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Solexa Sequencing Analysis of Chicken Pre-Adipocyte MicroRNAs

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MicroRNAs (miRNAs) are small non-coding RNAs that play important roles in a variety of biological processes. Studies of miRNAs in mammals suggest that many are involved in lipid metabolism and adipocyte differentiation, but little is known about miRNA expression profiles during chicken adipogenesis. In this study, the Solexa sequencing approach was used to sequence a small RNA library prepared from Arbor Acres broiler pre-adipocytes, and more than 10^6 short sequence reads were obtained. From these, 159 known chicken miRNAs and 63 novel miRNAs were identified using a bioinformatics approach. Fifty-nine of these miRNA genes were further organized into 27 compact miRNA genomic clusters, and 34 new chicken mirtrons were also discovered, among which there were 27 mirtron candidates. These findings should serve as a foundation for future research on the functional roles of miRNAs in chicken adipocyte differentiation.

Key words: Solexa sequencing; chicken; pre-adipocyte; microRNA (miRNA)

MiRNAs regulate gene expression post-transcriptionally by binding to specific sequences in the 3′ UTRs of target mRNAs.[1] The first two miRNAs to be discovered, lin-4 and let-7 in *Caenorhabditis elegans*, were reported to play important roles in controlling the timing of embryo development.[2,3] Since the discovery of miRNAs, their importance has been understood and research efforts have been targeted to identifying more miRNAs in different organisms. The number of miRNAs in the miRBase (http://www.mirbase.org/cgi-bin/browse.pl) has increased rapidly in recent years. Many miRNAs have been identified throughout the genomes of various organisms, and elucidation of the precise function of miRNAs has been the subject of much research.

In mammals, many miRNAs have been reported to be associated with adipogenesis and lipid metabolism. The expression level of miR-143 is increased in differentiating human white adipocytes, and inhibition of miR-143 suppresses adipocyte differentiation.[4] In 3T3-L1 adipogenesis miR-143 is also upregulated, as are the miR-17-92 cluster and let-7 miRNAs. Overexpression of the miR-17-92 cluster accelerates adipogenic differentiation of 3T3-L1 cells. By contrast, overexpression of let-7 miRNA inhibits clonal expansion and terminal differentiation of 3T3-L1 cells.[5–7] The miR-27 gene family, including miR-27a and miR-27b, is downregulated during 3T3-L1 adipocyte differentiation, and overexpression of miR-27a or miR-27b inhibits adipocyte differentiation of 3T3-L1 cells.[8]

Adipose tissue plays a crucial role in energy reservation and has been identified as an endocrine organ that secrets various biologically active substances.[5,9,10] Excessive accumulation of lipids in the adipose tissue of broilers can reduce animal carcass yields and feed efficiency, and cause difficulties in dealing with these fats and subsequently bring about environment pollution. Excessive accumulation of lipids in the abdominal adipose tissue is a big challenge for the broiler industry, and broilers are an important global food source. Strategies to deal with this problem have become a focal point for breeders and researchers. MiRNAs play an important role in adipocyte differentiation and might therefore provide new strategies for dealing with the excessive accumulation of lipids in adipose tissue. However, to date, miRNA expression profiles during chicken adipocyte differentiation have not been reported.

Deep sequencing technology has been used to identify the miRNA expression profiles of chicken embryos and immune organs.[11–13] To date, there is little information on the expression profile of a single type of cell in chickens, except for chicken embryo fibroblasts,[14] and in particular pre-adipocytes. In this study, we used the Solexa sequencing approach to identify the miRNA expression profile in chicken pre-adipocytes. Our findings should provide a basis for future research on the role of miRNA in chicken pre-adipocyte growth and development.

Experimental

Culture of chicken primary preadipocytes. Chicken preadipocytes were cultured by previously described methods,[15,16] with a few modifications. Briefly, abdominal adipose tissue was excised from a total of 10 11-d-old male AA broilers, then digested in 2 mg/mL of collagenase type I (Invitrogen, Carlsbad, CA) for 65 min at 37 °C with shaking. Digestion was followed by filtration through a 20-μm screen and centrifugation at 300 *g* for 10 min at room temperature. The pellet (including preadipocytes) was suspended and seeded at a density of 1×10^5 cells/cm^2 in the medium and maintained at 37 °C in a humidified, 5% CO_2 atmosphere until confluence (3 to 4 d). Cells were passaged once and harvested when confluent.

Small RNA library construction and sequencing. Total RNA was prepared using Trizol reagent (Invitrogen) following the manufactur-

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Abbreviations: miRNA, microRNA; nt, nucleotides; snoRNA, small nucleolar RNA; UTR, untranslated regions

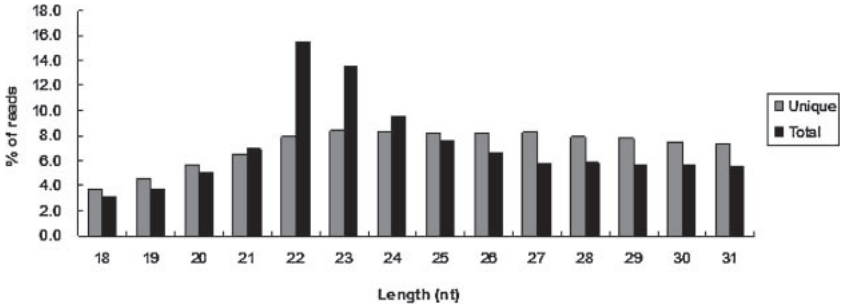

Fig. 1. Distribution of the Reads Length.
The lengths of all the reads detected in chicken pre-adipocytes are labeled on the *x*-axis, and the percentage of reads is labeled on the *y*-axis. For reads of the same length, percentages in all unique reads are shown by gray bars, and percentages in all reads are shown by black bars.

