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Expressed microRNA associated with high rate of egg production in chicken ovarian follicles

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Summary

MicroRNA (miRNA) is a highly conserved class of small noncoding RNA about 19–24 nucleotides in length that function in a specific manner to post-transcriptionally regulate gene expression in organisms. Tissue miRNA expression studies have discovered a myriad of functions for miRNAs in various aspects, but a role for miRNAs in chicken ovarian tissue at 300 days of age has not hitherto been reported. In this study, we performed the first miRNA analysis of ovarian tissues in chickens with low and high rates of egg production using high-throughput sequencing. By comparing low rate of egg production chickens with high rate of egg production chickens, 17 significantly differentially expressed miRNAs were found ($P < 0.05$), including 11 known and six novel miRNAs. We found that all 11 known miRNAs were involved mainly in pathways of reproduction regulation, such as steroid hormone biosynthesis and dopaminergic synapse. Additionally, expression profiling of six randomly selected differentially regulated miRNAs were validated by quantitative real-time polymerase chain reaction (RT-qPCR). Some miRNAs, such as gga-miR-34b, gga-miR-34c and gga-miR-216b, were reported to regulate processes such as proliferation, cell cycle, apoptosis and metastasis and were expressed differentially in ovaries of chickens with high rates of egg production, suggesting that these miRNAs have an important role in ovary development and reproductive management of chicken. Furthermore, we uncovered that a significantly up-regulated miRNA—gga-miR-200a-3p—is ubiquitous in reproduction-regulation-related pathways. This miRNA may play a special central role in the reproductive management of chicken, and needs to be further studied for confirmation.

Keywords egg-laying, illumina sequencing, Luhua chicken, reproduction regulation, RT-qPCR

Introduction

MicroRNAs (miRNA), which are extensively expressed in a variety of organisms and tissues, comprise a class of endogenous small noncoding single-stranded RNA about 19–24 nucleotides (nt) in length (Bartel 2004). Mature miRNAs from the transcripts of miRNA genes are produced through processing from the one arm of fold back precursors (pre-miRNAs) to form approximately 70-nt hairpin secondary structures (Lee *et al.* 2004; Kim 2005; Carthew & Sontheimer 2009). Cellular endogenous miRNA plays important roles in regulating various biological and metabolic processes, including organogenesis, cell proliferation, differentiation, development, hematopoiesis, apoptosis, tumorigenesis and many other cellular processes (Lim *et al.* 2003; Bartel 2004; Mansfield *et al.* 2004). According to an assessment, one miRNA can regulate the expression of hundreds of mRNAs, and the expression of one mRNA can be regulated by hundreds of miRNAs (Krek *et al.* 2005). In other words, miRNA construct networks of sophisticated regulator control systems in organisms and play very significant roles.

Genome-wide miRNA expression has been explored in gonads of cattle (Huang *et al.* 2011), mice (Ro *et al.* 2007; Mishima *et al.* 2008), sheep (McBride *et al.* 2012) and pigs (Li *et al.* 2011). These studies revealed that miRNAs have a significant function in the development of mammalian gonads. Some miRNAs have been identified in chicken, and expression has been reported in several processes, such as embryo development (Darnell *et al.* 2006; Glazov *et al.* 2008; Hicks

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et al. 2008, 2010; Bannister *et al.* 2009; Rathjen *et al.* 2009; Wang *et al.* 2009; Li *et al.* 2012), immune organ function (Hicks *et al.* 2009), germ cell development (Burnside *et al.* 2008; Lee *et al.* 2011) and disease (Yu *et al.* 2008; Tian *et al.* 2012; Wang *et al.* 2013). The chicken (*Gallus gallus*) is an important agricultural and avian-model species, and it is a major source of protein worldwide. However, a role for miRNAs in chicken ovarian development has not hitherto been reported clearly. The characteristics of ovarian tissue are highly related to reproductive and economic traits of chicken, so it is necessary to identify and characterize the miRNA in ovarian tissue.

In recent years, the development of next-generation sequencing, which is known as deep sequencing or high-throughput sequencing technology, has provided a powerful, highly reproducible and cost-efficient tool for transcriptomic research (Morozova & Marra 2008). In previous studies, the catalog of chicken miRNA expression during embryonic development was characterized using deep sequencing (Glazov *et al.* 2008), providing a strong background of information for analysis of tissue-specific miRNA signatures. In addition, the genome sequence is available for chicken, enhancing its use as a model for functional genomics studies during development. Thus, by means of miRNA sequencing, we aimed to identify relevant expressive miRNA associated with high rates of egg production and to extend the repertoire of known miRNAs in chicken ovarian tissue. Moreover, we can screen suitable miRNAs to use them as molecular markers in the application of genetic selection in the breeding programs of chicken.

The Luhua chicken breed (*Gallus gallus domesticus*), originally found mainly in Wenshang county of Shandong province, China, has been recognized as a commercial dual-purpose egg–meat type chicken (Ruan & Zheng 2011; Hu *et al.* 2013). It is a common strain especially selected for its superior reproductive performance such as high rates of egg production. In poultry breeding programs, egg number at 300 days of age is usually used as the most valuable indicator of total egg production potential (Li *et al.* 2013). In the present study, we sampled the ovarian tissues of three low-rate egg production (LP) and three high-rate egg production (HP) Luhua chickens at age 300 days. Comprehensive miRNA profiles of ovarian tissue from LP and HP chickens were generated, and a comparative analysis of miRNA data was performed. This miRNA data will be very useful in further understanding the functions of chicken ovarian tissues and will contribute to the investigation of the regulatory mechanism of miRNA in avian species.

