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PRELIMINARY REPORT

High-throughput Sequencing Analysis of miRNA Expression in Embryonic Chicken Breast Muscle

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Abstract

The embryonic period is a critical period for the development of muscle and adipose tissue in chickens. MicroRNAs (miRNAs) have been shown to play important roles in various biological processes, but little is known about miRNAs in chicken embryos. To investigate functional miRNAs regulating the meat quality of adult chickens, embryonic breast muscle tissues of Cobb broilers (CB) and rose-crowned chickens (RC) were collected and analyzed with high-throughput small RNA deep sequencing. The results showed that 842 known miRNAs and 598 novel miRNAs were identified from the four sequencing libraries, of which 592 were differentially expressed (DE) miRNAs ($P<0.05$). Taking Cobb broilers as a reference, 321 miRNAs were upregulated, and 271 miRNAs were downregulated. Real-time PCR confirmed that the trends in differentially expressed miRNAs were completely consistent with the sequencing results. We identified 120,666 target genes of the differentially expressed miRNAs, and functional enrichment analysis revealed that these genes were mainly involved in the Wnt signaling pathway, Adherens junction, Butirosin and neomycin biosynthesis and the Hedgehog signaling pathway. Furthermore, regulatory networks of interactions among miRNAs and their targets were constructed, and fatty acid binding protein 5 *(FABP5)* was confirmed as a target of miR-3532-5p by dual-luciferase assay. The results of this study enriched the relevant data on chicken muscle miRNA expression, and they laid a foundation for further analysis of the miRNA-mediated regulation of genes and the link between miRNA and chicken meat quality traits.

Keywords: *Cobb broilers, Rose-crowned chicken, miRNA, Meat quality, Embryo, High-throughput sequencing*

Embriyonik Tavuk Göğüs Kasında miRNA Ekspresyonunun Yüksek Verimli Dizi Analizi

Öz

Embriyonik dönem, tavuklarda kas ve yağ dokusunun gelişimi için kritik bir dönemdir. MikroRNA (miRNAs)'ların çeşitli biyolojik süreçlerde önemli roller üstlendikleri gösterilmiştir, fakat tavuk embriyolarında miRNA'lar hakkında çok az şey bilinmektedir. Yetişkin tavuklarda et kalitesini düzenleyen işlevsel miRNA'ları araştırmak için Cobb broylerlerinin (CB) ve gül taçlı tavukların (RC) embriyonik göğüs kas dokuları toplandı ve yüksek verimli küçük RNA dizi analizleri gerçekleştirildi. Sonuçlar, 592'si farklı olarak eksprese olan (DE) miRNA'lar ($P<0.05$) olmak üzere 842 bilinen miRNA ve 598 yeni miRNA'nın dört sekans kütüphanesinden tanımlandığını gösterdi. Cobb broylerleri referans alındığında, 321 miRNA'nın ekspresyonu artmış ve 271 miRNA'nın ekspresyonu azalmıştır. Real-time PCR analizi, farklı olarak eksprese olan miRNA'lardaki eğilimlerin dizileme sonuçlarıyla tamamen tutarlı olduğunu doğruladı. Farklı olarak eksprese olan miRNA'ların 120,666 hedef genini belirledik ve fonksiyonel zenginleştirme analizi, bu genlerin esas olarak Wnt sinyal yolağı, Adherens bağlantısı, Butirosin biyosentezi, neomisin biyosentezi ve Hedgehog sinyal yolağında yer aldığını ortaya çıkardı. Ayrıca, miRNA'lar ve hedefleri arasında düzenleyici etkileşim ağları oluşturuldu ve ikili lusiferaz analizi ile yağ asidi bağlayıcı protein 5 (FABP5), miR-3532-5p'nin bir hedefi olarak doğrulandı. Bu çalışmaya ait bulgular, tavuk kası miRNA ekspresyonu ile ilgili verileri zenginleştirdi ve genlerin miRNA aracılı regülasyonunun ve miRNA ile tavuk eti kalite özellikleri arasındaki bağlantının daha fazla analizi için bir temel oluşturdu.