Table 1. Overview of Sequencing Reads

Class	No. of unique reads	%	No. of total reads	%
Reads in total	348,832		1,147,787	
Matched perfectly to chicken genome	121,946	35.0[1]	572,490	49.9[2]
Matched to known *G. gallus* pre-miRNAs	1,042	0.9[3]	148,644	26.0[4]
No matches to known *G. gallus* pre-miRNAs	120,904	99.1[3]	423,846	74.0[4]
Total potential reads (except for other non-coding RNAs)	59,195	48.5[3]	272,201	47.5[4]

[1]Percentage in total unique reads; [2]percentage in total reads; [3]percentage in unique reads that matched perfectly to chicken genome; [4]percentage in total reads that matched perfectly to chicken genome.

er's instructions. The concentration and purity of the total RNA was determined by measuring the absorbance at 260 nm and the A_{260}/A_{280} ratio. RNA samples were stored at $-80\,^{\circ}C$ until further use.

Small RNAs, ranging from 18–31 nt, were enriched by denaturing polyacrylamide gel electrophoresis, and ligated to 5′ and 3′ proprietary adapters. The ligation product was reverse-transcribed into cDNA, which was then amplified by 18 PCR cycles and subjected to Illumina's Solexa proprietary sequencing-by-synthesis method.

Analysis of sequencing data. After removal all the repeated reads and the adaptor sequences, the sequencing results were mapped onto the *G. gallus* genome within Ensembl (http://www.ensembl.org) using SOAP.[17] Perfectly matching reads were then mapped onto the *G. gallus* miRNA precursor of the Sanger miRBase (release 15.0)[18] to identify known *G. gallus* miRNAs. The reads which could not be matched to known chicken miRNA precursors were searched against the metazoan mature miRNAs of the Sanger miRBase (15.0)[18] using the program Patscan[19] to identify conserved miRNAs. Two mismatches are permitted in the identification of homologs of known miRNAs. To identify degenerated fragments of mRNA or other non-coding RNAs, such as rRNA, tRNA, and snoRNA, unique reads were screened against *G. gallus* non-coding RNA (except miRNA), and the predicted genes within the Ensembl genome. Unique reads that had over 20 perfect matches on the genome were also removed.[20]

To analyze further the RNA secondary structure of the remaining reads, the software Einverted of Emboss was used to find inverted repeats (step loops or hairpin structures).[21] The secondary structures of these inverted repeats were predicted using RNAfold[22] and evaluated by MirCheck.[23] Non-conserved unique reads that were sequenced only once were removed. Reads that passed MirCheck[23] were considered new miRNAs, and their predicted precursors were considered new miRNA genes.

Results

Overview of sequencing results

To identify miRNAs in chicken pre-adipocytes, a small RNA library from Arbor Acres broiler pre-adipocytes was sequenced using the Solexa sequencing approach. A total of 1,147,787 high-quality reads were obtained. After removal of all of the repeated sequences, a total of 348,832 unique clean reads, ranging from 18 to 31 nucleotides (nt) in length, were obtained. The length distribution peaked at 22 and 23 nt (Fig. 1), consistently with that commonly expected for miRNAs. About half of the total reads (49.9%) perfectly matched the chicken genome, and 26% of these matched known chicken miRNAs (Table 1). These results indicate that many known chicken miRNAs exist in our small RNA library. However, when we considered the number of unique reads, we found that the 26% of all the reads represented only a small percentage (0.9%) of the unique reads, and about half of the unique reads (48.5%) were unclassified small RNAs. Hence there might be many unidentified miRNAs or other types of small regulatory RNAs in our library.

Known chicken miRNAs

We mapped the reads that could be matched perfectly to the chicken genome onto the *Gallus gallus* miRNA precursor of the Sanger miRBase (Release 15.0)[18] using the short oligonucleotide alignment program (SOAP),[17] to identify known *G. gallus* miRNAs, according to the following two criteria: First, a unique sequence must be perfectly mapped onto the precursor. Second, the start position of the alignment must be between +2 and −2 nt of the mature miRNA on the precursor. We identified 159 known chicken miRNAs, which belonged to 115 miRNA families and were derived from 174 predicted miRNA precursors. Among the 159 known miRNAs, only 14 were miRNA*s (Table 2, miRNA originates from the arm opposite the annotated mature miRNA-containing arm of the precursor), which is consistent with the current understanding of miRNA biogenesis and strand preference. Nine of these miRNA*s (miR-10a*, 126*, 1329*, 146c*, 1729*, 181a*, 199*, 22*, and 99a*) were simultaneously sequenced with their corresponding miRNAs (upper section of Table 2), and the

remaining five (miR-140*, 1560*, 1677*, 1684*, and 1685*) were only sequenced with the miRNA* type (lower section of Table 2). Since the number of sequencing reads can reflect the expression abundance of individual miRNAs in a specific sample, we compared the sequencing times of individual miRNAs. Of the 159 known miRNAs identified, miR-222 and let-7 family members were frequently sequenced in our small RNA library, indicating that they play important roles in chicken pre-adipocyte proliferation and differentiation. There were 58 miRNAs sequenced more than 100 times, and 31 miRNAs sequenced between 10 and 100 times. The top 10 most abundantly expressed miRNAs are listed in Table 3.

