



Identification and abundance of miRNA in chicken hypothalamus tissue determined by Solexa sequencing

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ABSTRACT. We used Solexa sequencing technology to identify and determine the abundance of miRNAs and compared the characteristics and expression patterns of miRNA of 1-day-old and 36-week-old chicken hypothalamuses. We obtained 17,825,753 and 10,928,745 high-quality reads from 36-week-old and 1-day-old chickens, respectively. Three hundred and seventy-one conserved miRNAs were expressed in both libraries. Among the conserved miRNAs, 22 miRNAs were up-regulated and 157 miRNAs were down-regulated in the 36-week-old chicken hypothalamus tissues. The abundance of sRNAs between 1-day-old and 36-week-old chickens differed considerably. KEGG pathway analysis suggested that the target genes of highly expressed miRNAs in the chicken hypothalamus are associated with metabolism and development. This information on differential expression of miRNAs in the hypothalamus of 1-day-old and 36-week-old chickens will help us understand the molecular mechanisms of metabolism and development.

Key words: Chicken; miRNA; Solexa sequencing; Expression profiles; Hypothalamus

INTRODUCTION

MicroRNAs (miRNAs) are small, noncoding regulatory RNAs of 18-24 nt that play important roles in various biological processes, including the post-transcriptional regulation of gene expression during development. In higher eukaryotes, almost 1-3% of the genome encodes miRNA genes, and collectively, up to 30% of genes are believed to be regulated by these small pieces of RNA (Filipowicz et al., 2008). In animals, miRNAs modulate gene expression through various mechanisms, such as sequence-specific targeting of 3'-untranslated regions, exons or introns of mRNAs, or translational suppression of mRNA, and often lead to decreased mRNA stability (Eulalio et al., 2008), inhibition of translation (Zhang et al., 2006), or even upregulation of translation (Vasudevan et al., 2007). miRNAs operate highly complex regulatory networks. Current estimates indicate that each miRNA could target several hundred genes, and each gene can combine with numerous miRNAs (Krek et al., 2005). miRNAs display a spatiotemporal pattern of expression and various functions and therefore are crucial in the regulation of biological processes such as development, viral defense, metabolism, apoptosis, neuron development, DNA methylation, and chromatin modification in various stages (Reinhart et al., 2000; Ambros, 2004; Miska, 2005; Glazov et al., 2008). Studies of miRNA in mammals have suggested that many brain-associated miRNAs are expressed and have a wide range of functions. Some examples are miR-9, which regulates the precise specifications of sensory organ precursors (Li et al., 2008) and affects neural lineage differentiation in embryonic stem cell-derived cultures (Krichevsky et al., 2006), and miR-125b, which is a critical player in the regulation of cell proliferation in chicken embryos (Hicks et al., 2008). Hypothalamic neural circuits play a critical role in the integration of peripheral signals that convey information about energy and nutrient status, which are interpreted and used to modulate chicken feeding behavior and energy expenditure to maintain body weight and energy stores at set levels (Richards and Proszkowiec-Weglarz, 2007).

Many studies have demonstrated that miRNAs have a temporal or tissue-specific expression pattern, and most chicken miRNAs have been identified from whole embryos (Glazov et al., 2008; Shao et al., 2008), somites (Rathjen et al., 2009), and primary fibroblast cells (Burnside et al., 2008). However, relatively few studies of miRNA expression and function in the later stages of development of the chicken have been reported. Furthermore, the relatively small overlap in the miRNAs described by previous reports indicates that these libraries are not saturated, and specific tissues contain other unidentified miRNAs. In other words, many chicken miRNAs are waiting to be discovered. Therefore, studies to identify miRNA expression profiles and gain insight into the potential role of miRNAs during chicken hypothalamus tissue development are required. Once these studies have been carried out, the function of specific miRNAs in chicken hypothalamus tissue was determined. We constructed 2 small RNA (sRNA) libraries from the hypothalamus of 1-day-old chickens, an age at which the organism begins to grow rapidly and display increased energy demands, and 36-week-old chickens, which have stopped growing. We further compared the characteristics and expression patterns of the sRNAs of 1-day-old and 36-week-old chickens.

MATERIAL AND METHODS

Animal collection and RNA isolation

Healthy male chickens were purchased from the experimental farm of Henan Agri-

culture University (Zhengzhou, China). The hypothalamus was isolated and collected from 1-day-old and 36-week-old chicken and quickly frozen in liquid nitrogen and stored at -80°C . Total RNA from the hypothalamus tissue was isolated with Trizol (TaKaRa) according to manufacturer instructions. The total RNA was quantified using a NanoDrop 2000 spectrophotometer and assessed for quality control using an Agilent 2100 Bioanalyzer.