Materials and methods

Experimental animals

Eight hundred Luhua chickens from the Experimental Chicken Farm of Sichuan Agricultural University were used in this study. A total of 200 LP chickens and 200 HP chickens were bi-directionally selected according to their number of eggs at 250 days of age. Six reproductive traits (body weight at first egg, weight of first egg, age at first egg, number of eggs at 300 days of age, body weight at 300 days of age and egg weight at 300 days of age) were recorded for both groups. The average number of eggs at 300 days of age (mean $\pm$ SEM) was 111 ± 2.1 and 144 ± 4.3 for LP and HP chickens respectively. Egg production cycles of these chickens were also recorded every day. According to their similar reproductive traits and regular egg production cycle (about 2 hours prior to ovulation), three LP (B4, B5, B7) and three HP (A2, A3, A9) chickens (Table S1) were selected for tissue collection. Experimental procedures were approved by the Committee on the Care and Use of Laboratory Animals of the State-level Animal Experimental Teaching Demonstration Center of Sichuan Agricultural University (Approval ID: S20141010) and performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (China, 1988) for animal experiments. All efforts were made to minimize the suffering of the chickens.

Tissue collection, RNA isolation and small RNA sequencing

Six chickens were sacrificed by exsanguination at 300 days of age, and the ovarian tissues, which contained the entire collection of various-sized follicles (including hierarchical and all prehierarchical follicles), were collected about 2 hours prior to ovulation. The egg yolks of the follicles were removed in the process of sample collection. Samples were quickly stored in RNAlater (Ambion®), frozen in liquid nitrogen and stored at −80 °C until RNA extraction. Total RNA was isolated from these tissues using an RNeasy Mini Kit (Qiagen), in accordance with the manufacturer's instructions. The quantity and purity of total RNA were monitored by a Qubit® 3.0 Fluorometer (Thermo Fisher Scientific) and formaldehyde–agarose gel electrophoresis and then stored at −80 °C.

To identify ovarian miRNAs in chickens, complementary DNA (cDNA) libraries for small RNA from chicken ovarian tissues were constructed according to published miRNA cloning protocols (Lagos-Quintana *et al.* 2001; Lau *et al.* 2001) and sequenced using an HiSeq2500 (Illumina) followed by NEXTflexTM Small RNA-seq Kits (BIOO Scientific Corp.).

Global annotation of miRNA sequences

The raw data, consisting of 17–35 nt sequence adaptors, first went through a data cleaning process to remove low quality adaptors, having quality values less than 20, 5′ and 3′ primer contaminants, N adaptors, polyA adaptors, sequences without insert tags and adaptors shorter than

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17 nt and longer than 35 nt. Subsequently, standard bioinformatic analyses were carried out to align or annotate the clean adaptors. The clean reads were mapped to the ensemble chicken genome galGal4 using NCBI MEGABLAST, and rRNA, tRNA, miscRNA, snRNA and snoRNA were discarded from the small RNA sequences; the remaining sequences were again searched against the miRBase 21.0 database of known Gallus gallus miRNA sequences with zero or one mismatch (http://www.mirbase.org/) were continued (Ambros *et al.* 2003; Griffiths-Jones 2004; Griffiths-Jones *et al.* 2006, 2008). The sequences matching *Gallus gallus* miRBase were considered known miRNA sequences, and the expression of all miRNAs was assayed. Next, after filtering known miRNA sequences, the remaining sequences were BLAST searched against the *Gallus gallus* genome. The sequences matching chicken genome sequences (except for one to three 5′- or 3′-end nt) were used to predict novel miRNAs by MIRDEEP2 (https://www.mdcberlin.de/8551903/en/research/research_teams/systems_biology_of_gene_regulatory_elements/projects/miRDeep) using default parameters. These sequences were considered as a potential novel miRNA, and expression of all miRNAs was assayed.

Hierarchical cluster analysis of differentially expressed miRNAs

Differential expression for known and novel miRNAs were analyzed using EDGER (http://www.bioconductor.org/packages/release/bioc/html/edgeR.html). Reads per million miRNA mapped values were used to represent miRNA expression levels. *P*-values were calculated using right-tailed Fisher's exact test. $P < 0.05$ and $|\log_2 \text{fold change}| \geq 1$ were used to screen differentially expressed miRNAs. A hierarchical cluster analysis was performed for known and novel miRNAs with similar expression patterns (Zhang *et al.* 2009).

MiRNA target gene prediction and functional annotation

The miRNA targets were predicted by analyzing the putative miRNA binding sites in the libraries. The miRNA target prediction software MIRDB (http://mirdb.org/miRDB/index.html) (Wong & Wang 2014) was used to predict the binding sites of differentially expressed miRNA. The TargetScan principle (http://www.targetscan.org/) was also applied in the prediction procedures. The intersection of the two prediction programs was selected for this study.

The BLAST2GO program was used to conduct gene ontology (GO) annotations and GO functional classifications (Conesa *et al.* 2005) of these predicted miRNA target genes. In GO terms, *P*-value ≤ 0.001 was used to identify the significantly enriched GO terms. These genes were also submitted to the KEGG (Kyoto Encyclopedia of Genes and Genomes) database for enrichment analyses. The *P*-value denotes the significance of the pathway correlated to the conditions. The lower the *P*-value, the more significant the metabolism pathway (*P*-value cut-off was 0.05).