Novel chicken miRNAs

To identify potential novel miRNAs from the small RNA library of chicken pre-adipocytes, we used the following approach and criteria: (i) Reads that matched known chicken miRNAs (miRBase Release 15.0)[18] and other non-coding RNAs were excluded. (ii) Reads that perfectly matched the chicken genome more than 20 times were excluded.[20] (iii) Non-conserved unique reads that were sequenced only once were removed. (iv) To be considered as new miRNAs, reads had to be entirely within the arm of the hairpin, and it was necessary that the hairpin does not contain large internal loops or bulges.[24]

We discovered 57 new miRNA genes, which encoded 63 mature miRNAs. Nine of the 63 novel miRNAs were conserved among metazoans, such as *Bos taurus* and *Mus musculus*. Among these nine conserved miRNAs, four were homologous to *G. gallus* miRNAs (Table 4). Genomic analysis of the nine conserved miRNAs showed that five of them were located in intergenic regions, three were located within the introns of protein-coding genes (TRB2, KCTD15, and DNAH10), and one overlapped with the RCJMB04_2b20 gene by six nucleotides at its 3′ terminus (Table 4).

The miRNAs generated from the remaining 48 new miRNA genes were not conserved among metazoans. Six of these 48 miRNA genes had the potential to produce mature miRNAs from both arms of their predicted precursors (Table 5), but their 3′ arm products had a higher sequencing frequency than their 5′ arm products, indicating that the predominant mature miRNA might originate in the 3′ arm of their precursors. The 3′ arm products of these six miRNA genes were sequenced 48–307 times, while the 5′ arm products were sequenced only 2–3 times. MiRNAs deriving from the other 42 miRNA genes were mostly sequenced 10–100 times, suggesting that they are expressed at relatively low levels in chicken pre-adipocytes.

Table 2. MiRNA*s and Corresponding miRNAs Detected in Chicken Pre-Adipocytes

MiRNA	Sequence	No. of reads
gga-miR-10a	UACCCUGUAGAUCCGAAUUUGU	50
gga-miR-10a*	AAAUUCGUAUCUAGGGGAAUA	3
gga-miR-126	UCGUACCGUGAGUAAUAAUGCGC	1
gga-miR-126*	CAUUAUUACUUUUGGUACGCG	1
gga-miR-1329	UACAGUGAUCACGUUACGAUGG	5
gga-miR-1329*	CCUCGUAGCUUGAUCACGAUAU	1
gga-miR-146c	UGAGAACUGAAUUCCAUGGACUG	541
gga-miR-146c*	AGUCCAUGGUAUUCAGUUCUCU	19
gga-miR-1729	AUCCCUUACUCACAUGAGUAGUC	2
gga-miR-1729*	CUACUCGGUGAGUAAGGAUAGC	1
gga-miR-181a	AACAUUCAACGCUGUCGGUGAGU	2,181
gga-miR-181a*	ACCAUCGACCGUUGAUUGUACC	4
gga-miR-199	CCCAGUGUUCAGACUACCUGUUC	4
gga-miR-199*	UACAGUAGUCUGCACAUUGG	1,077
gga-miR-22	AAGCUGCCAGUUGAAGAACUGU	741
gga-miR-22*	AGUUCUUCAGUGGCAAGCUUUA	432
gga-miR-99a	AACCCGUAGAUCCGAUCUUGUG	1,875
gga-miR-99a*	CAAGCUCGCUUCUAUGGGUCU	4
gga-miR-140*	CCACAGGGUAGAACCACGGAC	1,383
gga-miR-1560*	GCGGCGCGAGCAGAGAGGCGCU	2
gga-miR-1677*	UCCUGCACCGCUGAAGUCAAU	2
gga-miR-1684*	AGCUCUGCUUCCUCAUACAUAC	1
gga-miR-1685*	UGGAGUCACUACCAGUGCUGUG	1

MiRNA*s simultaneously sequenced with their corresponding miRNAs are in the upper section, and miRNA*s sequenced only with the miRNA* type are in the lower section. All miRNA*s are in bold font. *miRNA originating from the arm opposite the annotated mature miRNA-containing arm of the precursor.