Construction of sRNA libraries and Solexa sequencing

Two sRNA libraries were constructed for the hypothalamus tissue from the 1-day-old and 36-week-old stages. For Solexa sequencing, 2 sets of samples were pooled: one from six 1-day-old chickens and the other from six 36-week-old chickens. From each set, 20 mg of total RNA was used for the library construction following the protocol supplied with the Small RNA Sample Prep Kit, with minor modifications. In brief, fractions (18-30 bp) were isolated from the total RNA using 15% criterion Tris-borate-ethylenediaminetetraacetic acid (TBE)-urea polyacrylamide gels (Invitrogen). Subsequently, a 5'-adaptor (GUUCAGAGUUCUACAGUC CGACGAUC) was ligated to the purified sRNAs, and further purification of the ligation products (36-50 bp) was carried out on Novex 15% TBE-urea gel. The 5'-ligation products were ligated to a 3'-adaptor (UCGUAUGCCGUCUUCUGCUUGUdT), and the final products (62-75 bp) contained both 5'- and 3'-adaptors. These ligation products were then reverse transcribed using a Superscript RT kit. The cDNA was amplified using the forward primer (5'-CAAGCAGAAGACGGCATACTGA-3') and the reverse primer (5'-AATGATACGGCGAC CACCGACAGTTTCAGAGTTCTACAGTCCGA-3') and polymerase chain reaction (PCR). The amplification products were extracted from 6% TBE-urea gel. The purified DNA fragments were used for cluster analysis and sequencing on an Illumina Genome Analyzer at BGI-Shenzhen (China). The Solexa sequences were trimmed of their adaptor sequences, and sequences longer than 30 nt and shorter than 18 nt were discarded. All high-quality sequences, even those with only a single-unique read, were processed for computational analysis.

In silico analysis of Solexa reads

Solexa clean reads were aligned against the chicken genome using the Short Oligonucleotide Alignment Program (SOAP) (Li et al., 2008). Sequences with a perfect match or 1 mismatch were retained for further analysis. To analyze the RNA secondary structure further, we extracted 100 nt of the genomic sequence flanking each side of these sequences, predicted the secondary structures using mFold 3.1 (Zuker, 2003), and analyzed them with MIREAP (<http://sourceforge.net/projects/mireap>). MIPRED was used to determine whether the sequences were pre-miRNA-like hairpin sequences. Stem-loop hairpins were considered typical only when they fulfilled 3 criteria: mature miRNAs present in 1 arm of the hairpin precursors, which lack large internal loops or bulges; secondary structures steady, with a free energy of hybridization lower than -20 kcal/mol, and location in intergenic regions or introns. Any sequence that fulfilled these criteria was considered an miRNA precursor locus in the porcine genome and against sRNAs derived from ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), small nuclear RNAs (snRNAs), and small nucleolar RNA (snoRNAs) deposited at Rfam (<http://www.sanger.ac.uk/Software/Rfam/>).

To understand the molecular function of the 12 most abundant differentially expressed

miRNAs in adipose tissue, we predicted miRNA target gene sites using the algorithms in PicTar (http://pictar.mdc-berlin.de/cgi-bin/new_PicTar Vertebrate.cgi) (Krek et al., 2005) and TargetScan (5.1) (Ulitsky et al., 2010), and the genes were classified according to KEGG functional annotations using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) bioinformatics resources (<http://david.abcc.ncifcrf.gov/>) (Dennis et al., 2003; Huang et al., 2009).

Differential expression of conserved miRNA

Expression of conserved miRNAs from the 2 sets of samples was normalized to assess significant difference. For each sample, if the normalized expression of a given miRNA (NEM) was zero, its expression value was modified to 0.01. If the NEM was lower than one read in both samples, it was removed in the following analysis of differential expression. For each miRNA, the fold change and P value were calculated from the normalized expression level using the formula shown below:

NEM = miRNA sequencing count / total count of clean reads \times 1,000,000

Fold-change formula: fold-change = \log_2 (NEM of 36-week-old / NEM of 1-day-old chickens)

P value formula:

$$p(x|y) = \binom{N_2}{N_1} \frac{(x+y)!}{x!y! \left(1 + \frac{N_2}{N_1}\right)^{(x+y+1)}} \quad \begin{aligned} C(y \leq y_{\min}|x) &= \sum_{y=0}^{y \leq y_{\min}} p(y|x) \\ D(y \geq y_{\max}|x) &= \sum_{y \geq y_{\max}}^{\infty} p(y|x) \end{aligned}$$

where N_1 and x represent the total count of clean reads and normalized expression level of a given miRNA in the sRNA libraries of 1-day-old chicken hypothalamus tissue. N_2 and y represent the total count of clean reads and normalized expression level of a given miRNA in the sRNA library of 36-week-old chicken hypothalamus tissue.

