Novel miRNA identification and comparative profiling of miRNA regulations revealed important pathways in Jinding duck ovaries by small RNA sequencing

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Abstract

Functional studies have revealed miRNAs play pivotal roles in ovulation and ovary development in mammals, whereas little is known about the miRNA function in ducks. In this study, miRNA deep sequencing in the ovary tissues was carried out to obtain the miRNA profile from ovaries before oviposition (BO) and after oviposition (AO) in Jinding duck. Overall, an average of 23,128,075 and 26,020,523 reads were identified in the BO and AO samples, respectively, and 6739 miRNAs were identified from them through further mapping and analysis. Besides, 1570 miRNAs were identified as differentially expressed miRNAs compared with BO, including 493 miRNAs up-regulated and 1077 down-regulated in AO. Moreover, 2291 target genes were predicted from 443 significantly differentially expressed miRNAs. In addition, GO and KEGG pathway analysis indicated that target genes were enriched in some basic cell metabolism pathways as well as the productive pathways such as MAPK signaling pathway, gonadotropin-releasing hormone signaling pathway, TGF-beta signaling pathway which had been significantly changed. Our results helped to replenish the duck miRNA database and illustrate the potential mechanism of miRNA function in duck ovary development and reproduction process.

Keywords Duck ovaries · Oviposition · miRNA · Small RNA sequencing

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Introduction

Jinding duck is an important commercial food for poultry industry. The egg production directly impacts the economic benefits of the duck industry. However, the poor egg-laying performance of Jinding Duck has strongly inhibited the duck industry development. Many factors were reported to affect the egg laying rate, such as hormone secretion, circadian rhythm, viral pathogens, microbe infection etc. (Zhao et al. 2018; Tao et al. 2017; Zhang et al. 2019). For instance, daidzein was suggested to exert divergent effects on the egg-laying performance of Shaoxing ducks under different physiological conditions (Zhao et al. 2005).

MicroRNAs (miRNAs) are 18–25 nts small non-coding RNAs which negatively regulate the mRNA transcription levels through the binding of their 3'UTRs. Numerous miRNAs have been identified in various species, including homo sapiens, mus musculus, rattus norvegicus, caenorhabditis elegans et al. (Kozomara et al. 2019; Kozomara and Griffiths-Jones 2014). Increasing evidences have revealed that miRNAs play important roles

in organ differentiation and development, cell proliferation, apoptosis, etc. (Hwang and Mendell 2007; Song and Tuan 2006; Wahid et al. 2010; Jay et al. 2007; Liu et al. 2013; Li et al. 2011). miR-222 was reported to regulate follicular atresia, ovarian development and female reproduction by targeting THBS1 (Zhu et al. 2019). miR-432 was shown to play important roles in ovarian development through the binding of RPS6KA1 (Gu et al. 2019). Let-7 members were found to be correlated with ovary development through negatively regulating expression of their target genes (Lan et al. 2019). Song et al. (2014) reported that miR-2 and miR-133 differentially expressed during oocytes meiotic maturation and targeted the crab cyclin B. Let-7, miR-2, miR-184 and miR-276 were shown to participate in gonadal development and function (Meng et al. 2018). He et al. (2015) also elucidated several miRNAs to regulate the reproductive organ development and differentiation. In conclusion, we suspect that the process of duck laying eggs was also regulated by miRNA. Unfortunately, the expression profile of miRNA in duck ovaries is not clear. Thus, this study is aimed to expand the miRNA expression profile in duck ovaries and provide some theoretical basis for improving the performance of duck eggs.

In this study, global miRNA sequencing was applied to investigate the miRNA expression profile in ovaries of BO and AO Jinding ducks. Several miRNAs and their regulated pathways were identified and validated to participate in this process. Our data will significantly help the understanding of miRNA function and the potential gene regulation mechanism in duck ovary differentiation and development.

Results

Small RNA sequencing analysis in the ovaries of laying Jinding ducks before and after oviposition

Small RNA (sRNA) libraries from laying Jinding ducks were constructed from the ovaries before (BO) and after oviposition (AO), each of which included three different biological replicates. The sRNA libraries were then sequenced through Illumina Hiseq X Ten platform. In the ovary samples before laying, a total of 29,166,900, 24,267,426 and 15,949,900 reads were generated, respectively (Table 1). When Jinding ducks came into the laying period, a total of 24,370,769, 26,600,866 and 27,089,935 raw reads were obtained respectively from the sRNA deep sequencing of the ovary samples (Table 1). According to the filtering criteria, more than 94% of total clean reads (AO-1, 22,993,050 reads; AO-2, 25,376,643 reads; AO-3, 25,548,267 reads; BO-1, 27,595,691 reads; BO-2, 23,015,561 reads; BO-3, 15,098,158 reads) were used for further analysis. The GC content of clean reads occupied 43 to 45% in the sRNA libraries. The quality score valued by FASTQC revealed the good sRNA sequencing qualities (Supplemental Figure 1).

