GREB1* were beginning to express and also associated with sex-biased in our study (data not shown). The previous studies showed that exposure of ZW females to aromatase inhibitors caused complete and permanent gonadal and total-body sex conversion to males, and the aromatase was encoded by *CYP19A1* gene (Elbrecht and Smith, 1992; Abinawanto et al., 1996; Vaillant et al., 2001; Hudson et al., 2005), demonstrating the critical importance of *CYP19A1* gene in female sex differentiation. Wang and Gong showed that transcription of *CYP19A1* was

directly regulated by SF-1 (*NR5A1*) in the theca cells of ovary follicles in chicken, not the transcription factor FOXL2 (Wang and Gong, 2017). However, our study found that *NR5A1* did not begin to express at E6 (data not shown). Therefore, the upstream regulator of *CYP19A1* gene has not yet been identified in chicken during gonadal differentiation. In our ceRNA network, *CYP19A1* gene was the target of miR-7b and miR-302b-5p. It seems likely that miR-7b and miR-302b-5p directly activate *CYP19A1* expression. Furthermore, *FSHR* gene was the target of miR-7b, miR-211, and miR-204, and *GREB1* gene was the target of miR-7b, miR-211, miR-204, and miR-6606-5p. Thus, these miRNAs may also play crucial roles in ovarian development, and we speculated that they might directly activate transcription according to the previous reports (Hwang et al., 2007; Dragomir et al., 2018). In addition, the side-biased gene *STK31*, *CORIN*, and *TDRD9* were the target of many side-biased miRNAs as well as sex-biased miRNAs. *STK31* gene was the target of miR-7b, miR-211, miR-6606-5p, and miR-302a, *CORIN* gene was the target of miR-7b, miR-211, miR-204, miR-6606-5p and miR-2954, and *TDRD9* gene was the target of miR-7b, miR-211, miR-204, miR-6606-5p, miR-2954, miR-302b-5p, and miR-302c-5p, suggesting that these genes may play crucial roles in ovarian development too.

CONCLUSION

The present study not only identified the candidate sexual dimorphic miRNA, lncRNA, and mRNA but also the candidate side-biased miRNA, lncRNA, and mRNA. Most sex-biased miRNAs, lncRNAs, and mRNAs resided on sex chromosomes, and the side-biased were on autosome chromosomes. Moreover, the network of DE miRNA–DE lncRNA–DE mRNA interactions were obtained. These data have provided a detailed view of the transcriptional changes that occur when sexual differentiation begins and could facilitate the study of the molecular mechanisms of side-specific gonadal development in birds.

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SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at <http://doi.org/10.1016/j.psj.2019.10.019>.

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