Confirmation of differentially expressed miRNAs using real-time quantitative PCR (qPCR)

To determine the expression of miRNAs, qPCR was performed using a Hairpin-it miRNAs qPCR Quantitation Kit according to manufacturer protocols (Shanghai GenePharma Co., Ltd., China). The specific stem-loop RT primers of miR-21, miR-92, and U6 were obtained commercially from a Hairpin-it miRNAs qPCR Quantitation Kit. cDNA was synthesized from total RNA using various gene-specific primers according to the protocol for the qPCR assay (Shanghai GenePharma Co., Ltd.). The reaction volume contained 2 μ L 500 ng/ μ L total RNA, 1.2 μ L 1 μ M specific stem-loop RT primers, 2 μ L 5X RT buffer, 0.2 μ L 200 U/ μ L Reverse Transcriptase (TaKaRa). The mixture was incubated for 30 min at 42°C and for 5 s at 85°C and then held at 4°C. Real-time PCR amplification was performed in a 20- μ L reaction mixture that contained 10 μ L SYBR Green PCR master mix, 0.4 μ L 5 μ M miR-specific primer set, 0.2 μ L 5 U/ μ L Taq DNA polymerase, 2 μ L RT product, and 7.4 μ L double-distilled H₂O. The mixture was incubated at 95°C for 3 min. This incubation was followed by 40 cycles at

94°C for 15 s and 62°C for 40 s. All of the samples were analyzed in triplicate, and the average value was used for quantification. The expression levels of miR-21, miR-92, and U6 in each sample were measured in terms of threshold cycle value. The data were analyzed using the relative quantification ($2^{-\Delta\Delta C_t}$) method. U6 was used as internal standard to rectify miRNA expression in the hypothalamus tissue of 1-day-old chickens.

RESULTS

Solexa sequencing of sRNA from chicken hypothalamus tissue

To identify and quantify miRNA in chicken hypothalamus tissue, we used Solexa sequencing. First, we obtained 17,825,753 high-quality reads from the 36-week-old chicken hypothalamus library and 10,928,745 high-quality reads from the 1-day-old chicken hypothalamus library. The low-quality reads were removed according to the quality value of the bases. The adaptor sequence was trimmed at the 3'-terminus, the 5'-adaptor contaminants formed by ligation were cleaned, and reads containing one or more N or runs of 8 or more adenines were removed. A total of 16,414,456 (corresponding to 1,613,639 unique sequences) and 10,657,965 (corresponding to 471,567 unique sequences) high-quality reads 18-30 nt long were obtained from the 36-week-old and 1-day-old chicken hypothalamus libraries, respectively. Next, high-quality reads were mapped to the chicken genome using the SOAP program. Allowing no mismatches and considering only the sequences represented by at least 2 reads, we obtained a total of 11,820,292 (corresponding to 701,109 unique sequences) and 87,92,656 (corresponding to 286,368 unique sequences) sequence reads that could be mapped to the chicken genome for the 36-week-old and 1-day-old chicken hypothalamus libraries, respectively. These unique sequences contained 94,642 common sequences, 376,925 sequences specific to the 36-week-old chickens and 1,518,997 sequences specific to the 1-day-old chickens (Table 1).

Table 1. Summary of common sequences and specific sequences between 1-day-old and 36-week-old chickens.

Category	Unique sRNA	%	Total sRNA	%
Total sRNAs	1,990,564	100.00%	27,072,421	100.00%
Common sRNAs	94,642	4.75%	24,239,664	89.54%
1-day-specific sRNAs	376,925	18.94%	451,002	1.67%
36-week-specific sRNAs	1,518,997	76.31%	2,381,755	8.80%

No significant difference was found in the number of RNAs with high-quality reads between the 2 libraries, and the majority of reads had lengths of 22-23 nt. For example, in the library from 1-day-old chickens, almost half of the high-quality reads (44.35%) were 22 nt long, followed by 23 nt (26.37%); however, in the library from 36-week-old chickens, the length distribution peaked at 23 nt (23.06%), followed by 22 nt (20.24%; Figure 1). All high-quality reads (at least 18 nt long) were divided into categories according to their biogenesis and annotation (Figure 2A and B). Of the reads in the 36-week-old chicken library, 27.81, 3.37, 0.29, and 1.16% were fragments of rRNA, tRNA, snRNA, and snoRNA, respectively. By contrast, in the 1-day-old chicken library, 6.34, 1.06, 0.08, and 0.32% were fragments of rRNA, tRNA, snRNA, and snoRNA, respectively. In the 1-day-old and 36-week-old chicken libraries, 74.44 and 29.25%, respectively, were annotated to the conserved miRNAs. These results indicate that the proportions of sRNA types in the 2 libraries were different.

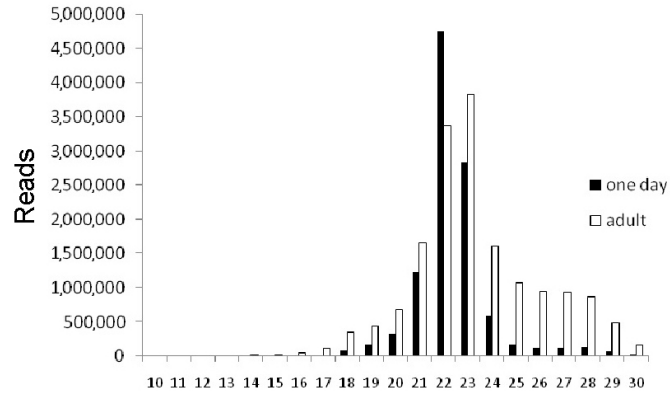


Figure 1. Size histogram of sequence short RNAs in the dataset.