Identification of known and novel miRNAs in Jinding duck ovaries

To identify the miRNAs in duck ovaries, the high-quality reads were mapped to mallard (*Anas platyrhynchos*) genome and Rfam database. Although more than 93% of total reads were annotated by the alignment of reference genome (Table 1), and more than 71% of total reads were classified as non-coding RNAs based on Rfam database

Table 1 Summary of sRNA sequencing data of each library from three duck ovary samples before oviposition and three samples after oviposition)

Category	After oviposition (AO)			Before oviposition (BO)		
	AO-1	AO-2	AO-3	BO-1	BO-2	BO-3
Raw reads	24,370,769 (100%)	26,600,866 (100%)	27,089,935 (100%)	29,166,900 (100%)	24,267,426 (100%)	15,949,900 (100%)
Clean reads	22,993,050 (94.35%)	25,376,643 (95.40%)	25,548,267 (94.31%)	27,595,691 (94.61%)	23,015,561 (94.84%)	15,098,158 (94.66%)
GC content	43%	45%	44%	44%	44%	44%
Reads mapped to the reference genome	22,822,735 (93.65%)	25,150,951 (94.55%)	25,339,301 (93.54%)	27,318,131 (93.66%)	22,712,875 (93.59%)	14,860,146 (93.17%)
Reads mapped to Rfam database	22,097,236 (90.67%)	24,403,333 (91.74%)	24,708,246 (91.21%)	22,935,336 (78.63%)	19,633,717 (80.91%)	12,456,217 (78.10%)

(Supplemental Table 1), little reads were identified as conserved known miRNAs when compared with miRBase because of the insufficiency of known miRNAs in mallard (Table 2, supplemental Figure 2). Only four known mature miRNAs (apl-mir-11588, apl-mir-11589, apl-mir-11590 and apl-mir-11591) of mallard were identified in the two stages (BO and AO), which showed low expression level (Table 2). Therefore, the remaining clean reads that were unmapped to miRBase were then adopted to align with known miRNAs from chickens (*Gallus gallus*) and zebra finch (*Taeniopygia guttata*) (Li et al. 2015) and to identify novel miRNAs by miRDeep2 (Friedlander et al. 2012). More than 5000 and 10,000 reads were mapped to chicken and zebra finch miRNAs, respectively, and most of the reads (76.75%–85.97% of the total reads) were identified as the potential novel miRNAs (Supplemental Figure 2).

Among 6739 miRNAs identified in this study, a total of 5576 miRNAs were common to BO and AO libraries, of which 650 miRNAs were specific in ovary tissues before oviposition and fewer miRNAs (513) were expressed specifically after oviposition (Fig. 1). The expression levels of miRNAs were normalized using the TPM values. Interestingly, the TPM values of miRNA expression levels before oviposition (total TPM value = 253,180) were much lower than miRNAs expressed after oviposition (total TPM value = 351,169) (Supplemental Table 2). This suggested that the expression levels of a wide range of miRNAs were increased after laying eggs. The miRNAs with top 10 highest expression levels accounted for 83.28% in AO library and 72.08% in BO library (Table 3), among which chr13_23635_mature@@gga-miR-143-3p and chr13_23636_mature@@gga-miR-143-3p with the highest expression levels in both stages represented 32.95% and 32.94% in AO respectively and 21.56% and 21.54% in BO, respectively. However, the TPM value of them in AO was about twice as much as BO, which suggested the significant function during duck laying period. Besides, 5 of the top 10 expressed miRNA members (chr1_472_mature@@tgu-let-7i-5p, chr12_23289_mature@@tgu-let-7g-5p, chr12_23333_mature@@tgu-let-7f-5p, chr12_23334_mature@@tgu-let-7f-5p and chrAADN04005513.1_33647_mature@@tgu-let-7f-5p) in AO library belonged to let-7 family which had been reported to be correlated with ovary development (Lan et al. 2019).

Fig. 1 Venn plot of the predicted miRNAs in AO (after oviposition) and BO (before oviposition) libraries

Differential expression, target prediction and QPCR vilification of miRNAs in AO and BO ovaries

In comparison with BO library, 1570 miRNAs were significantly changed in the ovary of laying ducks at the value of $\log_2$ fold changel > 1 and $FDR < 0.05$ (Supplemental Table 3). The 493 miRNAs were up-regulated and 1077 were down-regulated in AO (Fig. 2a), of which 1032 miRNAs with extremely low expression level (lower than 100 reads count in one library at least) were detected on account of the high sensitivity of deep sequencing technology. By making a hierarchical cluster, more down-regulated miRNAs were detected than up-regulated miRNAs during laying period, and the heatmap showed good repeatability and samples from BO or AO stages were characteristic of two separate clusters, suggesting different miRNA-regulation patterns in BO and AO of Jinding ducks (Fig. 2b). Among the miRNAs which included more than 100 reads in at least one library, 270 miRNAs were down-regulated with more than fourfold, while only 3 miRNAs (chr5_16981_mature, chr12_23064_mature, chrAADN04001189.1_32565_mature) were up-regulated with more than fourfold, which provided the evidence that lots of genes were activated for egg-laying performance.