Table 3. The Top 10 Most Abundantly Expressed miRNAs in Chicken Pre-Adipocytes

MiRNA	Sequence	No. of reads
miR-222	AGCUACAUCUGGCUACUGGGUCUC	25,589
miR-30d	UGUAAACAUCCCCGACUGGAAG	14,928
miR-26a	UUCAAGUAAUCCAGGAUAGGC	14,211
let-7c	UGAGGUAGUAGGUUGUAUGGUU	10,930
let-7d	AGAGGUAGUGGGUUGCAUAGU	8,550
let-7a	UGAGGUAGUAGGUUGUAUAGUU	8,425
let-7j	UGAGGUAGUAGGUUGUAUAGUU	8,396
let-7f	UGAGGUAGUAGAUUGUAUAGUU	7,584
let-7b	UGAGGUAGUAGGUUGUGUGGUU	4,295
miR-30a-5p	UGUAAACAUCCUCGACUGGAAG	4,042

Table 4. Novel Conserved miRNAs Discovered in Chicken Pre-Adipocytes

Name[1]	Hairpin position (strand[2])	Location	No. of reads	Conserved with
Con-1	chrZ:7867831–7868050(+)	Intergenic	1	bta-miR-1777a[3]
Con-2	chr3:35991414–35991649(+)	Intron of TRB2	1	bta-miR-1777a
Con-3	chr10:22232839–22232934(+)	Overlap RCJMB04_2b20	1	bta-miR-1777a
Con-4	chr11:11466076–11466274(+)	Intron of KCTD15	6	gga-miR-1576[4]
Con-5	chr20:11164006–11164158(+)	Intergenic	1	gga-miR-1576
Con-6	chr1:104482874–104483102(−)	Intergenic	1,154	mmu-miR-2133[5]
Con-7	chrUn_random:13277363–13277447(−)	Intergenic	4	gga-miR-1684
Con-8	chr15:4916703–4916821(−)	Intron of DNAH10	56	mmu-miR-1959
Con-9	chr9:24436105–24436324(−)	Intergenic	1	gga-miR-1648*

[1]Names of self-named miRNAs; con, novel conserved miRNA genes; [2]strand orientation, + forward strand, − reverse strand; [3]bta, *Bos taurus*; [4]gga, *Gallus gallus*; [5]mmu, *Mus musculus*; *miRNA originating from the arm opposite the annotated mature miRNA-containing arm of the precursor.

Table 5. Novel miRNAs Identified in Chicken Pre-Adipocytes Which Generated from Both Arms of the Predicted Precursors

Name[1]	Hairpin position (strand[2])	3′ arm sequence	No. of reads	5′ arm sequence	No. of reads
b-1	chr1:104459387–104459435(−)	UGGACCGGCGCA AGACGAACU	307	UUCGUAUUGUGCCG CUAGAGG	3
b-2	chr1:104459513–104459605(−)	AGGCUGGCCGCC GGAAUACUCCA	48	AACUGAGUGUCCCG CGGGGCCCGAAGC	2
b-3	chr1:104483075–104483298(−)	CGGCUCCGGGAC GGCUGGG	89	CGGCCGGCGUCCGG CCCCCGUCGGGC	2
b-4	chr18:3449288–3449367(−)	AAUUCGUGAAGC GUUCCAU	63	AAUUGGAACGAUA CAGAGAAG	3
b-5	chr28:2331283–2331362(+)	AAUUCGUGAAGC GUUCCAU	63	AAUUGGAACGAUA CAGAGAAG	3
b-6	chr28:2376689–2376768(+)	AAUUCGUGAAGC GUUCCAU	63	AAUUGGAACGAUA CAGAGAAG	3

[1]Names of self-named miRNAs; b, miRNA genes could generate miRNAs from both arms of the predicted precursors; [2]strand orientation, + forward strand, − reverse strand.

Table 6. MiRNA Clusters Discovered in Chicken Pre-Adipocytes

Cluster	Chromosome location (strand[1])	No. of reads[2]
mir-29b-1-mir-29a	**Chr1:** 3235312–3235392; 3236329–3236417(+)	115; 1,115
let-7a-3-let-7b	**Chr1:** 73421272–73421347; 73422101–73422185(+)	8,425; 4,295
mir-99a-let-7c	**Chr1:** 102424333–102424413; 102425086–102425169(+)	1,875; 10,930
b1-b2[3]	**Chr1:** 104459387–104459435; 104459513–104459605(−)	310; 52
Con6[4]**-b3**	**Chr1:** 104482874–104483102; 104483075–104483298(−)	1,154; 91
mir-222-1-mir-222-2-mir-221	**Chr1:** 114216027–114216124;114218422–114218519; 114218926–114219024(+)	25,589; 3,315
mir-92-mir-19b-mir-20a-mir-17	**Chr1:** 152248070–152248147; 152248183–152248269; 152248306–152248403; 152248781–152248865(−)	603; 2; 5; 32
mir-16-1-mir-15a	**Chr1:** 173700351–173700434; 173700493–173700575(−)	110; 20
mir-194-mir-215	**Chr3:** 19924487–19924561; 19924793–19924897(+)	3; 2
mir-20b-mir-106	**Chr4:** 3970047–3970131; 3970359–3970439(−)	4; 152
mir-16c-mir-15c	**Chr4:** 4048689–4048759; 4049055–4049130(−)	191; 19
mir-181a-1-mir-181b-1	**Chr8:** 2001561–2001664; 2001750–2001838(+)	2,181; 671
mir-16-2-mir-15b	**Chr9:** 23742791–23742884; 23742966–23743056(−)	110; 360
mir-3529-mir-7-2	**Chr10:** 14823529–14823619(+); 14823525–14823623(−)	1; 199
let-7d-let-7f-let-7a-1	**Chr12:** 6301452–6301554; 6302497–6302583; 6302911–6303000(−)	8,550; 7,584; 8,425
mir-1609-1-mir-1609-2	**Chr13:** 17399772–17399865; 17401478–17401571(+)	2; 2
mir-301-mir-130a	**Chr15:** 406313–406405; 408399–408481(−)	8; 468
mir-181a-2-mir-181b-2	**Chr17:** 10218497–10218587; 10220137–10220221(+)	2,181; 671
mir-219-mir-2964	**Chr17:** 5577817–5577901(+); 5577814–5577902(−)	112; 6
mir-301b-mir-130c	**Chr19:** 7144739–7144828; 7145027–7145120(−)	3; 130
mir-21-h-10[5]	**chr19:** 7322072–7322168; 7322074–7322161(+)	3,471; 73
mir-1b-mir-133c	**Chr23:** 4663912–4663975; 4664051–4664129(+)	1; 18
h-16-let-7a-2	**chr24:** 3380944–3381112; 3380993–3381064(+)	14; 8,425
mir-34b-mir-34c	**Chr24:** 5684900–5684983; 5685637–5685710(+)	143; 180
let-7j-let-7k	**Chr26:** 1442697–1442779; 1442897–1442979(−)	8,396; 103
mir-29c-mir-29b-2	**Chr26:** 2511658–2511746; 2512569–2512648(−)	1,113; 115
mir-23b-mir-27b-mir-24	**ChrZ:** 41157406–41157491; 41157642–41157738; 41158175–41158242(+)	159; 64; 175