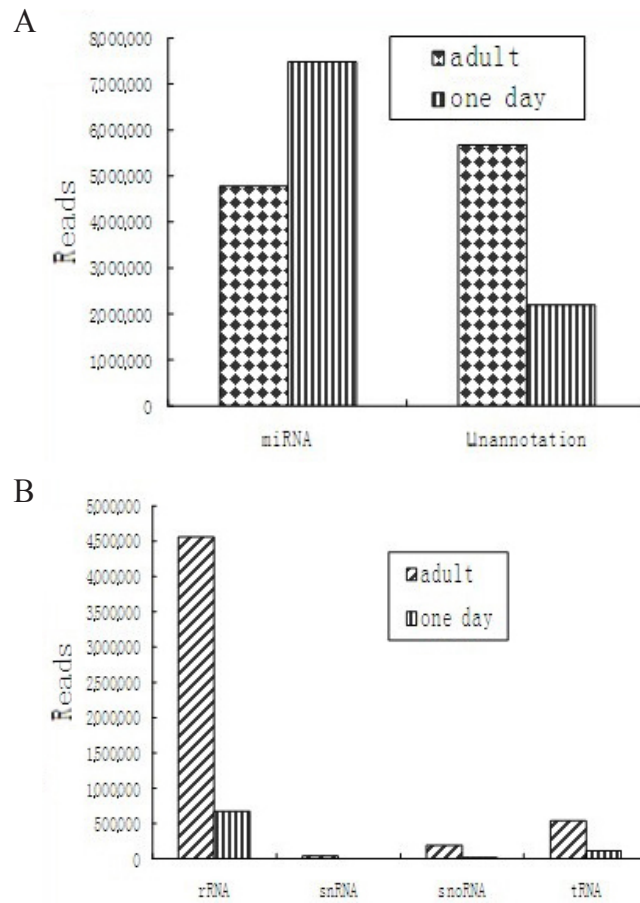


Figure 2. Different expression levels of sRNAs between 1-day-old and adult chickens. **A.** Reads different of miRNA and unannotated sequences in two libraries. **B.** Reads different of ribosomal RNA (rRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), and transfer RNA (tRNA) sequences in two libraries.

Computational analysis of conserved miRNAs

To assess the efficiency of the Solexa sequencing technology for miRNA detection, high-quality reads of sRNA sequences were used for a Basic Local Alignment Search Tool (BLAST) search against the miRBase database of miRNA sequences (release 14.0; <http://microrna.sanger.ac.uk/sequences/>). We found 371 conserved miRNAs in the 36-week-old and 1-day-old chicken libraries. There were 265 miRNAs that overlapped in the libraries, and 82 and 24 miRNAs were found to be expressed specifically in 1-day-old and 36-week-old chicken hypothalamus tissues, respectively. However, some of the other miRNAs were absent in the 2 libraries, which suggests that miRNA expression is specific to the phase or tissue. Interestingly, the conserved chicken miRNA reads of 1-day-old chickens were much more numerous than those of the 36-week-old chickens, which implies that the library from 1-day-old birds contained more mature miRNA.

In animals, miRNAs can be grouped as 1 cluster on parts of the same chromosome. The clustered miRNAs may have overlapping functions in regulating a similar set of genes, such as miR-17-92 in mice. The 371 expressed miRNAs identified in this study were classified into groups according to their genomic locations on chromosomes (inter-miRNA distance <10 kb) based on the results of miRBase 14.0 analysis. In total, 104 of the identified miRNAs were found to be in close proximity in 36 clusters (Table 2). Each cluster contained at least 2 miRNAs. Of 38 miRNA gene clusters, 37 were detected in 1-day-old chicken hypothalamus tissue and 32 were detected in the tissue from 36-week-old birds (see Table 2). These clusters were distributed across 19 chicken chromosomes, with an average of 2.5 miRNA genes per cluster. Cluster were the biggest clusters containing 6 miRNAs located on chromosome 1. Such clustered miRNAs can be transcribed together as polycistronic transcripts with similar expression patterns, and they may be functionally related (Li et al., 2011). Only 4 clusters appeared to be downregulated in the 1-day-old and 36-week-old chickens.

Expression analysis of conserved miRNAs

The number of reads for individual miRNAs in the 2 libraries generally reflected their relative abundance and expression level. Some of the miRNAs identified had more than 1000 reads, showing that our sRNA libraries were highly enriched with mature miRNAs. Others had fewer than 10 reads. Although these conserved miRNAs were sequenced at varying frequencies, some miRNAs dominated the miRNA library (Table 3). For example, in the 1-day-old chicken hypothalamus tissue library, the most abundant miRNA was let-7a, corresponding to 10.54% of all clean reads. The next most abundant miRNAs were let-7j and miR-9, with 10.50 and 9.0% of all clean reads, respectively. In the 36-week-old chicken hypothalamus tissue library, the most abundant miRNA was miR-125b, corresponding to 7.20% of all clean reads.

The miRNA reads from the 2 libraries were sequenced from high to low. We found that 16 miRNAs—miR-125b, miR-181a, miR-30d, miR-103, let-7a, let-7j, miR-140*, miR-100, let-7c, let-7bk, let-7k, miR-146c, miR-9, let-7f, miR-9*, miR-21 were coexpressed in 20 of the highly expressed miRNAs between the 2 libraries. In the 1-day-old chicken hypothalamus tissue library, the 16 most abundant miRNA reads constituted 68.75% of

the total clean reads, but in the 36-week-old chicken hypothalamus library, the 16 most abundant miRNA reads constituted 27.93% of the total clean reads. These results indicate that many chicken miRNAs are waiting to be discovered in 36-week-old chicken hypothalamus tissue.