Validation of miRNA expression by real-time PCR (RT-qPCR)

To validate the reliability of the Illumina analysis, we randomly tested the expression of eight miRNAs, including six differentially expressed miRNAs and two similarly expressed miRNAs. Reverse transcription (RT) real-time PCR was used to quantify the expression of eight mature miRNAs. The RT-qPCR primers were designed using PRIMER 5.0 (http://downloads.fyxm.net/Primer-Premier-101178.html) and are listed in Table S2 (miRNA-specific primers were synthesized by Shanghai Biological Technology Co. and universal primers were provided by miRcute miRNA qPCR Detection kit, Aidlab).

Briefly, the total RNA of each sample was reverse-transcribed with miRNA-specific RT primers using the First-strand cDNA Synthesis Kit (Thermo Scientific Fermentas). First, 2 μl of 10× poly(A) polymerase buffer, 2 μl of 10× rATP Solution, 0.4 μl of E. coli Poly(A) Polymerase (5 U/μl) and 2 μl of total RNA (10 $\text{pg} \cdot \mu\text{l}^{-1}$ ~1.5 $\mu\text{g} \cdot \mu\text{l}^{-1}$) were added into ice-cold RNase-Free reaction tubes and supplemented with RNase-Free ddH_2O to a total volume of 20 μl. After brief centrifugation, the mixture was incubated for 50 min at 37 °C. Second, 3 μl of the Poly (A) reaction mixture obtained in the first step was added to a solution containing 2 μl of 25 μmol RT Primer, 4 μl of 5× RT Buffer Reaction Mix, 0.8 μl of TUREscript H^- RTase (200 U/μl) and 10.2 μl of RNase-Free ddH_2O. After brief centrifugation, the reaction solutions were incubated at 42 °C for 50 min, at 70 °C for 15 min and then held at −20 °C.

The expression of mature miRNAs was detected using the SYBR green method. RT-qPCR was performed in a 96-well plate using the Bio-Rad iQ5 Real-time PCR Detection System, according to the protocol. In a 20-μl reaction mixture, 1.0 μl of cDNA was used as a template, with 10 μl of SYBR Select Master Mix (Applied Biosystems), 0.4 μl of specific forward primer and 0.4 μl of universal primer, with the following program: 94 °C for 3 min, followed by 42 cycles of 94 °C for 20 s and the optimal temperature for 40 s. 5.8S rRNA, which has relatively stable expression in most tissues, was used as an endogenous control (Elela & Nazar 1997), and the expression level of 5.8S rRNA was used to normalize the RT-qPCR results for each miRNA. All reactions were run in three technical replicates, including negative controls without template. Fold changes of miRNA expression were calculated using the $2^{-\Delta\Delta Ct}$ method (versus 5.8S rRNA) (Schmittgen & Livak 2008). All of the data were expressed as the mean ± standard deviation, and a statistical analysis using Student's *t*-tests was performed with SPSS 16.0 (SPSS Inc.).

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Accession code

The Illumina HiSeq 2500 sequencing data for the Luhua chicken ovarian miRNA sequences has been deposited in the NCBI Sequence Read Archive (SRA, http://www.ncbi.nlm.nih.gov/Traces/sra) with accession no. SRP062266.

Results

Overview of miRNA sequencing

A total of 30.20 million and 30.37 million raw reads were obtained from ovarian tissues of LP and HP chickens respectively. After filtering the low-quality sequences, 28 501 792 and 28 004 558 clean reads from the HP (93.83% clean) and LP (92.72% clean) chicken ovary libraries respectively were selected for further analysis (Table S3). The total and unique read length distributions of LP and HP chickens are shown in Fig. S1. Among the selected reads, the size distribution of small RNAs for sequencing was similar in six samples. The majority of these total small RNAs ranged from 20–24 nt, and unique reads were distributed in mainly 21–23 nt; there was no obvious difference between the LP and HP chickens. The results are consistent with the typical size range of small RNAs and indicated that the clean reads included a large number of miRNA sequences.

Annotation of small RNA sequences

Small RNA sequences identified by Illumina small RNA deep sequencing were compared with known mature miRNAs and precursors in miRBase 21.0. The unique and all small RNAs were annotated as miRNA, snRNA, tRNA, rRNA, snoRNA, miscRNA (repeat and polII-transcribed) and unannotated for the LP and HP chickens (Table S4). The HP chickens had a larger number of small RNAs and unique small RNAs than did the LP chickens. Most small RNAs in both groups were miRNAs (82%) and unannotated (16%) for all small RNAs, whereas for unique small RNAs, HP chickens had the greatest proportion of miRNAs (41%) and LP chickens had only 33% of miRNAs as the second major class (Fig. S2). This revealed that HP chickens produce more unique miRNAs, but the difference is not significant ($P > 0.05$). Proportions of the remaining categories of small RNAs, including tRNA, rRNA, snRNA, miscRNA or snoRNA, were relatively lower (<1.2%).

Expression of known and novel miRNAs in chicken ovaries

In the present study, 562 known miRNAs were identified in all samples including 507 in HP and 496 in LP chickens (Table S5). Among the 562 sequenced mature miRNAs, 441 (78.5%) unique miRNAs were expressed in all LP and HP chickens; however, 66 (11.7%) and 55 (12.5%) were specifically expressed in HP and LP chickens respectively. In the miRNA expression profile, the read numbers of the top 10 miRNAs accounted for 81.93% of the total reads from LP chickens and 78.3% of the total reads from HP chickens. The miRNA expression profile revealed that most miRNAs were expressed by a small portion of miRNA genes. Interestingly, we found that the miRNAs with the 19 highest expression levels were the same for both groups. Among them, miR-99a-5p and gga-miR-26a-5p displayed more than 7 500 000 reads and had the highest expression level, followed by gga-miR-199-5p and gga-miR-10a-5p, which displayed about 2 500 000 reads each. Some miRNAs (such as gga-miR-222b-3p and gga-miR-1729-5p) displayed fewer than about 1 000 reads, thereby indicating that expression varied significantly among different miRNAs; this result is consistent with previous studies (Gu *et al.* 2014).