[1]Strand orientation, + forward strand, − reverse strand; [2]number of reads of miRNAs is listed in order of cluster name; [3]b, miRNA genes could generate miRNAs from both arms of the predicted precursors in Table 5; [4]Con, novel conserved miRNA genes in Table 4; [5]h, non-conserved new miRNA genes.

MiRNA gene cluster analysis

MiRNAs are often clustered in the genome and aligned in the same orientation, allowing them to be transcribed polycistronically, thus regulating the expression levels of other genes cooperatively.[1)] Altuvia *et al.* used 3,000 nt as the maximal distance for two miRNA genes in intergenic regions to be considered as clustered, and for miRNA genes hosted in pre-mRNA, those encoded within the same non-coding element (intron or UTR-exon) were considered clustered. They found that 42% of the known human miRNA genes are clustered.[25)] Using this method, we organized 59 miRNA genes into 27 compact miRNA clusters comprising 23 pairs, three triplets, and one tetrad (Table 6). Of these 27 miRNA clusters, 23 were composed of known chicken miRNAs, and the members of eight clusters (mir-29b-1-mir-29a, let-7a-3-let-7b, mir-99a-let-7c, mir-222-1-miR-222-2-mir-221, let-7d-let-7f-let-7a-1, mir-181a-2-mir-181b-2, mir-181a-1-mir-181b-1, and let-7j-let-7k) were frequently sequenced in this library. Genomic analysis of all of these clusters showed that almost 1/3 of them resided on chromosome 1. The sequencing frequency of different members in the same cluster differed greatly for some clusters, such as let-7j-let-7k, perhaps due to the different transcriptional regulatory mechanisms of these miRNAs, while these miRNAs are located in different transcription units. Or it may that they are transcribed from the same polycistron but their maturation is differentially controlled.[26)] Detailed information on the miRNA clusters is given in Table 6.

Table 7. Mitrons Identified in Chicken Pre-Adipocytes

Gene symbol	Ensembl transcript ID/Intron number	No. of reads	Position[1]	Genomic location (strand[2])
HSD11B1L	**ENSGALT00000020974/Intron5**	**3**	**5p**	**chr28:35549–35627(+)**
SEC14L2	**ENSGALT00000012726/Intron5**	**2**	**5p**	**chr15:11139963–11140035(−)**
GLIPR1	**ENSGALT00000016620/Intron5**	**1**	**3p**	**chr1:39669671–39669752(+)**
IPI00575891.3	**ENSGALT00000006408/Intron4**	**1**	**3p**	**chr4:1555532–1555602(−)**
ARFGAP2	**ENSGALT00000013425/Intron1**	**1**	**3p**	**chr5:25349187–25349316(+)**
ADAM8	**ENSGALT00000005445/Intron14**	**1**	**5p**	**chr6:10456383–10456472(−)**
MRTO4	**ENSGALT00000006360/Intron1**	**1**	**5p**	**chr21:4679924–4679994(+)**
LOC769550	ENSGALT00000036815/Intron1	9	5p, 3p	chr1:104460067–104460182(−)
USP48	ENSGALT00000022876/Intron7	4	3p	chr21:6723075–6723348(+)
ARPC4	ENSGALT00000010814/Intron2	3	5p	chr12:11779864–11779984(+)
PTK7	ENSGALT00000014020/Intron9	2	3p	chr3:4335124–4335236(+)
Thrombospondin-1	ENSGALT00000015678/Intron9	2	5p	chr5:31934486–31934827(−)
BTRC	ENSGALT00000033197/Intron7	2	5p	chr6:24279884–24280354(−)
PTK	ENSGALT00000004233/Intron14	2	5p	chr8:3836023–3836095(−)
NADK	ENSGALT00000035171/Intron5	2	3p	chr21:1967278–1967512(+)
DSTYK	ENSGALT00000035232/Intron9	2	5p	chr26:1828537–1828880(−)
LOC422654	ENSGALT00000041397/Intron3	1	5p	chr4:52472541–52472621(+)
ANXA5	ENSGALT00000037754/Intron1	1	5p	chr4:55512600–55512698(+)
ADAM13	ENSGALT00000025839/Intron14	1	5p	chr4:93132755–93132852(−)
FNBP4	ENSGALT00000023534/Intron13	1	5p	chr5:12256–12351(−)
FN1	ENSGALT00000005663/Intron16	1	5p	chr7:4378261–4378646(−)
LOC771339	ENSGALT00000035416/Intron2	1	3p	chr8:7732028–7732458(+)
IPI00589771.2	ENSGALT00000013533/Intron11	1	3p	chr10:22246447–22246673(−)
DHX37	ENSGALT00000004688/Intron26	1	3p	chr15:4535367–4535579(+)
NOTCH-1	ENSGALT00000003743/Intron8	1	5p	chr17:8528750–8528841(+)
IPI00595338.1	ENSGALT00000004814/Intron1	1	3p	chr18:5895625–5895968(+)
VEZF1	ENSGALT00000008870/Intron5	1	3p	chr19:8687090–8687150(+)
CCNL1	ENSGALT00000002357/Intron2	1	5p	chr21:2197286–2197569(+)
LEPRE1	ENSGALT00000007719/Intron5	1	5p	chr21:6574687–6574841(+)
chAnk1	ENSGALT00000005683/Intron18	1	3p	chr22:2673369–2673620(−)
BSDC1	ENSGALT00000005386/Intron10	1	5p	chr23:5458665–5458815(−)
MACF1	ENSGALT00000005857/Intron30	1	3p	chr23:5651904–5652086(+)
PANX3	ENSGALT00000001383/Intron1	1	3p	chr24:310615–310736(−)
IPI00583097.4	ENSGALT00000001087/Intron13	1	5p	chr28:963998–964086(−)