Table 2. Clusters of the known miRNA genes.

Clusters No.	Precursor ID	Chromosome	Clusters No.	Precursor ID	Chromosome
1	gga-mir-17	1	18	gga-mir-130c	19
	gga-mir-18a	1		gga-mir-301b	19
	gga-mir-19a	1	19	gga-mir-124a	2
	gga-mir-20a	1		gga-mir-1714	2
	gga-mir-19b	1		gga-mir-30d	2
2	gga-mir-92	1	20	gga-mir-30b	2
	gga-mir-130a	15		gga-mir-1b	23
	gga-mir-301	15	21	gga-mir-133c	23
	gga-mir-454	15		gga-mir-30e	23
	gga-mir-130b	15		gga-mir-30c-1	23
3	gga-mir-216c	3	23	gga-mir-100	24
	gga-mir-216b	3		gga-let-7a-2	24
	gga-mir-216	3	24	gga-mir-34b	24
	gga-mir-217	3		gga-mir-34c	24
4	gga-mir-222-1	1	25	gga-let-7k	26
	gga-mir-222-2	1		gga-let-7j	26
	gga-mir-221	1	26	gga-mir-29b-2	26
gga-mir-3529	10	gga-mir-29c		26	
5	gga-mir-7-2	10	27	gga-mir-15c	4
	gga-mir-1720	10		gga-mir-16c	4
	gga-let-7a-1	12	28	gga-mir-10b	7
gga-let-7f	12	gga-mir-1713		7	
gga-let-7d	12	29		gga-mir-375	7
gga-mir-1a-1	20		gga-mir-1788	7	
7	gga-mir-1773	20	30	gga-mir-181a-1	8
	gga-mir-133a-2	20		gga-mir-181b-1	8
	gga-mir-200b	21	31	gga-mir-181a-2	17
gga-mir-200a	21	gga-mir-181b-2		17	
8	gga-mir-429	21	32	gga-mir-15b	9
	gga-mir-106	4		gga-mir-16-2	9
	gga-mir-18b	4	33	gga-mir-194	3
gga-mir-20b	4	gga-mir-215		3	
9	gga-mir-23b	Z	34	gga-mir-206	3
	gga-mir-27b	Z		gga-mir-133b	3
	gga-mir-24	Z		gga-mir-1727-1	1
10	gga-mir-29b-1	1	35	gga-mir-1727-2	1
	gga-mir-29a	1		gga-mir-219	17
11	gga-let-7a-3	1	36	gga-mir-2964	17
	gga-let-7b	1		gga-mir-196-2	2
12	gga-mir-99a	1	37	gga-mir-1732	2
	gga-let-7c	1		gga-mir-449c	Z
13	gga-mir-15a	1	38	gga-mir-449b	Z
	gga-mir-16-1	1		gga-mir-449	Z
14	gga-mir-1574	10	38	gga-mir-302b	4
	gga-mir-204-2	10		gga-mir-302c	4
15	gga-mir-193b	14	38	gga-mir-1811	4
	gga-mir-365-1	14		gga-mir-302a	4
16	gga-mir-144	19	38	gga-mir-302d	4
	gga-mir-451	19		gga-mir-367	4

Table 3. The most abundant expression of miRNA between two library.

miRNA	1 day		36 weeks	
	Reads	% ^a	Reads	% ^a
gga-miR-125b	101,643	0.9537	1,182,594	7.2046
gga-miR-181a	94,438	0.8861	653,932	3.9839
gga-miR-30d	290,848	2.7289	574,225	3.4983
gga-miR-103	233,389	2.1898	420,957	2.5646
gga-let-7a	1,123,369	10.5402	354,659	2.1607
gga-let-7j	1,118,819	10.4975	351,372	2.1406
gga-miR-140*	83,021	0.7790	203,814	1.2417
gga-miR-100	173,405	1.6270	174,838	1.0651
gga-let-7c	550,941	5.1693	133,123	0.8110
gga-let-7b	494,703	4.6416	113,300	0.6902
gga-let-7k	597,346	5.6047	100,481	0.6121
gga-miR-146c	241,815	2.2689	97,067	0.5914
gga-miR-9	959,746	9.0050	94,669	0.5767
gga-let-7f	528,745	4.9610	80,291	0.4891
gga-miR-9*	471,123	4.4204	25,751	0.1569
gga-miR-21	263,778	2.4749	22,651	0.1380
合计	7,327,129	68.748	4,583,724	
27.9249				

^a% = miRNA reads/total clean reads.