A total of 470 novel miRNAs were predicted in this study, and 389 and 384 novel miRNAs were identified in HP and LP chickens respectively (Table S6). Among the 470 predicted novel miRNAs, 303 (64.5%) unique miRNAs were expressed in both groups; however, 86 (18.3%) and 55 (17.2%) were specifically expressed in HP and LP chickens respectively. The novel miRNA expression profile also revealed that most novel miRNAs were expressed by a small portion of miRNA genes. Moreover, we found that the sequencing frequencies of novel miRNAs were much lower compared to those of known miRNAs. The same expression pattern has also been reported in other species (Chi *et al.* 2011; Xu *et al.* 2013), which suggests that novel miRNAs are usually weakly expressed whereas known miRNA genes are highly expressed.

Hierarchical cluster analysis of differentially expressed miRNAs

We first performed a correlation analysis to assess the variance of miRNA expression across replicates. All samples showed high reproducibility in the same group, with Pearson correlation coefficients >0.85 (Fig. 1). Known and novel miRNAs were compared between the two groups to identify differentially expressed miRNAs. The results of the analysis demonstrated that there were 17 significantly differentially expressed miRNAs, which were divided into two categories, the upper part comprising eight up-regulated miRNAs and the lower half nine down-regulated miRNAs in HP chickens (Table 1 & Fig. 2). In the cluster analysis, miRNAs that showed similar patterns of differential expression in different sample pairs were clustered together. Based on miRBase 21.0, these 17 miRNAs comprised five clusters, of which one cluster contained eight miRNAs, one cluster contained five miRNAs, one

cluster contained two miRNAs and two clusters contained one miRNA (Fig. S3).

Target gene prediction and gene functional annotation

Target prediction is an important way to determine the functions of miRNAs. We predicted a total of 1305 target genes among the differentially expressed miRNAs (Table S7). To probe the biological roles of the differentially expressed miRNAs, all of the predicted targets in this study were mapped to terms in the GO and KEGG databases. A total of 128 significantly enriched GO terms and 18 KEGG pathways regulated by 11 differentially expressed known miRNAs were identified (Table S8). Among the top 30 significantly GO terms for biological process, most target genes were associated with biological regulation, single-organism cellular process, single-organism process and regulation of metabolic process (Fig. 3). For cellular component, target genes were significantly associated with intracellular part. For molecular function, target genes were significantly related to protein binding and ion binding. Results of the KEGG analysis showed that these target genes were involved mainly in endocytosis, miRNAs in cancer, glycerol phospholipid metabolism, thyroid hormone signaling pathway and ubiquitin mediated proteolysis pathways (Fig. 4). Involvement in cancer pathways suggest that the differentially expressed miRNAs play a regulatory role in cell proliferation and cell cycle progression. Notably, a specific enrichment of genes was found in some reproduction regulation pathways, such as steroid hormone biosynthesis, dopaminergic synapse, GnRH signaling pathways, oxytocin signaling pathway, oocyte meiosis, calcium signaling pathways, progesterone-mediated oocyte maturation, endocrine and other factor-regulated calcium reabsorption and MAPK signaling pathway (Table S9).

Table 1 miRNAs significantly differentially expressed between low- and high-rate egg production chickens.

miRNA	logFC	*P*-value	Up-/down-regulated
gga-miR-34b-5p	3.73965	0.00003	Up
gga-miR-34c-5p	2.56597	0.00021	Up
gga-miR-34b-3p	1.74087	0.02398	Up
gga-novel-18-star	4.34580	0.03305	Up
gga-miR-34c-3p	1.93322	0.03626	Up
gga-miR-200a-3p	1.29779	0.03646	Up
gga-novel-31-mature	2.27375	0.03687	Up
gga-miR-1641	2.61869	0.04540	Up
gga-miR-1744-3p	−3.16434	0.00086	Down
gga-novel-280-mature	−2.55983	0.00944	Down
gga-miR-1655-5p	−1.85425	0.02031	Down
gga-novel-73-star	−2.66111	0.02283	Down
gga-miR-216b	−2.75966	0.02540	Down
gga-miR-1734	−3.19824	0.03786	Down
gga-novel-79-mature	−4.02767	0.03809	Down
gga-novel-81-mature	−4.02779	0.03823	Down
gga-miR-7465-3p	−1.71936	0.04816	Down