Based on the Miranda and RNAhybrid method, the 443 significantly differentially expressed miRNAs were then adopted to the target gene prediction (Supplemental Figure 3). A total of 2291 genes were predicted (Supplemental Table 4), of which chr22_27062_mature@@gga-miR-2127 and

Table 2 Statistics of the numbers of reads that were mapped to the known *Anas platyrhynchos* miRNAs

Known miRNAs	AO-1	AO-2	AO-3	BO-1	BO-2	BO-3
apl-mir-11588	26	17	7	8	15	10
apl-mir-11589	156	29	31	15	36	21
apl-mir-11590	19	4	7	164	150	71

Table 3 Top 10 expressed miRNAs in AO and BO samples respectively

AO			BO		
miRNA ID	Average TPM value	Proportion (%)	miRNA ID	Average TPM value	Proportion (%)
chr13_23635_mature@@gga-miR-143-3p	115695.1269	32.95	chr13_23635_mature@@gga-miR-143-3p	54578.25072	21.56
chr13_23636_mature@@gga-miR-143-3p	115669.7177	32.94	chr13_23636_mature@@gga-miR-143-3p	54534.54835	21.54
chr1_472_mature@@tgu-let-7i-5p	22167.63784	6.31	chr12_23289_mature@@tgu-let-7 g-5p	17682.38175	6.98
chr12_23289_mature@@tgu-let-7 g-5p	11953.03583	3.40	chr1_472_mature@@tgu-let-7i-5p	14242.19944	5.63
chr15_24811_mature@@gga-miR-143-3p	8042.877375	2.29	chrAADN04019721.1_35585_mature@@tgu-miR-26-5p	8776.11342	3.47
chr12_23333_mature@@tgu-let-7f-5p	6991.410432	1.99	chr2_8898_mature@@tgu-miR-26-5p	8680.764504	3.43
chr12_23334_mature@@tgu-let-7f-5p	6980.42307	1.99	chr7_19631_mature@@tgu-miR-26-5p	8650.652467	3.42
chrAADN04005513.1_33647_mature@@tgu-let-7f-5p	6957.384212	1.98	chrZ_31248_star@@tgu-miR-27-3p	5866.783533	2.32
chr27_28140_mature@@gga-miR-10a-5p	5903.661058	1.68	chr27_28140_mature@@gga-miR-10a-5p	4844.801592	1.91
chrZ_31248_star@@tgu-miR-27-3p	4664.740552	1.33	chr1_1650_mature@@tgu-let-7c-5p	4604.912426	1.82

Fig. 2 The differentially expressed miRNAs. **a** Volcano plot of the differentially expressed miRNAs. **b** Heat map of the differentially expressed miRNAs. The red indicates the up-regulated miRNAs, and the green indicates the down-regulated miRNAs

chr2_7146_mature@@gga-miR-2127 were associated with 68 and 53 genes, respectively and showed multi-biological functions involved in miRNAs originated gga-miR-2127 family. To validate miRNA expression patterns predicted by small RNA-sequencing, four miRNAs (chr16_25018_miR-143-3p, chr18_25741_miR-193b-5p, chr1_1594_miR-7482-5p, chr12_23328_miR-1457) were selected randomly for Quantitative real-time PCR verification (QPCR) (Fig. 3). The expression level of chr18_25741_miR-193b-5p in the development stage of AO was significantly up-regulated (P value=0.000)

Fig. 3 Expression validation with 5 replicates of 4 miRNAs in the ovaries before (BO) and after oviposition (AO) by qPCR. The expression detection of the 4 miRNA (chr16_25018_miR-143-3p, chr18_25741_miR-193b-5p, chr1_1594_miR-7482-5p, chr12_23328_miR-1457) were performed five technical replications, respectively. The calculation of expression was carried out by $2^{-\Delta\Delta Ct}$ method and normalized to U6 small nuclear RNA which was the endogenous reference gene. The P values (P) were calculated by t test, and shown above the respective columns

when compared with the expression in BO samples, while chr1_1594_miR-7482-5p and chr12_23328_miR-1457 were expressed lower in AO than BO. This observation was consistent with the differential analysis using miRNA-seq reads (Supplemental Table 3). However, significant differences were difficult to detect for chr16_25018_miR-143-3p (Fig. 3), which suggested that chr16_25018_miR-143-3p of miR-143 family in ovaries of AO was as important as BO.

GO and pathway analysis of targets of differentially expressed miRNAs

To further investigate the functions of miRNAs in AO and BO libraries, GO and pathway analysis were performed using Fisher's exact test and KEGG database at a P value < 0.05 respectively. The GO enrichment analysis categorized 756 target genes to the GO terms of biological process (BP), molecular function (MF) and cellular component (CC), and 131, 57 and 36 terms were found to be enriched in each main category respectively (Supplemental Table 5). The top 15 enriched terms in BP, MF and CC category were related to various metabolic process of proteins such as the transport (retrograde transport endosome to Golgi (0042147), fatty acid transport (0015908), vacuolar transport (0007034), protein localization to endosome (0036010), regulation of protein localization to cell surface (2000008), extracellular vesicular exosome (0070062)), synthesis [endocytic vesicle (0030139), pre-mRNA binding (0036002)) and degradation (protein polyubiquitination (0000209), endomembrane system (0012505), endosome (0005768), early endosome (0005769), polyubiquitin binding (0031593)] (Fig. 4, supplemental Table 5). Protein polyubiquitination (GO:0000209) corresponding to 10 obviously changed genes were the most enriched in the main category of BP (Supplemental Table 5). The KEGG analysis showed 258 related

Fig. 4 GO analysis of the targeted genes. Top 15 GO enrichment terms of the targeted genes predicted by differentially expressed miRNAs in the biological process (BP), the molecular function (MF) and the cellular component (CC) GO enrichment analysis

pathways corresponding to 376 target genes (Supplemental Table 6). The most enriched pathway was endocytosis which was related to 30 target genes. Although highly enriched pathways were involved in basic metabolic processes such as “Endocytosis, Synaptic vesicle cycle, Biosynthesis of amino acids, Lysosome and Spliceosome” (Fig. 5), some pathways of lower enrichment-levels, such as MAPK signaling pathway, Gonadotropin-releasing hormone (GnRH) signaling pathway, TGF-beta signaling pathway, estrogen signaling pathway and the glycolysis/gluconeogenesis pathway, were nonnegligible.