Expression levels of conserved miRNAs from the 2 types of samples were normalized to determine whether a significant difference existed. We analyzed 371 conserved miRNAs, if the normalized expression of an miRNA was zero, its expression value was modified to 0.01. If the normalized expression of an miRNA was lower than 1 read in both samples, it was removed from subsequent differential expression analyses. The results revealed significant differences in the level of expression of conserved miRNA between the 1-day-old and the 36-week-old phases. The majority of miRNA showed more than a 1-fold difference between the 2 libraries. We found 179 miRNAs that had significant differences in expression between the 2 libraries (174 miRNA had more than a 1-fold difference, $P < 0.01$; 5 miRNAs, $0.01 < P < 0.05$; Table S1); 157 of them appeared to be downregulated from the 1-day-old to the 36-week-old chickens, whereas 22 seemed to be upregulated in the same order. Forty-two miRNAs displayed similar levels in both libraries, including miR-103, miR-30d and so on (Table S2).

Confirmation of differentially expressed miRNAs

We used stem-loop quantitative RT-PCR to confirm the expression pattern of differentially expressed miRNAs in chicken hypothalamus. The changed directions of differentially expressed miRNAs between the qPCR and Solexa sequence analyses in 2 miRNAs (miR-21 and miR-92) were generally consistent (Figure 3), although the expressed miRNAs levels were different between qPCR and Solexa sequence analyses.

miRNA target prediction and KEGG pathway analysis

Modulation of the target genes of miRNAs is one of the most critical steps in the regulation of gene expression. To understand the primary functions of the differentially expressed

miRNAs in the 2 libraries, we chose 12 miRNAs with various levels of expression, more than 1000 reads, and a fold change of >2 (excluding let-7) from the 2 hypothalamus tissue libraries (Table S3). The target gene of each differentially expressed miRNA was predicted using the algorithms in PicTar (Krek et al., 2005) and TargetScan (Ulitsky et al., 2010). The predicted target genes were used to identify gene ontology biological terms and for KEGG pathway analysis using the DAVID tool. The results showed that 2253 unique target gene sites were predicted by TargetScan and 3315 target sites by PicTar, which corresponded to 1394 genes. The 973 target genes at the intersection of these 2 algorithms were used for KEGG functional analysis. In our study, these 12 miRNAs predicted 973 target genes, which suggests that each of these miRNAs has more than one predicted target gene and that some have more than 500 target genes. Similarly, some unigenes have more than one miRNA target site.

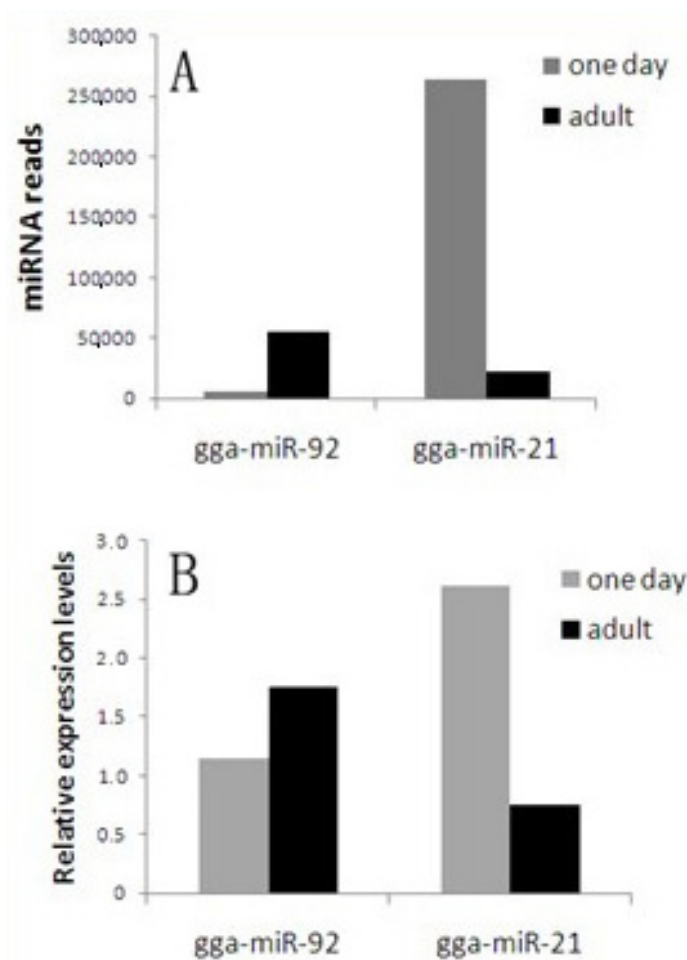


Figure 3. Histogram of the miR-21, miR-92 expression by deep sequence and qPCR. **A.** Differentially expressed miRNA by deep sequence. **B.** Differentially relative expressed miRNA by real-time RT-PCR. miRNA expression in hypothalamus of 1-day-old chicken as rectify. Results of the mean of qRT-PCR from 3 repeats of each sample were presented and results of the mean of histogram qPCR assays without standard deviation were showed.