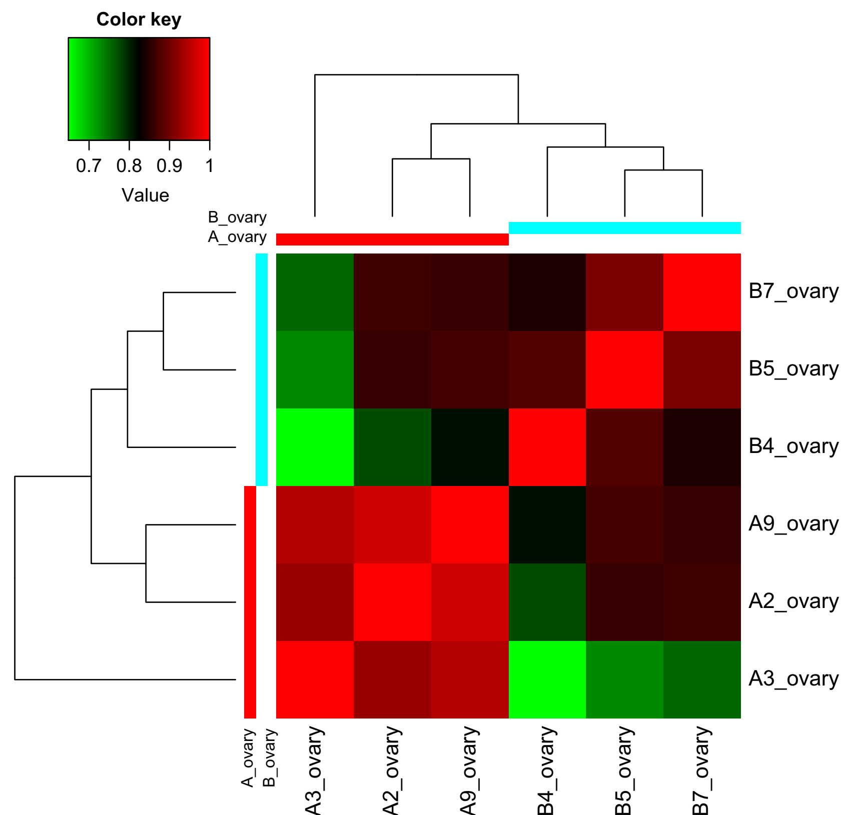

Figure 1 Correlation analysis of miRNA expression across samples of LP and HP chickens.

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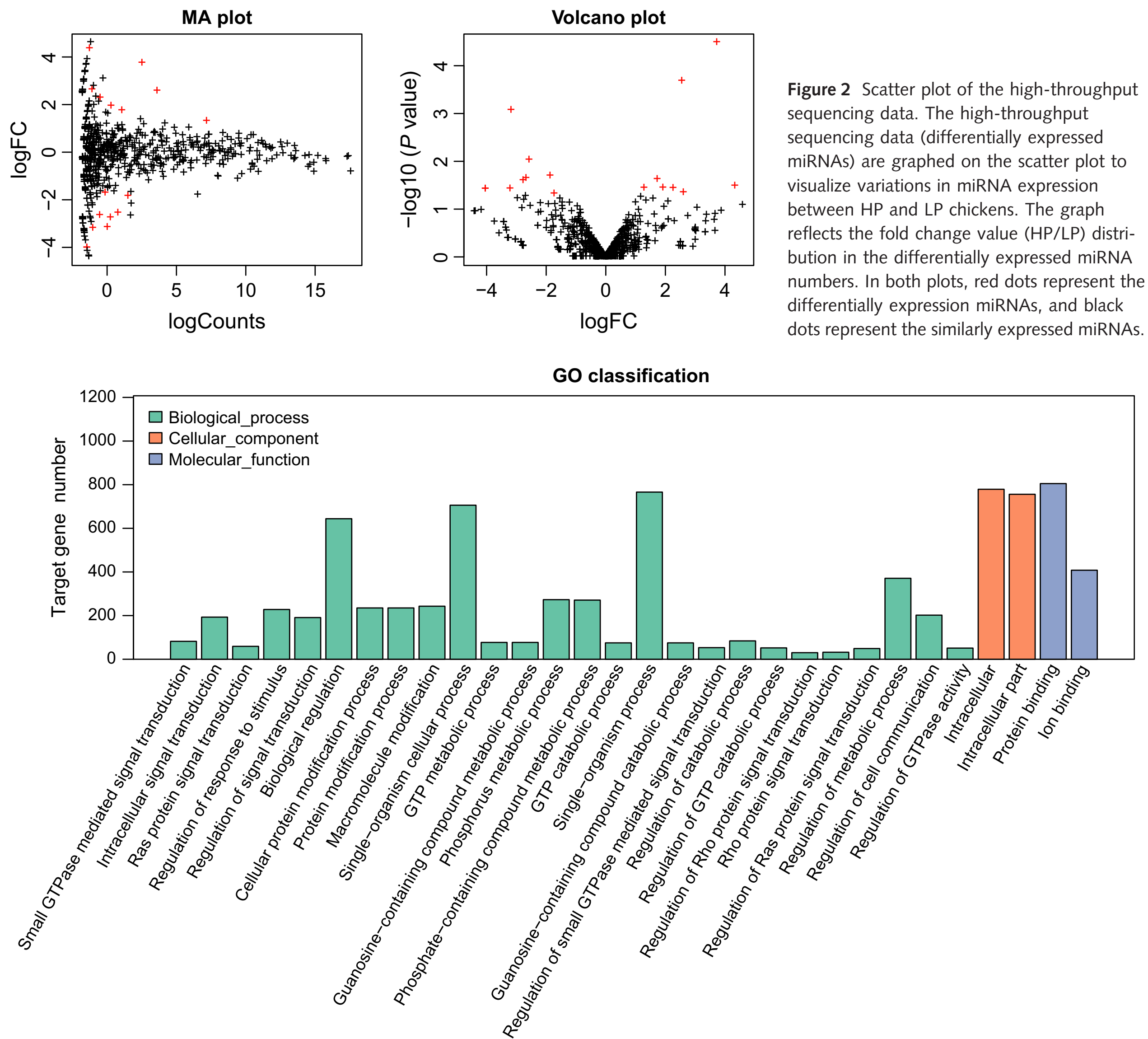

Figure 2 Scatter plot of the high-throughput sequencing data. The high-throughput sequencing data (differentially expressed miRNAs) are graphed on the scatter plot to visualize variations in miRNA expression between HP and LP chickens. The graph reflects the fold change value (HP/LP) distribution in the differentially expressed miRNA numbers. In both plots, red dots represent the differentially expression miRNAs, and black dots represent the similarly expressed miRNAs.