Anahtar sözcükler: *Cobb broyleri, Gül taçlı tavuk, miRNA, Et kalitesi, Embriyo, Yüksek verimli dizi analizi*

INTRODUCTION

Poultry meat is one of the edible meat proteins accepted by consumers all over the world. With the continuous improvement of people's living standards, the demand for poultry products has gradually changed from an increase in quantity to an increase in quality. Cobb broilers (CB) is a special-purpose commercial matching variety introduced,

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which has the advantages of fast growth rate, high feed conversion rate and high slaughter rate. The Rose-crowned chicken (RC) is a Chinese native chicken breed with good meat quality and unique flavor. Previous studies in our lab found that there are significant differences in the growth rate and muscle quality between the two chicken breeds, and the content of inosinic acid and amino acid in breast muscle and leg muscle of RC are higher than that of CB [1]. Therefore, these two breeds are ideal animal models for studying the genetic mechanism of chicken quality. Meat quality is influenced by many factors, among which the content of intramuscular fat (IMF) affects the juiciness, tenderness and flavor of meat [2-4]. Therefore, an in-depth understanding of the differentially expressed genes and their molecular regulatory mechanisms that affect intramuscular fat would be beneficial for controlling intramuscular fat content and improving muscle quality.

MicroRNAs (miRNAs) are a class of endogenous non-coding RNAs that regulate post-transcriptional regulation of gene expression through base pairing with complementary sequences in the 3' untranslated region of mRNA [5]. An increasing number of studies have indicated that miRNAs have important regulatory roles in various biological and metabolic processes, such as cell proliferation, differentiation, apoptosis and lipid metabolism [6-8]. Several miRNAs have been identified to be responsible for adipogenesis or muscle development [9,10]. Gga-miR-140-5p was found to promote intramuscular adipocyte differentiation in chicken muscle via targeting retinoid X receptor gamma [11]. Furthermore, miR-130a [12], miR-223 [13], miR-125b [14], miR-15a [15], miR-143a-3p [16], etc. were found to be associated with adipocyte differentiation and lipid metabolism. Therefore, miRNAs may play an important role in affecting meat quality characteristics. Studies showed that the development of muscle and intramuscular fat in the embryonic stage plays a decisive role in the meat production and meat quality of poultry after hatching [17,18]. In our previous study, a remarkable difference in the meat traits and flavor between Rose-crowned chicken and Cobb broilers were found during the embryonic period. We speculated that miRNAs in embryonic stage might serve as important regulatory factor of chicken meat quality.

Fatty acid-binding proteins (FABPs) are approximately 14-15 kDa, mainly cytoplasmic proteins, which can reversibly bind saturated and unsaturated long-chain fatty acids with high affinity [19]. FABP5 is a class of intracellular lipid carrier that can transport fatty acids to PPARγ [20], and plays an important regulatory in lipid metabolism. FABP5 was reported to be a molecular regulator of fat synthesis [21], deposition [22], and obesity [23]. At the same time, FABP5 is also regulated by miRNAs, such as miRNA-122 regulates liver metabolism in the chicken by targeting to inhibition expression of FABP5 [24].

The aim of this study was to identify the miRNAs affecting the differences in meat quality between Rose-crowned chicken and Cobb broilers using small RNA deep sequencing. Differentially expressed miRNAs and their target genes were identified to elucidate the regulatory patterns of miRNAs and their network. Subsequently, based on combined analysis of miRNAs and potential target mRNAs, the candidate miRNAs involved in intramuscular adipocyte differentiation were further characterized. Our results may provide a theoretical basis for the subsequent study of the molecular mechanisms of muscle development and fat formation in chicken embryos.

Material and Methods

Ethics Statement

This study was approved by the Medical Ethics Committee, First Affiliated Hospital, Medical College, Shihezi University (A2016-095, 9 March 2016). All samples were collected in strict accordance with the committee's guidelines.

Sample Collection and RNA Isolation

Embryonic Cobb broilers (CB) and rose-crowned chicken (RC) were used for high-throughput sequencing. Thirty fertilized RC and CB eggs were incubated at 37°C and 60% humidity, then ED 8 breast muscle tissues were collected by surgery at a clean bench. Meanwhile, the embryonic brain was collected for gender identification, using a method reported by Milos Vucicevic [25]. Total RNA was extracted from each breast muscle using TRIzol (Invitrogen, Carlsbad, CA, USA). The quality and concentration of all RNA samples were determined by 1.5% agarose gel electrophoresis and by analyzing the ratio of absorbance at A260/280, and then the samples were divided into four mixed RNA pools: CBM (male cobb broilers) and RCM (male rose-crowned chicken), CBF (female cobb broilers) and RCF (female rose-crowned chicken), each with 3 biological replicates.

Small RNA Library Construction and Deep Sequencing

Total RNA (3 μg per sample) was used as input material for generating each small RNA library. Four small RNA libraries were constructed using NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (NEB, USA) following the manufacturer's recommendations. The quality and quantity of the cDNA library were assessed using Qubit2.0 (Life Technologies) and Agilent 2100 (Agilent Technologies) systems. Finally, the assessed cDNA libraries were sequenced using an Illumina HiSeq2500 sequencing platform at Novogene (Beijing, China).