Typical mirtrons are in bold font (upper section), and mirtron candidates with atypical hairpin structure are in regular font (lower section). [1]Position of mirtrons within the precursors, 5p, 5′ arm, 3p, 3′ arm; [2]strand orientation, + forward strand, − reverse strand.

Mirtrons

There are two miRNA generating pathways: the canonical pathway and the alternative pathway. The canonical miRNA pathway generates miRNAs *via* sequential cleavage by two RNase III enzymes, Drosha and Dicer, while the alternative pathway uses the intron splicing machinery to bypass Drosha cleavage in miRNA maturation. These miRNA-generating introns are called mirtrons,[27–29] and their characteristic feature is that the miRNA hairpin-like precursor is directly adjacent to the splice sites, which means that mature miRNAs often start directly at the boundary of the intron.[11] We used the approach described by Berezikov *et al.*[29] to scan for mirtrons in our small RNA library, and discovered 34 new chicken mirtrons, of which there were 27 mirtron candidates with atypical hairpin structures (Table 7, lower section). Analysis of the evolutionary conservation of these newly identified chicken mirtrons showed no conservation with the mirtrons of mammals,[29] and none of the 39 chicken mirtrons reported by Glazov *et al.*[11] in chicken embryos were detected in our chicken pre-adipocyte library.

MiRNA target gene prediction and gene ontology analysis

A search for miRNA target genes was performed using the approach described for the Sanger miRbase.[30] All identified miRNA sequences were used to query the *G. gallus* predicted genes within Ensembl for the identification of potential target sequences using miRanda,[31] using the parameters: score cutoff ≥ 140, energy cutoff ≤ -7.0, gap opening $= -9.0$, gap extension $= -4.0$, 5′ scaling $= 4$. Target genes were then annotated using Interproscan.[32] All predicted target genes were subjected to gene ontology (GO) annotation (http://www.ebi.ac.uk/GOA/chicken_release.html) and were analyzed using the Web Gene Ontology Annotation Plot (WEGO).[33] We conducted these analyses with all of the miRNAs detected in our library and focused on biological process analysis. The results indicate that most of the predicted genes were involved in cellular process, metabolic process, and biological regulation (Fig. 2).

To determine whether any of these identified miRNAs regulate chicken adipocyte differentiation and lipid metabolism, we conducted a reverse search for miRNAs regulating genes important in adipocyte differentiation and lipid metabolism, such as PPARγ and FABP4, and selected out the corresponding miRNAs. The results are listed in Table 8.

Discussion

In this study, the Solexa deep sequencing method was used to investigate the miRNA expression profile of

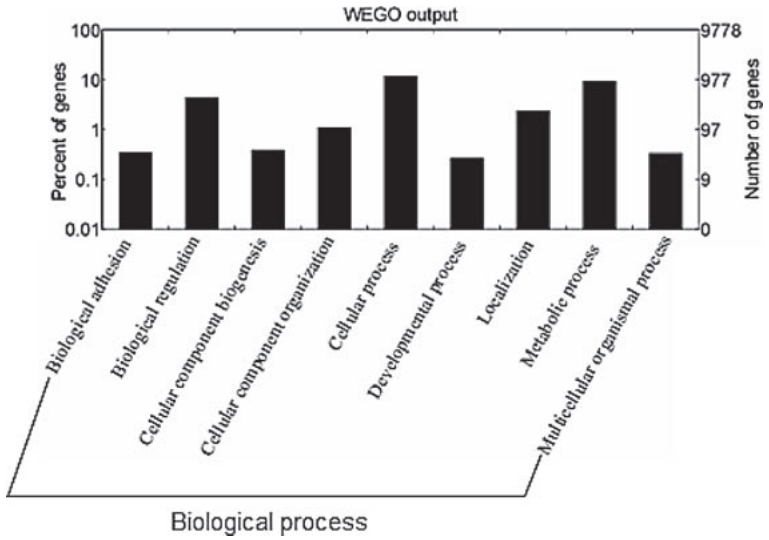

Fig. 2. WEGO Analysis of the Precdicted Target Genes of All MiRNAs Detected in Our Library.