Figure 3 Selected top 30 GO categories of predicted target genes regulated by the differentially expressed miRNAs.

Verification of miRNA and targeted gene expression

RT-qPCR detection assays were used to confirm the expression of certain differentially expressed miRNAs in the LP and HP chickens. The expression of eight randomly selected miRNAs was verified by real-time PCR (Table 2 & Fig. 5). The expression of gga-miR-34b-3p, gga-miR-34c-3p and gga-miR-200a-3p were up-regulated, whereas gga-miR-1744-3p, gga-miR-216b and gga-miR-1655-5p were down-regulated and gga-miR-99a-5p and gga-miR-26a-5p showed no significant difference, which was in agreement with the high-throughput sequencing results (Table 1). The discrepancies with respect to ratio may be attributed to the essentially different algorithms and sensitivities between the two techniques.

We further investigated the potential roles of the 17 miRNAs in regulating targeted gene expression using RNA-seq. Thirty-nine predicted targets of 10 miRNAs showed evidence of differential expression between HP and LP chickens (Fig. S4). Among them, most genes have been demonstrated to play important roles in regulating organogenesis, development, tumorigenesis and many other processes (Moreau *et al.* 1997; Sarbassov *et al.* 2004; Kuo *et al.* 2011; Kanda *et al.* 2016). Our results suggest that these genes are also associated with reproductive traits of hens. These results provide invaluable insights into candidate genes for reproductive traits and selective breeding of chicken.

Discussion

The miRNA sequences we identified were generally close to 22 nt in length, in agreement with previous reports (Yuan *et al.* 2014; Li *et al.* 2015), and annotation of RNA

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distribution showed that the clean reads included a myriad of miRNA sequences. All of the above results suggest that the major clean reads mapped to known miRNAs in miRBase were highly enriched and that the deep sequencing data are representative of the miRNA expression profile of ovarian tissues and would be reliable for subsequent analyses and prediction of novel miRNAs.

In the ovary libraries, gga-miR-99a-5p and gga-miR-26a-5p were the two most frequently sequenced miRNAs (>7 500 000 reads). Previous studies demonstrated that mir-99a and mir-99b can inhibit proliferation of c-Src-transformed cells and prostate cancer cells by targeting mTOR (Oneyama *et al.* 2011; Sun *et al.* 2011). They were also identified as two novel target miRNA genes of transforming growth factor-b (TGF-β) and played important roles during TGF-β-induced epithelial to mesenchymal transition (EMT) of NMUMG cells (Turcatel *et al.* 2012). TGF-β is a secreted cytokine that regulates a variety of processes in development and cancer including EMT (Bierie & Moses 2006a,b). EMT is a key process during embryonic development and disease development and progression. miR-26a is reported to have anti-apoptotic effects on many cancers (Garzon *et al.* 2006; Saito & Jones 2006; Zhang *et al.* 2011). It was also found to play a role in normal tissue growth and development and to have an impact on cell proliferation and differentiation (Luzi *et al.* 2008; Wong & Tellam 2008). One study showed that miR-26a regulates osteoblast cell growth and differentiation in human adipose tissue-derived stem cells (Luzi *et al.* 2008). Among other miRNAs, we found that the let-7 miRNA family was another abundant cluster with let-7a-5p being the most abundantly expressed miRNA. The let-7 miRNA family was also found to be abundantly expressed in ovary and oocyte of bovines (Tripurani *et al.* 2010; Huang *et al.* 2011b; Miles *et al.* 2012), as well as in murine ovaries and testis (Reid *et al.* 2008). Furthermore, gga-miR-10a-5p, gga-miR-146c-5p, gga-miR-21-5p, gga-miR-148a-3p, gga-miR-126-3p and gga-miR-30d were abundant in our sequencing libraries, as has been shown in other animal gonads (Mishima *et al.* 2008; Md Munir *et al.* 2009; Tripurani *et al.* 2010; Kang *et al.* 2013). The significant biological functions of these miRNAs imply that they have important roles in the female reproductive physiology of chicken.

Among the significantly differentially expressed miRNAs, gga-miR-34b and gga-miR-34c (both including 3p and 5p) exhibited a significant increase in the HP ovary compared with