Discussion

miRNAs are critical to the post-transcriptional regulation of gene expression through sequence complementarity. In pace with the development of next-generation sequencing (NGS), genome-wide identification of miRNAs, increasingly, have been reported in plants (Yu et al. 2017; Guo et al. 2019; Bhan et al. 2019; Liu et al. 2018), mammals (Song et al. 2018; Li et al. 2018a, b; Markkandan et al. 2018), poultry (Wu et al. 2017b; Wang et al. 2018a; Chen et al. 2014; Luo et al. 2012), fishes (Bizuayehu and Babiak 2014; Andreassen and Hoyheim 2017), insects etc. (Belles 2017; Menzel et al. 2019). In poultry, egg-laying performance is

commercially important and determined by ovarian function. Recently, miRNAs in ovaries have been explored and found to be in association with the regulation of various biological process such as cell proliferation and differentiation (Xu et al. 2014), follicle development (Kang et al. 2013), reproduction processes (Xu et al. 2014; Wu et al. 2017a), steroid hormone biosynthesis (Wang et al. 2018b). In the present study, the genome-wide miRNA profile in ovaries of Jinding duck before and after oviposition was described based on sRNA-seq technology. Due to the lack of known miRNAs in the miRBase of ducks (mallard), sequenced data were mapped to the miRNA database of other related species including chickens and zebra finch, and then adopted to discover the novel miRNAs. We identified 6739 miRNAs containing abundant low-expressed miRNAs, within which 513 and 650 miRNAs were specific before and after oviposition respectively (Fig. 1), which revealed large-scale miRNA changes involving multiple gene expressions during duck laying period. The miRNAs obtained in this study enriched the miRNA database of ducks.

Half of the top 10 dominantly expressed miRNAs in egg-laying duck ovaries were the members of let-7 miRNA family, indicating its important functions in duck ovaries. This was consistent with the previous reported literatures that let-7 was shown to participate in follicular growth, ovulation mechanism, sexual maturity, oocyte differentiation

Fig. 5 KEGG pathway analysis of the differentially expressed miRNA targeted genes. The bar figure of the top 20 pathway enrichment terms of the target genes predicted by differentially expressed miRNAs. The ordinate represents the enrichment score, and the abscissa represents the path term enriched by the target gene of the differentially expressed miRNA. Red represents significant enrichment ($P < 0.05$)

and folliculogenesis (Kang et al. 2013; Tong et al. 2014). Among the top 10 miRNAs with the significantly changed expression levels (Table 3), miR-143 family was prevalent in both periods of duck ovary. Nevertheless, the TPM values of chr13_23635_mature@@gga-miR-143-3p and chr13_23636_mature@@gga-miR-143-3p in laying ducks were much higher than the period before laying. This result was in line with the findings reported by Xu et al. that miR-143 actively participated in ovarian function, including hormone secretion, reproduction processes et al. (Xu et al. 2014). The analysis of miRNA differential expression showed more numbers of miRNAs decreased in AO library (Fig. 2 and supplemental Table 3). In addition, the validation result of differentially expressed miRNAs (chr18_25741_mature@@tgu-miR-193b-5p, chr12_23328_mature@@gga-miR-1457 and chr1_1594_star@@gga-miR-7482-5p) by QPCR showed consistency between experiments and miRNA sequencing data analysis (Fig. 3). It has been reported that miR-193 actively participated in gonadal development, sexual differentiation and teleost reproduction (Fernandez-Perez et al. 2018; He et al. 2019). The increasing of miR-193 levels would induce the downregulation of c-kit expression therefore repressing cell proliferation and migration. In the current study, significantly elevating of miR-193 levels were observed in the AO duck ovaries. As a result, elevated miRs could bind to mRNAs and suppress its translation through the RNA-inducing silencing complex (RISC). These results provided the evidence that the differentially expressed miRNAs were involved in ovary development and egg-laying process. However, these are just the efforts we have made on the basis of bioinformatics, and the specific function of miRNAs and their target genes needs to be further studied on the mechanism in the future.