Sequence Analysis and Identification of miRNAs

Raw sequencing reads were processed by evaluating the sequencing quality, removing low-quality reads, adaptor sequences and reads smaller than 18 nt or longer than 35 nt. To analyze the distribution of small RNAs based on the reference sequence, all of the clean sequencing reads were mapped to the chicken genome using Bowtie2

(https://sourceforge.net/projects/bowtie-bio/files/). The reads mapped to the chicken genome were BLASTed against the non-coding RNA sequences in GenBank *(http://www.ncbi.nlm.nih.gov/)* and the RNA families in Rfam *(http://rfam.sanger. ac.uk/)* to identify miRNA types and numbers and sRNA fragments generated from mRNA degradation. Finally, the remaining sequences were then searched against the mature chicken miRNAs in miRBase (Release 21.0), and miREvo [26] and mirdeep2 [27] were used to identify known and unknown miRNAs.

Analysis of Differently Expressed miRNAs

The expression levels of miRNAs in the different libraries constructed were estimated based on the Illumina sequencing data according to the transcripts per million clean reads (TPM). The normalized expression values were calculated using the following formula: (read count x 1.000.000)/total miRNA read counts in the library. The DESeq2 program was used to analyse the DE miRNAs. miRNAs with |log2(fold change)| <5 and P<0.05 were identified as DE miRNAs.

Quantitative Real-Time PCR (qPCR)

The relative expression levels of eight randomly selected DE miRNAs were validated using quantitative real-time reverse transcription PCR (qRT-PCR). The *U6* gene was chosen as the reference gene for miRNA expression. The primers used are described in *Table 1*. Total RNA was extracted using TRIzol reagent, and then it was reverse transcribed using a PrimeScript RT reagent kit with gDNA Eraser (TaKaRa) following the manufacturer's instructions. A miScript SYBR Green PCR kit was used to perform qPCR and determine expression levels of miRNAs. All of the reactions were repeated in triplicate, and the relative expression levels were calculated using the $2^{-\Delta\Delta ct}$ method. P<0.05 were considered to indicate significant differences.

Target Gene Prediction, Pathway and Network Analysis

Target genes were predicted using miRanda *(http://www.microrna.org/microrn-a/home.do)* and RNAhybrid *(http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/)*. GO enrichment and KEGG pathway analysis were performed using GOseq *(http://www.geneontology.org/)* and Kobas 2.0 *(http://kobas.cbi.pku.edu.cn/help.do),* respectively.

Vector Construction

The 3′ untranslated region (UTR) of the *FABP5* gene, containing the gga-miR-3532-5p binding site, was amplified from chicken genomic DNA by PCR and subcloned into the XhoI-NotI site of a psiCHECK-2 vector (FABP5-3′-UTR-WT). Mutant FABP5-3′-UTR plasmids were generated by changing the gga-miR-3532-5p binding site from AGTGCAA to GTACGAA (FABP5-3′-UTR-Mut). The primers used were described in *Table 2*. Plasmid DNA was sequenced by Sangon Biotech (Shanghai, China) and extracted using an EndoFree Maxi Plasmid kit (TIANGEN, Beijing, China).

Luciferase Reporter Assay

Luciferase reporter experiments were performed in DF-1 cells. Cells were seeded in 12-well plates and cultured under routine conditions with 10% foetal bovine serum for 24 h. Then, the cells were cotransfected with 1000 ng of FABP5-3′UTR-WT or FABP5-3′UTR-Mut and 50 nM gga-miR-3532-5p mimic or negative control using Lipofectamine 2000, and the medium was replaced 6 h later. Forty-eight hours after transfection, the activities of firefly and Renilla luciferase were analyzed using a dual-luciferase reporter assay system (Promega) following the manufacturer's instructions.

Cell Transfection

The miR-3532-5p mimics and mimic NC were purchased from Ribobio (Guangzhou, China). Chicken preadipocyte line was cultured at 37°C in high-glucose medium supplemented with 10% fetal bovine serum and 100 μg/mL penicillin/streptomycin. Upon reaching 60-70%

Table 1. *Primer sequences for differential miRNA of qPCR verification*

miRNA	Primer Sequences (5′-3′)
gga-miR-140-5p	cgcgAGTGGTTTTACCCTATGGTAG
gga-miR-133a-3p	ttGGtCCCCttCAACCAGCtG
gga-miR-3532-5p	GTTGCACTGCAGCTGCTCTTGG
gga-miR-218-5p	cgcgTTGTGCTTGATCTAACCATGT
gga-miR-128-1-5p	CGGGGCCGTAACACTGTCT
gga-miR-1677-5p	TCCTGCACCGCTGAAGTCAAT
gga-miR-1306-3p	TGGACGTTGGCTCTGGTGG
novel_4	cgcTGAGATGAAGCACTGTAGCTC
U6-F	CAAATTGGCTAAGCGGGCCT
U6-R	CTAACAGCGTCGAGACTGCG