These 973 genes were classified into 33 pathways according to KEGG functional annotations using the DAVID program. These pathways in biological processes were enriched significantly ($P < 1E$; see Table 4). Long-term potentiation was identified as the KEGG pathway with the highest fold enrichment (3.91-fold). Many of these pathways are related to growth and development, including the mitogen-activated protein kinase (MAPK) signaling pathway, Wnt signaling pathway, and ubiquitin-mediated proteolysis. Most miRNA targets belong to the MAPK signaling pathway. Some pathways - for example, the MAPK signaling pathway (39 genes), pathways in cancer (35 genes), regulation of actin cytoskeleton (29 genes), focal adhesion (26 genes), Wnt signaling pathway (25 genes), neurotrophin signaling pathway (22 genes), ubiquitin-mediated proteolysis (22 genes), axon guidance (21 genes), and the calcium signaling pathway (21 genes) - contained more than 20 of the identified genes.

Table 4. KEGG pathways enriched for targets of the 12 most abundant miRNAs expressed in chicken hypothalamus.

Term	Count	P	Fold enrichment	Bonferroni
MAPK signaling pathway	39	3.27E-07	2.427297	4.31E-05
Long-term potentiation	16	8.09E-06	3.910035	0.001067
Wnt signaling pathway	25	8.32E-06	2.751266	0.001097
Neurotrophin signaling pathway	22	1.14E-05	2.948292	0.001503
Ubiquitin-mediated proteolysis	22	5.40E-05	2.668527	0.007104
Regulation of actin cytoskeleton	29	6.77E-05	2.24145	0.008898
Axon guidance	21	6.91E-05	2.705198	0.00908
Oocyte meiosis	19	7.79E-05	2.870321	0.010232

MAPK = mitogen-activated protein kinase.

DISCUSSION

Solexa sequencing is a high-throughput technology; it can identify sequences of up to 35 bp and yields 1-3 million reads/sample (Rathjen et al., 2009). Solexa sequencing is considered by some to be more powerful than other conventional technologies, which was our reason for selecting Solexa sequencing in the present study. We obtained 17,825,753 high-quality reads from 36-week-old chicken hypothalamus tissue and 10,928,745 high-quality reads from 1-day-old chicken hypothalamus tissue, respectively. The proportions of sRNA types in the 2 libraries were different. The 2 stages shared the same genome but exhibited different gene expression profiles, which suggested differential regulation of gene expression (Kang et al., 2004), and our study showed that miRNAs exert potentially different functions at different stages of development.

Solexa sequencing can be performed on any tissue/organ without prior information about sequences or secondary structure (Dostie et al., 2003). It can also detect miRNAs with very low copy numbers, which reflect either a low expression level in the tissue or a contribution from minor cell types such as blood or adipose or connective tissues in the biopsy specimen (Nielsen et al., 2010). Further experiments are in progress to identify novel miRNAs among the candidates reported herein. The expression of miRNAs in the 2 samples were normalized to obtain the expression of transcripts per million. If the normalized expression of a given miRNA was less than 1 in both samples, it was removed from future differential expression analysis. We found 179 miRNAs that had significant differences in expression between the 2 libraries.

Among the highly abundant miRNAs, some such as miR-9, miR-125b, and miR-21 reportedly have important modulation functions in nerve cell growth and physiological activity (Lagos-Quintana et al., 2002; Dostie et al., 2003) - for example, miR-9, which regulates the precise specification of sensory organ precursors (Li et al., 2008) and affects neural lineage differentiation in embryonic stem cell-derived cultures (Krichevsky et al., 2006), and miR-125b, which is a critical player in the regulation of cell proliferation in chicken embryos (Hicks et al., 2008). The miR-21 and miR-148a miRNAs are overexpressed in a wide variety of cancers and have been causally linked to cellular proliferation, apoptosis, and neuron migration (Chan et al., 2005). Our study uncovered 179 miRNAs that had significant differences in expression between the 2 libraries, and miR-9, miR-125b, miR-181a, and so on were expressed abundantly, although no report has described their functions. Such differences in expression may imply a functional role in the phase translation of the chicken, and we may find new approaches to control the feeding and energy metabolism of chickens through further study of these miRNAs. The similar levels of some miRNAs-miR-103 and miR-30d, for example - in the 2 libraries suggested that miRNAs with ubiquitous expression at developing embryonic stages and in the 36-week-old chickens may be involved in the regulation of more general biological processes (McDanel et al., 2009).

miRNAs have roles in regulating signaling networks, such as the MAPK signaling pathway, that are more important than those in cellular processes (Cui et al., 2006). The MAPK signaling pathway, which is a highly conserved energy sensor, regulates diverse cellular programs including growth, muscle, apoptotic responses, and apoptosis (Nielsen et al., 2010). The MAPK pathway is also related to other signaling pathways, such as 5'-adenosine monophosphate-activated protein kinase, Wnt, cell cycle, apoptosis signaling pathways, and others (Raman et al., 2007), which suggests that the genes involved in cell cycle progression and cell proliferation are targeted by miRNAs. Another pathway identified was ubiquitin-mediated proteolysis, which plays important roles in many cellular processes (Scheffner et al., 1993). The data also highlighted pathways associated with Wnt signaling, which are conserved throughout evolution and regulate cell proliferation, morphology, motility, and fate during embryonic development. The majority of the enriched KEGG pathways identified were also found to be overrepresented in a previous study of differentially expressed miRNA (Nielsen et al., 2010).