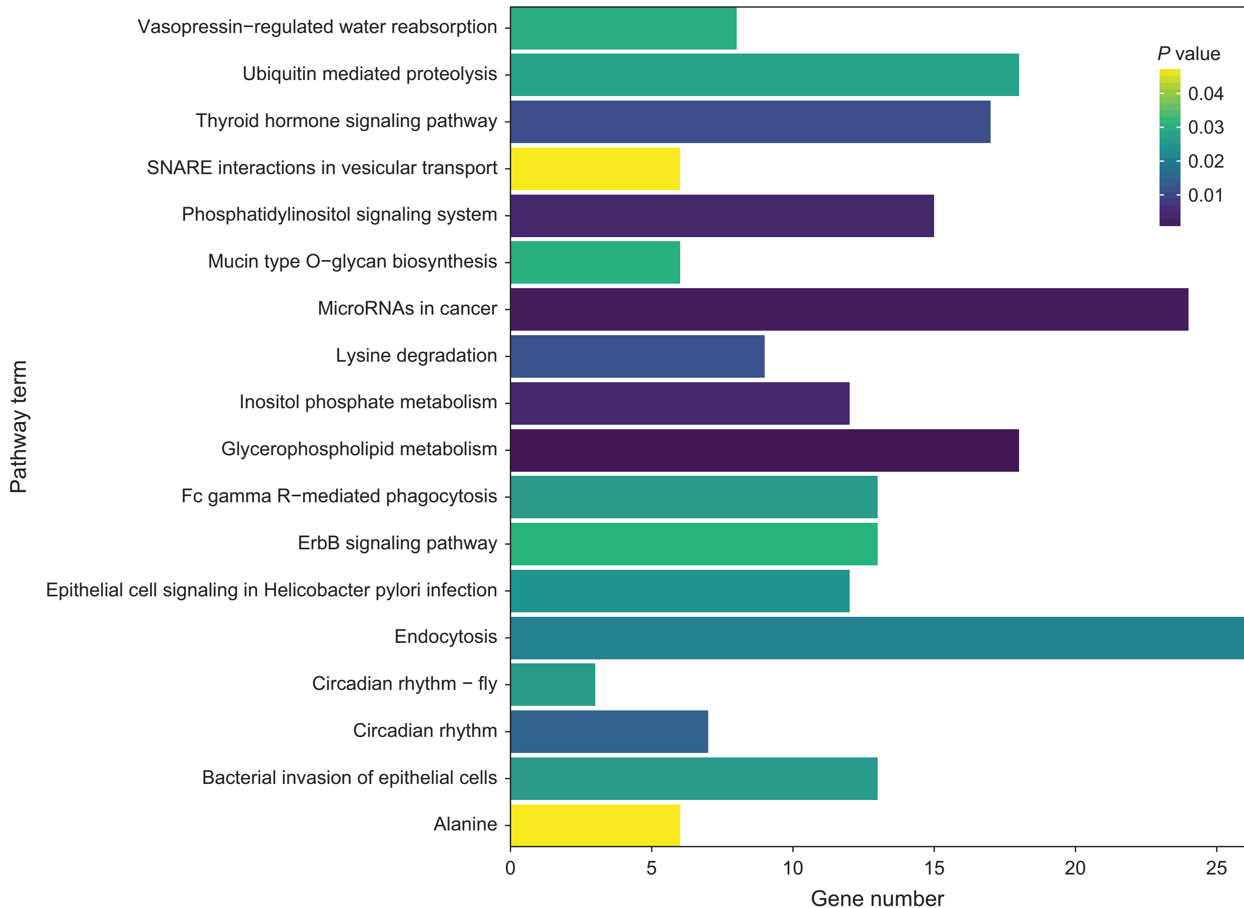

Figure 4 Top 18 pathways of predicted target genes regulated by the differentially expressed miRNAs.

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Table 2 Expression profile of eight randomly selected miRNAs.

miRNA	Fold change (high egg-laying/low egg-laying)		*P*-value	
	RNA-Seq	RT-qPCR	RNA-Seq	RT-qPCR
gga-miR-1744-3p	0.11	0.16	0.0009	0.0148
gga-miR-216b	0.15	0.03	0.0254	0.0002
gga-miR-1655-5p	0.28	0.09	0.0203	0.0049
gga-miR-99a-5p	0.88	1.06	0.6960	0.3723
gga-miR-26a-5p	0.86	0.78	0.6440	0.2766
gga-miR-34b-3p	3.34	3.49	0.0240	0.0017
gga-miR-34c-3p	3.81	3.75	0.0363	0.0026
gga-miR-200a-3p	2.46	2.34	0.0365	0.0027

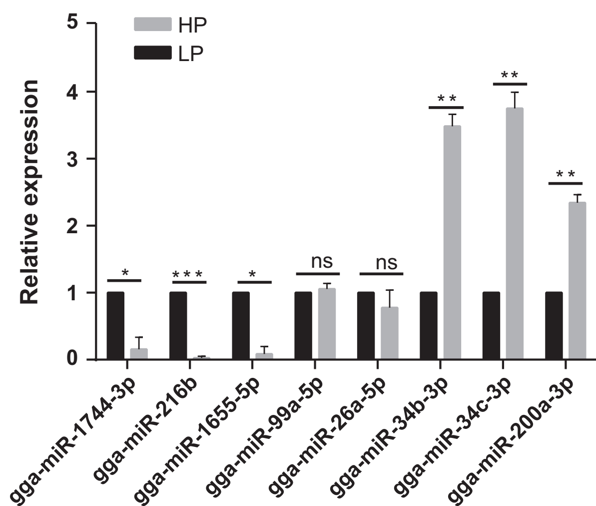

Figure 5 Validation of the miRNA expression profile by RT-qPCR. Relative expression of miRNAs was calculated according to the $2^{-\Delta\Delta Ct}$ method using 5.8S rRNA as an internal reference RNA. The error bar shows the standard deviation. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

the LP ovary. Previous studies identified the mir-34 family as a p53 target and a potential tumor suppressor for regulating processes such as proliferation, cell cycle, apoptosis and metastasis (Bommer *et al.* 2007; He *et al.* 2007a,b; Hermeking 2009). The expression of miR-34a, b and c appears to be correlated with p53 and was found in over 50% of human cancers, including pancreatic cancer (Ji *et al.* 2009), lung adenocarcinoma (Okada *et al.* 2014), breast cancer (Kato *et al.* 2009), gastric cancer (Ji *et al.* 2008), ovarian cancer (Corney *et al.* 2010) and so on. Some studies also revealed that, in *Drosophila*, miR-34 loss triggers a gene profile of accelerated brain aging, late-onset brain degeneration and a catastrophic decline in survival; miR-34 upregulation extends median lifespan and mitigates neurodegeneration induced by human pathogenic poly-glutamine disease protein; and miR-34 can regulate age-associated events and long-term brain integrity to modulate aging and neurodegeneration (Liu *et al.* 2012). In the present study, gga-miR-34b and gga-miR-34c both had a significant increase in HP ovary compared with LP ovary, and gga-miR-34b-5p had the highest level—a 13.36-fold increase—in HP ovary, implying that gga-miR-34 plays an important role in reproductive management in hens.