Note: *Lowercase letters in the primer sequences are protective bases*

Table 2. *Primer sequences of candidate target genes*

Gene	Primer Sequences	Product Length
gga-FABP5-F-W	*CCG*CTCGAGGAGTAGCTGAGTGCAATC	
gga-FABP5-F-M	*CCG*CTCGAGGAGTAGCTG**GTACG**AATC	308 bp
gga-FABP5-R-W	*ATTT*GCGGCCGCAGAGCATGAACTTTGAAT	

Note: *Italics are protective bases of restriction enzymes. Underlined letters are recognition sequences that introduce Not I and Xho I restriction enzymes. The points in bold are the base mutation sites. W stands for wild type and M stands for mutant*

confluence, the cells were transfected with a gga-miR-3532-5p mimic (50 nM) or a negative control (50 nM) using 5 μL of Lipofectamine 2000, and the medium was replaced 6 h later. After 24 h, the cells were used for RNA extraction.

Statistical Analyses

All experiments were carried out at least three times. Data are presented as the mean±standard deviation of the mean based on at least three replicates of each treatment. Data were analysed using SPSS 22.0 software (IBM, Chicago, IL, USA), and differences between groups were evaluated by one-way ANOVA; $P<0.05$ was considered significant.

Result

Overview of Small RNA Deep Sequencing Data

Four small RNA libraries were constructed as follows: CBM, RCM, CBF and RCF, with three replicates for each treatment. After quality control steps and adaptor removal were performed, a total of 11,540,611, 13,512,883, 11,660,807, and 12,108,093 clean reads were obtained from the four libraries *(Table 3)*. The size distribution of clean reads was assessed for all four groups. The small RNA sequence length was mainly concentrated at 21-24 nt, and the length of 22 nt was the most common size *(Fig. 1)*.

Identification of Differentially Expressed miRNAs

A Pearson correlation analysis showed that the correlation between samples was high, greater than 0.902 *(Fig. 2-a)*, indicating that the sample expression patterns were highly similar. The expression of miRNA was normalized by TPM calculation. The results showed that the expression level of 15 miRNAs in all samples was greater than 10000 TPM. These highly expressed miRNAs may be closely related to the proliferation and differentiation of embryonic cells. In total, 592 differentially expressed miRNAs were identified, including 518 known miRNAs and 74 novel miRNAs. Of these, 161 were upregulated, and 151 were downregulated in comparing RCM vs CBM; 160 were upregulated, and 120 were downregulated miRNAs in comparing RCF vs CBF *(Fig. 2-b)*. Among the identified miRNAs, 217 were found in all four libraries *(Fig. 2-c)*. Clustering analysis indicated that high similarity was shown within three replicates for each group and among the significant differences in miRNA expression between the two breeds of chicken *(Fig. 2-d)*.

qRT-PCR Validation of the Sequencing Data

To verify the RNA-Seq data, 8 miRNAs identified as differentially expressed between the two groups were validated by qRT-PCR *(Fig. 3)*. Our results showed that the expression patterns of these 8 miRNAs were consistent with the RNA-Seq results, indicating that the deep sequencing results were reliable and appropriate for further analysis.