Similarly, the most significantly enriched KEGG pathway belonged to the basic metabolic pathways (Fig. 5), such as endocytosis, synaptic vesicle cycle, carbon metabolism, lysosome, spliceosome, inositol phosphate metabolism, axon guidance and fc gamma R-mediated phagocytosis, indicating the regulating role of differentially expressed miRNA in protein transportation, cell signaling transduction and cell-cell interaction. Besides, several pathways including B cell receptor signaling pathway, bacterial invasion of epithelial cells, chronic myeloid leukemia and glioma pathways also suggested that the ducks were involved with immune system adjustment and regulation. Overall, these results revealed that these differentially expressed miRNAs were mainly related with cell metabolism, immune process and signal transduction. KEGG pathway analysis also indicated that the reproductive biological process had been significantly enriched. For example, 21 genes for differentially expressed miRNA were annotated in the MAPK signaling pathway. Nine genes in the Gonadotropin-releasing hormone (GnRH) signaling pathway, 7 genes in the TGF-beta signaling

pathway and 6 genes in the estrogen signaling pathway as well as 7 genes in the glycolysis/gluconeogenesis pathway for differentially expressed miRNA were annotated. Interestingly, we found that GRB2 and PRKCA have a synergistic effect on chrAADN04013263.1_34964_star, and MAP2K6 and HRAS have a synergistic effect on chr10_22399_mature, and these four target genes are simultaneously enriched in the GnRH signaling pathway (Supplemental Table 4). GnRH signaling pathway had been reported to affect the ability of the pituitary gonadotrope which was highly related with reproductive cycles and fertility (Bliss et al. 2010; Millar 2005). This suggests that the egg production cycle of the ovary is regulated by differentially expressed miRNAs in AO and BO, and may play a regulatory role through the GnRH signaling pathway. Moreover, TGF-beta signaling pathway served as multifunctional roles in regulating virtually all aspects of animal development and differentiation (Kandasamy et al. 2010). Estrogen signaling pathway had been revealed to promote the development of secondary sexual characteristics and sexual organ maturation in female animals (Velarde 2013; Li et al. 2013). These results implicated the potential regulatory roles of miRNAs in the reproduction process.

In conclusion, this study enlarged miRNA repertoire of the duck miRNA database and provided the overall miRNA expression in duck ovaries before and after oviposition. Great changes in miRNA expression levels were observed in egg-laying duck ovaries that were closely related with ovary development, hormone secretion and egg-laying performance. We found miR-143 and let-7 miRNA family were abundant in ovary tissues, and several reproductions associated miRNAs changed significantly. The miRNA target genes were not only involved in reproduction process, but also the cell metabolism, indicating their important roles in egg-laying period. This study provided the information for understanding the potential roles of miRNA regulation and mechanism of duck egg-laying process.

Materials and methods

Sample preparation and treatment

Jinding Duck was belong to *A. platyrhynchos* mallard, commonly known in the market as an egg duck. All Jinding ducks were raised together at Sichuan Animal Science Academy after birth and were guaranteed freedom of life, including water and food. Three ducks were randomly selected from BO groups and other three ducks were randomly selected from AO groups after ovulation. The age of BO and AO ducks are 14 and 30 weeks respectively. The ducks were executed through cervical dislocation. The ovary tissues were healthy and fresh, which were

Table 4 The primers used for QPCR

Name	Sequence
U6 forward	5'-AGAAAATTAGCATGGCCCCTG-3'
U6 reverse	5'-AACGCTTCACGAATTTGCGT-3'
chr16_25018_miR-143-3p forward	5'-CGCGCAGGGAGATGAAGC-3'
chr18_25741_miR-193b-5p forward	5'-ATATAGTATCTCGCCCCGAAAG-3'
chr1_1594_miR-7482-5p forward	5'-AGAGATGGGGAGCTGGGC-3'
chr12_23328_miR-1457 forward	5'-GCGCAGCCAGCCTGTAGT-3'
all-miRNAs reverse	5'-AGTGCGTGTCTGGAGTCG-3'

All-miRNAs reverse indicates that the four candidate miRNAs share the same reverse primer for QPCR

collected in BO and AO period and snap frozen in liquid nitrogen. Samples were stored at -80°C before further processment.

RNA isolation, small RNA library construction and sequencing

Total RNA extractions were performed according to the manufacturer's instructions (TRIzol Plus RNA Purification Kit, Cat.12183555, ThermoFisher). Briefly, the TRIzol Reagent was added to sample and separate the aqueous phase containing the RNA. Then DNA were removed by on-column DNase treatment. Finally, RNA was eluted by DNase, RNase-free water. The RNA concentration was measured by NanoDrop with the quality evaluated by gel electrophoresis. TruSeq Small RNA Sample Preparation Kit (Illumina) was used to prepare small RNA libraries. Total RNA was ligated by adaptors, reverse transcribed and unique indexes were incorporated during PCR amplification. After electrophoresis, the gel region containing miRNA fraction was excised. The samples were loaded on the Illumina Hiseq X Ten platform at Yingbiotech (Shanghai, China).

miRNA analysis

Fast-QC software (<http://www.bioinformatics.babB1-LC37h.am.ac.uk/projects/fastqc/>) was used to evaluate the sequencing data qualities, including base mass, length distribution and GC contents et al. BWA algorithm was used for miRNA mapping. The filtered clean reads were mapped to *A. platyrhynchos* miRNA, genome and Rfam database. But the mapping known miRNA number was too low. As a result, two species (*G. gallus*, *Zebra finch*) were used for miRNA prediction using miRDeep2 software (Friedlander et al. 2012). Differentially expressed miRNA analysis was performed by DEseq 2 algorithm, with the cutoff of $|\text{Log}_2\text{FC}| > 1$ and $\text{FDR} < 0.05$. Due to the poor correlation of A1 and B2 samples, 3vs3 was used for differentially expression analysis, miRNA target prediction, GO and pathway analysis.

miRNA target prediction

miRanda and RNAhybrid database were used for miRNA target prediction, with the cutoff of Score ≥ 150 and Energy < -20 for the previous one, and Energy < -25 for the latter one. The intersection results of the two databases were used as the final miRNA target prediction result.