All the miRNAs detected in our library were used to predict target genes with software miRanda. All the predicted target genes were subjected to GO annotation and WEGO analysis, with a focus on biological processes. The GO level was set to 3. The *x*-axis indicates different biological processes, the *y*-axis on the left indicates the percentages of genes involved in the biological processes and the *y*-axis on the right indicates the numbers of genes involved in various biological processes.

Table 8. MiRNA Prediction of Genes Important in Adipocyte Differentiation and Lipid Metabolism

Gene ensembl ID	Gene symbol	miRNA
ENSGALP00000007962	PPARγ	gga-miR-1677; gga-miR-19b
ENSGALP00000030221	FABP4	gga-miR-1740
ENSGALP00000001890	PPARGC1B	gga-miR-30a-5p
ENSGALP00000038124	FASN	gga-let-7d; gga-miR-1329; gga-miR-1456; gga-miR-1689; gga-miR-1699; gga-miR-199*; gga-miR-204; gga-miR-211; gga-miR-222; gga-miR-34a; gga-miR-34c; gga-miR-429; gga-miR-460b-3p
ENSGALP00000008899	ADIPOQ	gga-let-7d; gga-miR-1306; gga-miR-429
ENSGALP00000000131	ADIPOR1	gga-miR-103; gga-miR-106; gga-miR-107; gga-miR-16c; gga-miR-17-5p; gga-miR-205a; gga-miR-20b; gga-miR-455-5p; gga-miR-460b-3p
ENSGALP00000003376	ACOX1	gga-miR-103; gga-miR-107; gga-miR-1456; gga-miR-1583; gga-miR-1689; gga-miR-1699; gga-miR-1716; gga-miR-1729*; gga-miR-1740; gga-miR-20a; gga-miR-2131
ENSGALP00000011510	APOA1	gga-miR-1609; gga-miR-31; gga-miR-455-3p
ENSGALP00000030109	GATA2	gga-miR-106; gga-miR-17-5p; gga-miR-204; gga-miR-20a; gga-miR-20b; gga-miR-211
ENSGALP00000017274	ACSL1	gga-miR-460b-3p; gga-miR-146c*; gga-miR-1550-5p; gga-miR-1552-3p; gga-miR-1653; gga-miR-1674; gga-miR-17-3p; gga-miR-1729; gga-miR-199; gga-miR-218; gga-miR-221; gga-miR-31
ENSGALP00000036979	LPL	gga-miR-124a; gga-miR-140*; gga-miR-1555; gga-miR-1699; gga-miR-29b
ENSGALP00000032896	BMP7	gga-miR-1653; gga-miR-194; gga-miR-460b-3p
ENSGALP00000010614	PLIN1	gga-miR-1552-3p; gga-miR-22
ENSGALP00000013734	CD36	gga-let-7a; gga-let-7b; gga-let-7c; gga-let-7d; gga-let-7j; gga-miR-1329; gga-miR-17-3p
ENSGALP00000011452	CPT1A	gga-miR-181a; gga-miR-181b; gga-miR-24
ENSGALP00000037404	CPT-2	gga-let-7a; gga-let-7b; gga-let-7c; gga-let-7f; gga-let-7i; gga-let-7j; gga-miR-130a; gga-miR-130b; gga-miR-130c; gga-miR-1609; gga-miR-1656; gga-miR-1663; gga-miR-1698; gga-miR-1699; gga-miR-1705; gga-miR-205a; gga-miR-221; gga-miR-34a, gga-miR-34b; gga-miR-34c; gga-miR-429; gga-miR-456
ENSGALP00000039276	ACADL	gga-miR-133a; gga-miR-133c; gga-miR-146c*; gga-miR-17-5p; gga-miR-221
ENSGALP00000007948	SLC27A4	gga-miR-1653; gga-miR-193b; gga-miR-204; gga-miR-211
ENSGALP00000037108	DBI	gga-miR-1451; gga-miR-30c
ENSGALP00000037669	NR1H3	gga-miR-125b; gga-miR-1552-3p
ENSGALP00000009207	SCD	gga-miR-1699
ENSGALP00000011607	FADS2	gga-miR-1684*; gga-miR-1698; gga-miR-17-3p; gga-miR-1716
ENSGALP00000035951	CYP7A1	gga-miR-460a; gga-miR-1674
ENSGALP00000010786	EHHADH	gga-miR-122; gga-miR-128; gga-miR-135a; gga-miR-15b; gga-miR-15c; gga-miR-1689; gga-miR-2131; gga-miR-27b; gga-miR-460b-3p
ENSGALP00000009408	ACAA1	gga-miR-1454; gga-miR-1583; gga-miR-27b
ENSGALP00000017306	SCP2	gga-miR-107
ENSGALP00000008589	SORBS1	gga-mir-1563; gga-mir-16c
ENSGALP00000004130	RXRα	gga-miR-455-5p; gga-miR-1456; gga-miR-1609; gga-miR-1699; gga-mir-1716; gga-miR-184; gga-mir-2131; gga-miR-221; gga-miR-222

*miRNA originating from the arm opposite the annotated mature miRNA-containing arm of the precursor.

chicken pre-adipocytes, and 159 previously annotated miRNAs and 57 novel miRNA genes were identified. Genomic analysis revealed that there are 27 compact clusters that consist of 59 miRNA genes. MiRNA target gene prediction and GO analysis results showed that most of these target genes are involved in cellular processes, metabolic processes, and biological regulation. Seven new chicken mirtrons and 27 mirtron candidates were also discovered.