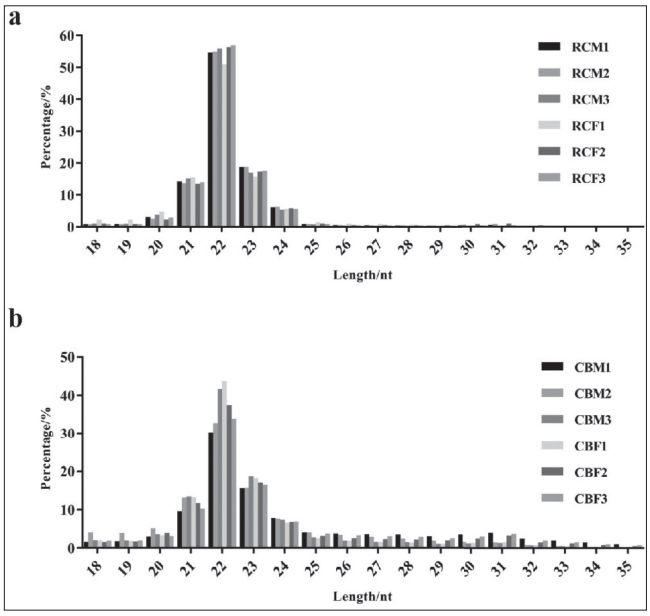

Fig 1. Length distribution of miRNA sequences in RC **(a)** and CB **(b)**

Table 3. *The information of raw data filtering*

Sample Name	Total Reads	Clean Reads	Q20/%	Q30/%	GC Content/%
RCM1	11168561	11050590 (98.94%)	97.50	94.11	49.29
RCM2	11194256	11055720 (98.76%)	97.51	94.15	49.22
RCM3	12647857	12515524 (98.95%)	97.52	94.15	49.35
RCF1	15928743	15725381 (98.72%)	98.84	97.74	49.63
RCF2	13499911	13337495 (98.80%)	98.90	97.88	49.07
RCF3	11594373	11475773 (98.98%)	97.59	94.31	49.19
CBM1	12532506	12318734 (98.29%)	96.89	93.34	51.80
CBM2	12317713	12100224 (98.23%)	96.49	92.51	52.10
CBM3	12450399	12272280 (98.57%)	98.52	96.79	51.42%
CBF1	12880091	12686254 (98.50%)	98.11	95.68	51.05%
CBF2	12151906	11977217 (98.56%)	96.91	93.40	51.44%
CBF3	11857817	11660807 (98.34%)	96.95	93.47	51.68%