GO and pathway analysis

Based on the Fisher's exact test (Beissbarth and Speed 2004; Al-Shahrour et al. 2004; Zeeberg et al. 2003; Draghici et al. 2003), GO analysis (<http://geneontology.org>) was performed to annotate and classify genes by Molecular Function (MF), biological process (BP) and cellular component (CC). KEGG pathway analysis (<http://www.genome.jp/Kegg/>) was employed to systematically analyze gene functions and associated high-level genomic functions.

QPCR validation

To validate the expression of the novel miRNAs, those sequencing counts exceeded 100 in at least one library were picked out for QPCR experiment, and the coefficient of variation in the higher expression library should be less than 0.2. For QPCR process, the assay was performed according to the manufacturer's instructions (Master Mix, Roche). Finally, the QPCR was performed on the ABI Q6 Real-Time PCR System (Applied Biosystems). The primers used in this analysis were showed in Table 4.

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Author contributions Conceptualization: MQ and ZZ. Data curation: CY and XX. Formal analysis: XJ and HD. Funding acquisition: MQ and ZZ. Investigation: CY, QL and HL. Methodology: XX, WG and CY. Software: HP and BX. Writing—original draft: CY and XX. Writing—review & editing: JC, XS, LY and CH. All authors read and approved the final manuscript.

Availability of data and materials The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Ethics approval The animal experiment in this study was approved via the animal care and ethical committee of Sichuan Animal Science Academy. All ducks were carried out on the guidelines of China legislations on the ethical use and care of laboratory animals.

Consent for publication The authors declare that there is no conflict of interest regarding the publication of this paper.