The above pathway analyses illustrate some of the possible roles of highly expressed miRNAs in the metabolism and development of the chicken. Based on the above analysis, the differential expression of miRNAs in the hypothalamus of 1-day-old and 36-week-old chickens might be useful in further analyses of the molecular mechanisms of metabolism and development.

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Supplementary material

REFERENCES

Ambros V (2004). The functions of animal microRNAs. *Nature* 431: 350-355.

- Burnside J, Ouyang M, Anderson A, Bernberg E, et al. (2008). Deep sequencing of chicken microRNAs. *BMC Genomics* 9: 185.
- Chan JA, Krichevsky AM and Kosik KS (2005). MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. *Cancer Res.* 65: 6029-6033.
- Cui Q, Yu Z, Purisima EO and Wang E (2006). Principles of microRNA regulation of a human cellular signaling network. *Mol. Syst. Biol.* 2: 46.
- Dennis G Jr, Sherman BT, Hosack DA, Yang J, et al. (2003). DAVID: database for annotation, visualization, and integrated discovery. *Genome Biol.* 4: 3.
- Dostie J, Mourelatos Z, Yang M, Sharma A, et al. (2003). Numerous microRNPs in neuronal cells containing novel microRNAs. *RNA* 9: 180-186.
- Eulalio A, Huntzinger E and Izaurralde E (2008). GW182 interaction with Argonaute is essential for miRNA-mediated translational repression and mRNA decay. *Nat. Struct. Mol. Biol.* 15: 346-353.
- Filipowicz W, Bhattacharyya SN and Sonenberg N (2008). Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat. Rev. Genet.* 9: 102-114.
- Glazov EA, Cottee PA, Barris WC, Moore RJ, et al. (2008). A microRNA catalog of the developing chicken embryo identified by a deep sequencing approach. *Genome Res.* 18: 957-964.
- Hicks JA, Tembhurne P and Liu HC (2008). MicroRNA expression in chicken embryos. *Poult. Sci.* 87: 2335-2343.
- Huang da W, Sherman BT and Lempicki RA (2009). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* 4: 44-57.
- Kang L, Chen X, Zhou Y, Liu B, et al. (2004). The analysis of large-scale gene expression correlated to the phase changes of the migratory locust. *Proc. Natl. Acad. Sci. U. S. A.* 101: 17611-17615.
- Krek A, Grun D, Poy MN, Wolf R, et al. (2005). Combinatorial microRNA target predictions. *Nat. Genet.* 37: 495-500.
- Krichevsky AM, Sonntag KC, Isacson O and Kosik KS (2006). Specific microRNAs modulate embryonic stem cell-derived neurogenesis. *Stem Cells* 24: 857-864.
- Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, et al. (2002). Identification of tissue-specific microRNAs from mouse. *Curr. Biol.* 12: 735-739.
- Li G, Li Y, Li X, Ning X, et al. (2011). MicroRNA identity and abundance in developing swine adipose tissue as determined by Solexa sequencing. *J. Cell Biochem.* 112: 1318-1328.
- Li R, Li Y, Kristiansen K and Wang J (2008). SOAP: short oligonucleotide alignment program. *Bioinformatics* 24: 713-714.
- McDanel TG (2009). MicroRNA: mechanism of gene regulation and application to livestock. *J. Anim. Sci.* 87: E21-E28.
- Miska EA (2005). How microRNAs control cell division, differentiation and death. *Curr. Opin. Genet. Dev.* 15: 563-568.
- Nielsen M, Hansen JH, Hedegaard J, Nielsen RO, et al. (2010). MicroRNA identity and abundance in porcine skeletal muscles determined by deep sequencing. *Anim. Genet.* 41: 159-168.
- Raman M, Chen W and Cobb MH (2007). Differential regulation and properties of MAPKs. *Oncogene* 26: 3100-3112.
- Rathjen T, Pais H, Sweetman D, Moulton V, et al. (2009). High throughput sequencing of microRNAs in chicken somites. *FEBS Lett.* 583: 1422-1426.
- Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, et al. (2000). The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature* 403: 901-906.
- Richards MP and Proszkowiec-Weglarz M (2007). Mechanisms regulating feed intake, energy expenditure, and body weight in poultry. *Poult. Sci.* 86: 1478-1490.
- Scheffner M, Huibregtse JM, Vierstra RD and Howley PM (1993). The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. *Cell* 75: 495-505.
- Shao P, Zhou H, Xiao ZD, He JH, et al. (2008). Identification of novel chicken microRNAs and analysis of their genomic organization. *Gene* 418: 34-40.
- Ulitsky I, Laurent LC and Shamir R (2010). Towards computational prediction of microRNA function and activity. *Nucleic Acids Res.* 38: e160.
- Vasudevan S, Tong Y and Steitz JA (2007). Switching from repression to activation: microRNAs can up-regulate translation. *Science* 318: 1931-1934.
- Zhang B, Pan X, Cobb GP and Anderson TA (2006). Plant microRNA: a small regulatory molecule with big impact. *Dev. Biol.* 289: 3-16.
- Zuker M (2003). Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res.* 31: 3406-3415.