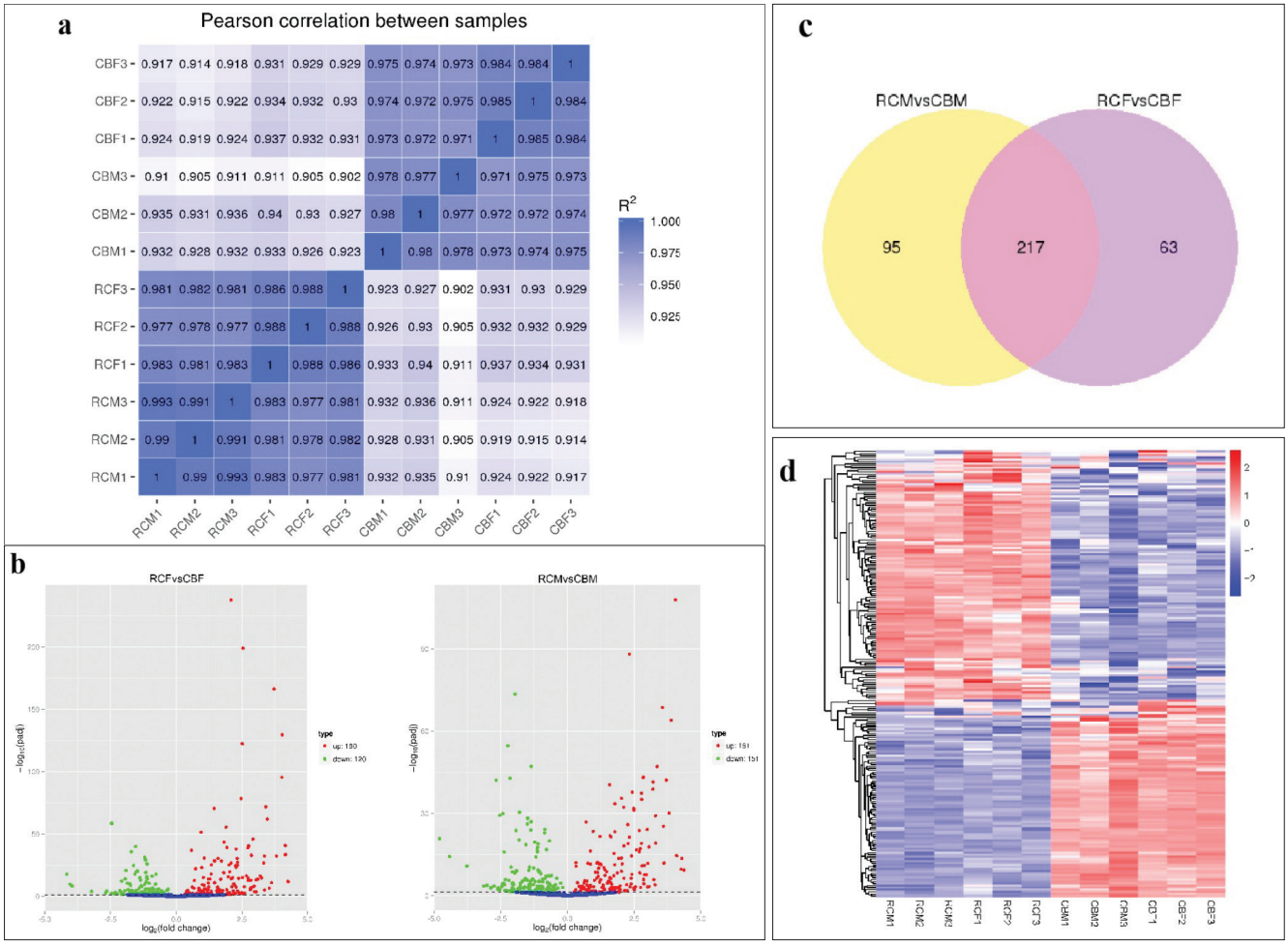

Fig 2. Overview of small RNA Deep Sequencing Data. **a-** Correlation analysis between samples, **b-** Volcano plot of DE miRNAs between two comparisons, **c-** Venn plot of DE miRNAs between two comparisons, **d-** Heat map of coexpressed miRNAs between the two groups

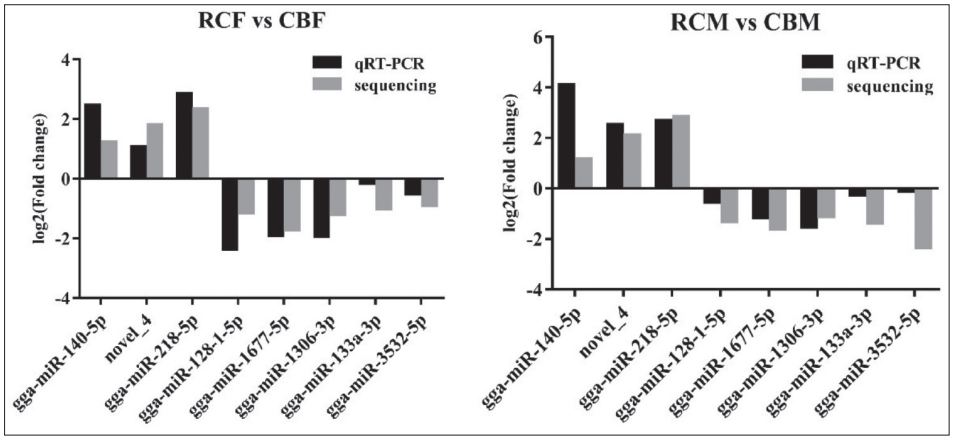

Fig 3. qRT-PCR validation of differentially expressed miRNAs. All experiments were carried out at least three times. log2 (Fold change) was the multiple of the difference in expression between samples to calculate the log2 value

Target Gene Prediction and Functional Enrichment Analysis

Using miRanda and RNAhybrid, a total of 120,666 consensus potential miRNA targets were identified for all differentially expressed miRNAs. For all potential targets, GO annotation and KEGG pathway analysis were performed to identify functional modules. All of the target genes were mainly enriched in biological metabolic processes, biosynthesis of cellular components, cell proliferation and differentiation, and embryonic development. Pathway analysis of all targets revealed that 9 KEGG pathways were significantly enriched ($P<0.05$), including 4 in RCM vs CBM and 5 in RCF vs CBF. The significantly enriched pathways included Ribosome, Wnt signalling pathway, Adherens junction, Butirosin and neomycin biosynthesis and Hedgehog signalling pathway

Table 4. *Top 5 KEGG signaling pathways of two comparisons*

Sample	KEGG	Rich Factor	P-Value	Gene Number
RCM vs CBM	Ribosome	0.4	0.02	48
	Wnt signaling pathway	0.370	0.011	44
	Adherens junction Butirosin and neomycin biosynthesis Hedgehog signaling pathway	0.397 1 0.4	0.022 0.029 0.05	27 5 18
RCF vs CBF	Ribosome	0.375	0.003	45
	Wnt signaling pathway	0.361	0.007	43
	Adherens junction Hedgehog signaling pathway Butirosin and neomycin biosynthesis	0.382 0.422 1	0.018 0.019 0.023	26 19 5