Previous studies reported that miR-216b attenuated nasopharyngeal carcinoma cell proliferation, invasion and tumor growth in nude mice and that it mediated its tumor suppressor function, at least in part, by suppressing downstream pathways of KRAS, such as in the PI3K-AKT and MEK-ERK pathways (Deng *et al.* 2011). Other studies also showed that miR-216b inhibits cell proliferation, migration and invasion of hepatocellular carcinoma by regulating insulin-like growth factor 2 mRNA-binding protein 2 and that it is regulated by the hepatitis B virus x protein (Liu *et al.* 2015). In the present study, gga-miR-216b was down-regulated 6.77-fold in HP ovary, suggesting that the suppressing expression of gga-miR-216b may be beneficial for the improvement of egg laying in hens.

Among the KEGG pathways, some pathways associated with endocytosis, cancer, Fc gamma R-mediated phagocytosis, snare interactions in vesicular transport, mucin type o-glycan biosynthesis, lysine degradation, ubiquitin mediated proteolysis, alanine and some signal transduction pathways, such as the phosphatidylinositol signaling system, thyroid hormone signaling pathway and ErbB signaling pathway, were all significantly enriched. This indicates a role of the differentially expressed miRNAs in the regulation of cell motility, cell proliferation, cell nutrition, nervous system development and function, communication between cells and the extracellular matrix. In addition, a small number of genes involved in the epithelial cell signaling pathways in *Helicobacter pylori* infection and bacterial invasion of epithelial cells suggests that the hens were involved in a stage of immune regulation.

We also found that specific enrichment of predicted targeted genes was involved in some reproduction-related pathways, such as steroid hormone biosynthesis, dopaminergic synapse and GnRH signaling pathways. Some of these genes have been demonstrated to play important roles in ovary development and reproductive management of hens; for example, *FSHB* produces a pituitary glycoprotein hormone, FSH, that plays a key role in the reproductive system of chicken, including steroidogenesis, folliculogenesis and follicular maturation (Choi *et al.* 2005). Moreover, we observed that all differentially expressed known miRNAs were involved in reproduction-related pathways, and biological functions of most miRNAs have not been reported. This provides us with a guideline to explore their unknown roles in reproductive management of hens. Furthermore, we uncovered a miRNA—gga-miR-200a-3p—that is ubiquitous in most reproduction-regulation-related pathways. The miR-200a family was reported to play a critical role in tumor progression and metastasis, specifically in the maintenance of the epithelial phenotype by targeting ZEB1, SIP1 and SIRT1, thus preventing the silencing of E-cadherin

(Bracken *et al.* 2008; Eades *et al.* 2011). In several mesenchymal phenotype breast cancer cell lines, the miR-200a family was found to be down-regulated (Gregory *et al.* 2008) and enforced expression of miR-200a family members prevented TGF-β-induced epithelial to mesenchymal transition (Eades *et al.* 2011). In breast cancer, in addition to targeting ZEB1 and SIP1, the miR-200 family has been shown to target phospholipase C-γ1 and BMI1, reducing EGF-driven motility and cancer stem cell self-renewal respectively (Shimono *et al.* 2009; Uhlmann *et al.* 2010). In the present study, gga-miR-200a-3p exhibited a significant 2.46-fold increase in HP ovary, implying that the miRNA may play a special essential role in ovary development and reproductive management of hens.

In previous work, genomic and transcriptomic studies have identified that some genes and miRNAs are related to egg production performance. Kang *et al.* (2009) revealed 18 known and eight unknown differentially expressed genes detected in ovarian tissues from the pre-laying to the egg-laying stages (Kang *et al.* 2009). Kang *et al.* (2013) identified 202 known miRNAs, 93 of which were found to be significantly differentially expressed in sexually immature and mature chicken ovaries. Luan *et al.* (2014) presented the transcriptomic profiles of ovarian tissue from Huoyan geese during the ceased and laying periods using RNA-Seq. In the present study, we performed the first miRNA analysis of low- and high-rate of egg production chicken ovarian tissues using high-throughput sequencing. Compared with other studies, we found some new significantly differentially expressed ovarian miRNAs, such as gga-miR-1744-3p, gga-miR-1655-5p, gga-miR-1734 and gga-miR-7465-3p, in the high egg-laying chickens. These newly identified miRNAs will be an important guideline for the future research.

Conflict of interest

All authors declared no conflict of interest exists.

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Supporting information

Additional supporting information may be found online in the supporting information tab for this article:

Figure S1 Length distribution of total small RNA in LP and HP chicken ovary libraries.

Figure S2 Small RNAs percentages of (A) HP and (B) LP chickens.

Figure S3 Cluster plots of the 17 differentially expressed miRNAs.

Figure S4 Thirty-nine differentially expressed predicted targets of 10 candidate miRNAs.

Table S1 Main reproductive traits of the selected LP and HP samples.

Table S2 Primer sequences for real-time PCR.

Table S3 Overview of miRNA-seq data of all samples.

Table S4 Identities of various small RNA sequences.

Table S5 Expression profile of known miRNAs in ovarian tissues of low and high rate of egg production chickens.

Table S6 Novel miRNAs expressed in ovarian tissues of low and high rate of egg production chickens.

Table S7 Prediction of the differentially expressed miRNA targets.

Table S8 GO and KEGG pathway annotations for the miRNA targets.

Table S9 miRNAs and their predicted target genes involved in the reproduction regulation process.

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