Among the known miRNAs identified, miR-222 was the most frequently sequenced miRNA, being sequenced 25,589 times. MiR-222 has been reported to inhibit endothelial cell proliferation in humans[34] and is down-regulated during 3T3-L1 pre-adipocyte differentiation, the development of primary fat cells, and human subcutaneous adipocyte differentiation.[35,36] These data indicate that miR-222 is a negative regulator of adipocyte differentiation in mice and humans, and potentially also in chickens.

Besides miR-222, there were many other frequently sequenced miRNAs in our library, including let-7 family members, miR-30s, miR-26a (top 10 listed in Table 3), miR-21, miR-103, and miR-181a. These miRNAs are also highly expressed in mammalian pre-adipocyte and adipose tissue. Let-7a and miR-21 are expressed abundantly at various differentiation stages (pre-adipocyte, differentiation day 1 and day 9) of 3T3-L1 cells,[5] and miR-30a-5p and miR-103 are upregulated in 3T3-L1 adipogenesis and primary adipocyte development.[35] In human adipose, miR-26a is the most abundant miRNA, and the let-7 family members are also highly expressed.[26] MiR-30s have been reported to be upregulated during human adipocyte differentiation.[36] The expression of miR-181a in human omental fat tissue has been reported to correlate with parameters of obesity, metabolism, and morphology, such as the mean adipocyte volume and lipid metabolism.[37] All these data confirm that these miRNAs also play important roles in adipocyte differentiation in chickens.

Besides the 159 known miRNAs, we also discovered 63 novel miRNAs in our small RNA library, which corresponded to 57 miRNA genes. Nine of the 63 novel miRNAs were found to be conserved among metazoans. There were four homologs of *G. gallus* miRNAs among the nine conserved miRNAs. These were similar to the sequences of the corresponding miRNAs published in miRBase,[18] but were located on different chromosomes from those of the published precursors. They might therefore be different types of the known miRNAs. The 54 new miRNAs, deriving from the 48 remaining novel miRNA genes, have not been identified in any other species and may therefore be chicken pre-adipocyte specific.

MiRNAs are often clustered in the genome, and they regulate their target genes synergistically. Based on miRBase (version 15.0),[18] 42 miRNA clusters are present in the chicken genome by the criterion of an inter-miRNA distance of less than 3,000 nt.[25] We discovered 27 miRNA clusters, including 59 miRNA genes, in our small RNA library of chicken pre-adipocytes. Twenty-three of these 27 clusters were composed of known miRNA genes, and four of them were composed of newly identified miRNA genes. Twenty-one of the 23 clusters composed of known miRNA genes were discovered in chicken embryos.[11] We also compared these 23 clusters with human and mice genes using the miRNA data in miRBase (version 15.0),[18] and found that 15 of them were conserved in *Homo sapiens* and *M. musculus*, except for the following eight clusters: mir-16c-mir-15c, mir-3529-mir-7-2, mir-1609-1-mir-1609-2, mir-301-mir-130a, mir-219-mir-2964, mir-301b-mir-130c, mir-1b-mir-133c, and let-7j-let-7k. One cluster, mir-17-92, comprised six miRNA genes, but we detected only four of these in chicken pre-adipocytes.

Mirtrons were first discovered in invertebrates, including flies and nematodes. These kinds of animals have a higher proportion of short introns with lengths typical of pre-miRNA hairpins than mammals.[27,28] Mirtrons have since been discovered in mammals. They exhibit several characteristics distinct from invertebrate mirtrons, such as 3′ and 5′ portion preference, different GC contents, and a hairpin end structure.[29] Glazov *et al.* first reported the discovery of 39 new chicken mirtrons, including 21 mirtron candidates, none of which had been identified in other vertebrates.[11] We discovered 34 new chicken mirtrons, including 27 mirtron candidates. These were not orthologous to the mirtrons previously discovered in mammals and chicken embryos.[11,29] Previous studies have found that flies, nematodes, and mammals have different sets of mirtrons, suggesting that mirtrons evolve quickly between different species, potentially independently.[29,38]

We also conducted a reverse search among all identified miRNAs for miRNAs that regulate chicken adipocyte differentiation and lipid metabolism. Many miRNAs were identified that were predicted to target important genes for adipocyte differentiation and lipid metabolism (Table 8), including the let-7 family members, miR-17s and miR-221. These might play important roles in chicken adipocyte differentiation and lipid metabolism.

In summary, we used the Solexa deep sequencing approach to determine the expression profiles of miRNAs in chicken preadipocytes. We identified not only previously annotated miRNAs, but also many novel miRNAs. Our findings provide information for future studies on chicken adipogenesis. Much work still needs to be done to determine which miRNAs are functional in chicken adipogenesis.

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