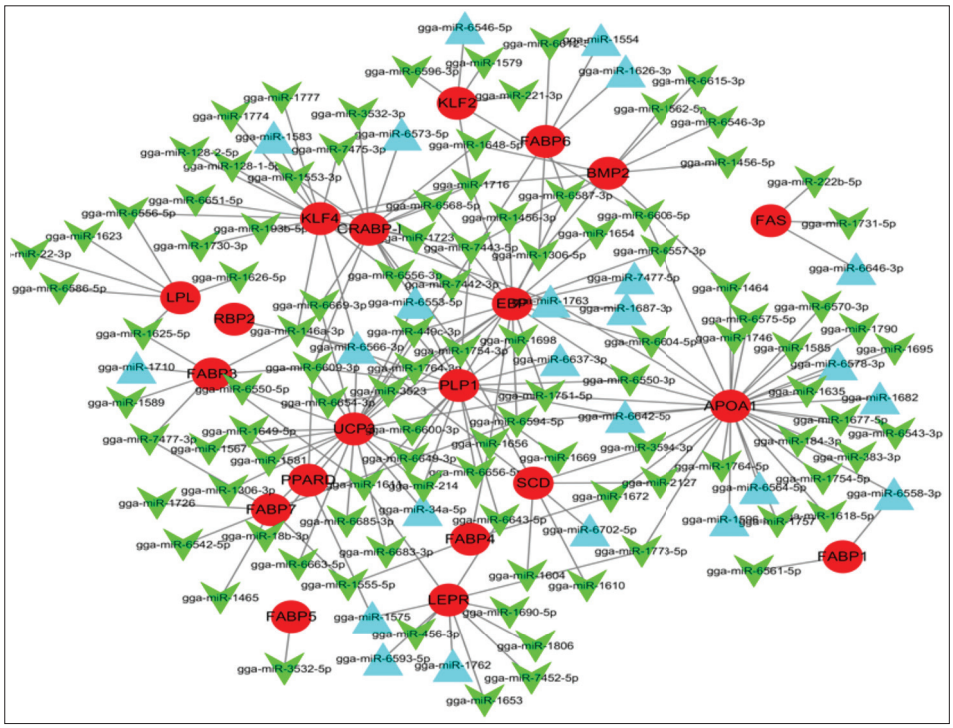

Fig 4. Interaction network of highly differentially expressed miRNAs and their potential targets. In this network, the target genes are displayed as red circles, the upregulated miRNAs are displayed as blue triangles, and the down regulated miRNAs are displayed as green arrows

(Table 4). According to the results of GO and KEGG analysis, we have identified key molecular players in the development of chicken breast muscle. In order to further understand the function of miRNA and their target genes, we focused on some of the differentially expressed miRNA and mRNA related with lipid metabolism, and a regulatory network for miRNA-mRNA interaction was constructed *(Fig. 4)*. In total, 33 up-regulated miRNA-mRNA pairs, and 160 down-regulated miRNA-mRNA pairs were identified. Among them, FABP5 as a potential target may be down-regulated by miR-3532-5p, this is the first discovery. EBP was the network core gene, a total of 19 miRNA-mRNA pairs were found, including gga-miR-1716, gga-miR-1751-5p and gga-miR -6604-5p, etc. gga-miR-1716 was also targets BMP2, CRABP-I, KLF2 and KLF4. APOA1 was targeted by 32 miRNAs, including 7 up-regulated and 25 down-regulated miRNAs. LPL was targeted by 7 down-regulated miRNAs, FAS was targeted by down-regulated gga-miR-222b-5p and gga-miR-1731-5p, and up-regulated gga-miR-6646-3p.

Validation of the miR-3532-5p Targeted FABP5 Gene

To verify the direct binding site between miR-3532-5p and *FABP5*, a 3'UTR fragment containing a seed region binding site was inserted into a psiCHECK-2 vector *(Fig. 5-a,b)*. Luciferase assays revealed that the luciferase reporter activity of FABP5-3'UTR-WT was significantly repressed by the miR-3532-5p mimic, while the luciferase activity of FABP5-3'UTR-MUT was not changed *(Fig. 5-c)*. Chicken preadipocytes were transfected with miR-3532-5p mimics, and the results show that overexpression of miR-3532-5p extremely significant downregulated *FABP5* mRNA expression *(Fig. 5-d)*. These results indicated that the predicted site of *FABP5* is a target of miR-3532-5p.

Discussion

Small RNA sequencing technology based on the Illumina high-throughput sequencing platform can directly assess