References

- Al-Shahrour F, Diaz-Uriarte R, Dopazo J (2004) FatGO: a web tool for finding significant associations of Gene Ontology terms with groups of genes. *Bioinformatics* 20(4):578–580. <https://doi.org/10.1093/bioinformatics/btg455>
- Andreassen R, Hoyheim B (2017) miRNAs associated with immune response in teleost fish. *Dev Comp Immunol* 75:77–85. <https://doi.org/10.1016/j.dci.2017.02.023>
- Beissbarth T, Speed TP (2004) Gostat: find statistically overrepresented Gene Ontologies within a group of genes. *Bioinformatics* 20(9):1464–1465. <https://doi.org/10.1093/bioinformatics/bth088>
- Belles X (2017) MicroRNAs and the evolution of insect metamorphosis. *Annu Rev Entomol* 62:111–125. <https://doi.org/10.1146/annurev-ento-031616-034925>
- Bhan B, Koul A, Sharma D, Manzoor MM, Kaul S, Gupta S, Dhar MK (2019) Identification and expression profiling of miRNAs in two color variants of carrot (*Daucus carota* L.) using deep sequencing. *PLoS One* 14(3):e0212746. <https://doi.org/10.1371/journal.pone.0212746>
- Bizuayehu TT, Babiak I (2014) MicroRNA in teleost fish. *Genome Biol Evol* 6(8):1911–1937. <https://doi.org/10.1093/gbe/evu1511>
- Bliss SP, Navratil AM, Xie J, Roberson MS (2010) GnRH signaling, the gonadotrope and endocrine control of fertility. *Front Neuroendocrinol* 31(3):322–340. <https://doi.org/10.1016/j.yfrne.2010.04.002>
- Chen F, Li J, Zhang H, Xu J, Tao Z, Shen J, Shen J, Lu L, Li C (2014) Identification of differentially expressed known and novel miRNAs in broodiness of goose. *Mol Biol Rep* 41(5):2767–2777. <https://doi.org/10.1007/s11033-014-3131-8>
- Draghici S, Khatri P, Bhavsar P, Shah A, Krawetz SA, Tainsky MA (2003) Onto-Tools, the toolkit of the modern biologist: onto-express, onto-compare, onto-design and onto-translate. *Nucl Acids Res* 31(13):3775–3781. <https://doi.org/10.1093/nar/gkg624>
- Fernandez-Perez D, Brieno-Enriquez MA, Isolser-Alcaraz J, Larriba E, Del Mazo J (2018) MicroRNA dynamics at the onset of primordial germ and somatic cell sex differentiation during mouse embryonic gonad development. *RNA* 24(3):287–303. <https://doi.org/10.1261/rna.062869.117>
- Friedlander MR, Mackowiak SD, Li N, Chen W, Rajewsky N (2012) miRDeep2 accurately identifies known and hundreds of novel microRNA genes in seven animal clades. *Nucl Acids Res* 40(1):37–52. <https://doi.org/10.1093/nar/gkr688>
- Gu B, Liu H, Han Y, Chen Y, Jiang H (2019) Integrated analysis of miRNA and mRNA expression profiles in 2-, 6-, and 12-month-old Small Tail Han Sheep ovaries reveals that oar-miR-432 down-regulates RPS6KA1 expression. *Gene* 710:76–90. <https://doi.org/10.1016/j.gene.2019.02.095>
- Guo J, Wang Q, Liu L et al (2019) Analysis of microRNAs, phased small interfering RNAs and their potential targets in *Rosa rugosa* Thunb. *BMC Genom* 19(Suppl 9):983. <https://doi.org/10.1186/s12864-018-5325-2>
- He L, Wang YL, Li Q, Yang HD, Duan ZL, Wang Q (2015) Profiling microRNAs in the testis during sexual maturation stages in *Eriocheir sinensis*. *Anim Reprod Sci* 162:52–61. <https://doi.org/10.1016/j.anireprosci.2015.09.008>
- He P, Wei P, Chen X, Lin Y, Peng J (2019) Identification and characterization of microRNAs in the gonad of *Trachinotus ovatus* using Solexa sequencing. *Comp Biochem Physiol Part D Genom Proteom* 30:312–320. <https://doi.org/10.1016/j.cbd.2019.03.010>
- Hwang HW, Mendell JT (2007) MicroRNAs in cell proliferation, cell death, and tumorigenesis. *Br J Cancer* 96(Suppl):R40–44
- Jay C, Nemunaitis J, Chen P, Fulgham P, Tong AW (2007) miRNA profiling for diagnosis and prognosis of human cancer. *DNA Cell Biol* 26(5):293–300. <https://doi.org/10.1089/dna.2006.0554>
- Kandasamy K, Mohan SS, Raju R et al (2010) NetPath: a public resource of curated signal transduction pathways. *Genome Biol* 11(1):R3. <https://doi.org/10.1186/gb-2010-11-1-r3>
- Kang L, Cui X, Zhang Y, Yang C, Jiang Y (2013) Identification of miRNAs associated with sexual maturity in chicken ovary by Illumina small RNA deep sequencing. *BMC Genom* 14:352. <https://doi.org/10.1186/1471-2164-14-352>
- Kozomara A, Griffiths-Jones S (2014) miRBase: annotating high confidence microRNAs using deep sequencing data. *Nucl Acids Res* 42(Database issue):D68–73. <https://doi.org/10.1093/nar/gkt1181>
- Kozomara A, Birgaoanu M, Griffiths-Jones S (2019) miRBase: from microRNA sequences to function. *Nucl Acids Res* 47(D1):D155–D162. <https://doi.org/10.1093/nar/gky1141>
- Lan T, Chen YL, Gul Y, Zhao BW, Gao ZX (2019) Comparative expression analysis of let-7 microRNAs during ovary development in *Megalobrama amblycephala*. *Fish Physiol Biochem* 45(3):1101–1115. <https://doi.org/10.1007/s10695-019-00624-7>
- Li T, Wu R, Zhang Y, Zhu D (2011) A systematic analysis of the skeletal muscle miRNA transcriptome of chicken varieties with divergent skeletal muscle growth identifies novel miRNAs and differentially expressed miRNAs. *BMC Genom* 12:186. <https://doi.org/10.1186/1471-2164-12-186>
- Li X, Gao Y, Guo LH, Jiang G (2013) Structure-dependent activities of hydroxylated polybrominated diphenyl ethers on human estrogen receptor. *Toxicology* 309:15–22. <https://doi.org/10.1016/j.tox.2013.04.001>
- Li Z, Zhang J, Su J et al (2015) MicroRNAs in the immune organs of chickens and ducks indicate divergence of immunity against H5N1 avian influenza. *FEBS Lett* 589(4):419–425. <https://doi.org/10.1016/j.febslet.2014.12.019>
- Li X, Li C, Ni W et al (2018a) Identification and comparison of microRNAs in pituitary gland during prenatal and postnatal stages of sheep by deep sequencing. *J Genet* 97(4):965–975
- Li Y, Li RH, Ran MX et al (2018b) High throughput small RNA and transcriptome sequencing reveal capacitation-related microRNAs and mRNA in boar sperm. *BMC Genom* 19(1):736. <https://doi.org/10.1186/s12864-018-5132-9>
- Liu Y, Li M, Ma J et al (2013) Identification of differences in microRNA transcriptomes between porcine oxidative and glycolytic skeletal muscles. *BMC Mol Biol* 14:7. <https://doi.org/10.1186/1471-2199-14-7>
- Liu W, Cheng C, Chen F, Ni S, Lin Y, Lai Z (2018) High-throughput sequencing of small RNAs revealed the diversified cold-response pathways during cold stress in the wild banana (*Musa itinerans*). *BMC Plant Biol* 18(1):308. <https://doi.org/10.1186/s12870-018-1483-2>
- Luo GZ, Hafner M, Shi Z et al (2012) Genome-wide annotation and analysis of zebra finch microRNA repertoire reveal

- sex-biased expression. *BMC Genom* 13:727. <https://doi.org/10.1186/1471-2164-13-727>
- Markkandan K, Ahn K, Lee DJ et al (2018) Profiling and identification of pregnancy-associated circulating microRNAs in dairy cattle. *Genes Genom* 40(10):1111–1117. <https://doi.org/10.1007/s13258-018-0668-2>
- Meng X, Zhang X, Li J, Liu P (2018) Identification and comparative profiling of ovarian and testicular microRNAs in the swimming crab *Portunus trituberculatus*. *Gene* 640:6–13. <https://doi.org/10.1016/j.gene.2017.10.026>
- Menzel P, McCorkindale AL, Stefanov SR, Zinzen RP, Meyer IM (2019) Transcriptional dynamics of microRNAs and their targets during *Drosophila neurogenesis*. *RNA Biol* 16(1):69–81. <https://doi.org/10.1080/15476286.2018.1558907>
- Millar RP (2005) GnRHs and GnRH receptors. *Anim Reprod Sci* 88(1–2):5–28. <https://doi.org/10.1016/j.anireprosci.2005.05.032>
- Song L, Tuan RS (2006) MicroRNAs and cell differentiation in mammalian development. *Birth Defects Res C Embryo Today* 78(2):140–149. <https://doi.org/10.1002/bdrc.20070>
- Song YN, Shi LL, Liu ZQ, Qiu GF (2014) Global analysis of the ovarian microRNA transcriptome: implication for miR-2 and miR-133 regulation of oocyte meiosis in the Chinese mitten crab, *Eriocheir sinensis* (Crustacea:decapoda). *BMC Genom* 15:547. <https://doi.org/10.1186/1471-2164-15-547>
- Song J, Jiang X, Hu Y et al (2018) High-throughput sequencing of putative novel microRNAs in rhesus monkey peripheral blood mononuclear cells following EV71 and CA16 infection. *Intervirology* 61(3):133–142. <https://doi.org/10.1159/000493798>
- Tao Z, Song W, Zhu C et al (2017) Comparative transcriptomic analysis of high and low egg-producing duck ovaries. *Poult Sci* 96(12):4378–4388. <https://doi.org/10.3382/ps/pex229>
- Tong XH, Xu B, Zhang YW, Liu YS, Ma CH (2014) Research resources: comparative microRNA profiles in human corona radiata cells and cumulus oophorus cells detected by next-generation small RNA sequencing. *PLoS One* 9(9):e106706. <https://doi.org/10.1371/journal.pone.0106706>
- Velarde MC (2013) Pleiotropic actions of estrogen: a mitochondrial matter. *Physiol Genom* 45(3):106–109. <https://doi.org/10.1152/physiolgenomics.00155.2012>
- Wahid F, Shehzad A, Khan T, Kim YY (2010) MicroRNAs: synthesis, mechanism, function, and recent clinical trials. *Biochim Biophys Acta* 1803(11):1231–1243. <https://doi.org/10.1016/j.bbamer.2010.06.013>
- Wang S, Zhang Y, Yuan X et al (2018a) Identification of differentially expressed microRNAs during preadipocyte differentiation in Chinese crested duck. *Gene* 661:126–132. <https://doi.org/10.1016/j.gene.2018.03.085>
- Wang Y, Yang HM, Cao W, Li YB, Wang ZY (2018b) Deep sequencing identification of miRNAs in pigeon ovaries illuminated with monochromatic light. *BMC Genom* 19(1):446. <https://doi.org/10.1186/s12864-018-4831-6>
- Wu N, Gaur U, Zhu Q et al (2017a) Expressed microRNA associated with high rate of egg production in chicken ovarian follicles. *Anim Genet* 48(2):205–216. <https://doi.org/10.1111/age.12516>
- Wu N, Zhu Q, Chen B, Gao J, Xu Z, Li D (2017b) High-throughput sequencing of pituitary and hypothalamic microRNA transcriptome associated with high rate of egg production. *BMC Genom* 18(1):255. <https://doi.org/10.1186/s12864-017-3644-3>
- Xu Q, Zhang Y, Chen Y et al (2014) Identification and differential expression of microRNAs in ovaries of laying and Broody geese (*Anser cygnoides*) by Solexa sequencing. *PLoS One* 9(2):e87920. <https://doi.org/10.1371/journal.pone.0087920>
- Yu Y, Jia T, Chen X (2017) The ‘how’ and ‘where’ of plant microRNAs. *New Phytol* 216(4):1002–1017. <https://doi.org/10.1111/nph.14834>
- Zeeberg BR, Feng W, Wang G et al (2003) GoMiner: a resource for biological interpretation of genomic and proteomic data. *Genome Biol* 4(4):R28. <https://doi.org/10.1186/gb-2003-4-4-r28>
- Zhang Y, Chen Y, Gu T et al (2019) Effects of *Salmonella enterica* serovar Enteritidis infection on egg production and the immune response of the laying duck *Anas platyrhynchos*. *PeerJ* 7:e6359. <https://doi.org/10.7717/peerj.6359>
- Zhao RQ, Zhou YC, Ni YD et al (2005) Effect of daidzein on egg-laying performance in Shaoxing duck breeders during different stages of the egg production cycle. *Br Poult Sci* 46(2):175–181. <https://doi.org/10.1080/00071660500064808>
- Zhao L, Niu Y, Lu T et al (2018) Metagenomic analysis of the Jinding duck fecal virome. *Curr Microbiol* 75(6):658–665. <https://doi.org/10.1007/s00284-018-1430-3>
- Zhu W, Yang M, Shang J et al (2019) MiR-222 inhibits apoptosis in porcine follicular granulosa cells by targeting the THBS1 gene. *Anim Sci J* 90(6):719–727. <https://doi.org/10.1111/asj.13208>