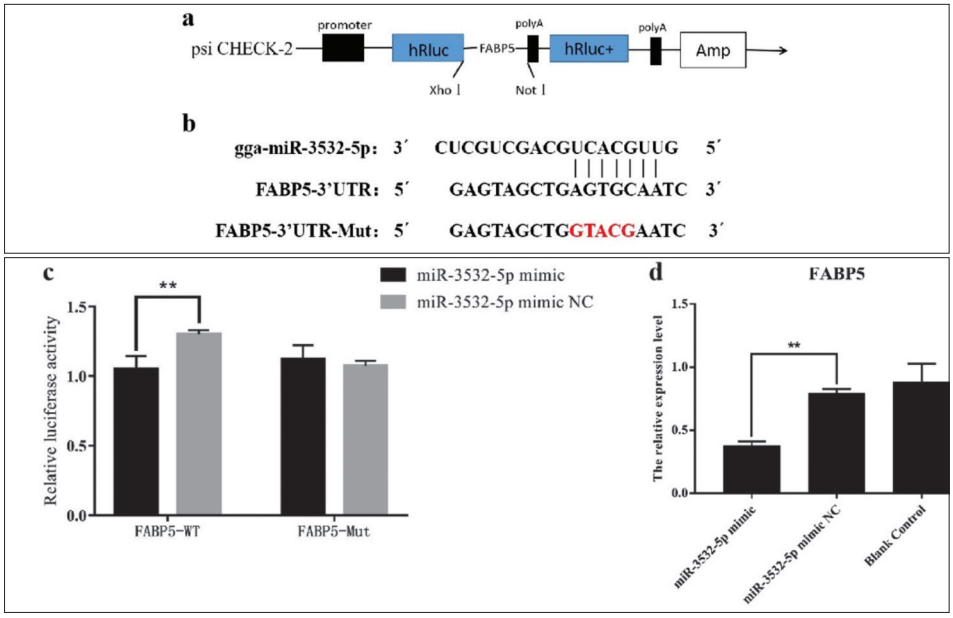

Fig 5. Validation of *FABP5* as a direct target of gga-miR-3532-5p. **a-** Target site of gga-miR-3532-5p within chicken *FABP5* mRNA 3'UTR and the mutation design of its 3'UTR, **b-** The sequence with the miR-3532-5p and *FABP5* binding sites was inserted into the psiCHECK-2 vector, **c-** miR-3532-5p mimics was transfected into DF1 cells along with FABP5-3'UTR-WT or FABP5-3'UTR-Mut (**P<0.01), **d-** The mRNA expression of *FABP5* is significantly reduced after overexpression miR-3532-5p

miRNAs in samples and has been used to identify known and predicted possible miRNAs [28]. The emergence of this method has greatly increased the discovery of new miRNAs. Many miRNAs have been identified as being associated with animal intramuscular fat deposition performance by high-throughput sequencing [11,29], but little is known about miRNAs in chicken embryos associated with adipogenesis.

In this study, the breast muscles on ED 8 from RC and CB were analyzed by high-throughput sequencing. To identify differentially expressed miRNAs that may play important regulatory roles in chicken meat quality, we compared the expression levels of miRNAs in the embryonic muscles of rose-crowned and Cobb broilers. A total of 842 known miRNAs were identified, and 598 new miRNAs were predicted. A total of 592 DE miRNAs were identified, of which 321 in the CB group were upregulated, and 271 were downregulated. MiRNAs are important regulatory factors in the process of fat deposition. Many of the miRNAs we have screened have been shown to regulate the proliferation and differentiation of adipocytes [30,31]. Previous studies showed that miR-128-3p inhibits the differentiation and generation of 3T3-L1 adipocytes by targeting *PPARG* and *Sertad2* [32]. Overexpression of miR-199a-5p promoted the proliferation of preadipocytes, and it was found that miR-199a-5p plays a role in the proliferation and differentiation of preadipocytes by downregulating the expression of Cav-1 [33]. The role of miRNA is mainly achieved by regulating the expression of target genes. In this study, miRanda and RNAhybrid were used to obtain 120,666 potential target genes for the differentially expressed miRNAs. MiRNAs affect cell signaling pathway transmission by regulating key genes in the signal transduction pathway to indirectly regulate adipocyte differentiation. The GO results show that the target genes are mainly concentrated in biological processes such as biological metabolism, cellular component biosynthesis, cell proliferation and differentiation, and embryonic development. Based on the GO annotation classification, KEGG pathway enrichment analysis was performed on the predicted target genes, and the results showed that a total of 5 signaling pathways were significantly enriched, of which the Wnt signaling pathway and the Hedgehog signaling pathway have been confirmed to play an important role in fat metabolism. According to the gene functions and their signaling pathway, we identified some candidates that affect fat formation and differentiation, including the *FABP* gene family, *FAS*, *APOA1*, and *LPL*.

In understanding the function of miRNA and its target genes and constructing a regulatory network of miRNA-mRNA interactions, miR-3532-5p may be an important regulator of intramuscular fat formation. Liu et al. showed that miR-3532 is closely related to the chicken reproductive process and ovarian steroidogenesis [34]. Wang et al.[35] mentioned that miR-3532 is a miRNA that is differentially expressed by chicken lung and trachea, but no further study on its target genes or its function was performed. To our knowledge, no previous studies have associated gga-miR-3532-5p with chicken IMF deposition. We used miRanda and RNAhybrid software to predict target genes, and we found that *FABP5* may be a target gene of miR-3532-5p. As a lipid carrier, *FABP5* is specific for the binding of fatty acids, preferentially binding long-chain unsaturated fatty acids and long-chain saturated fatty acids. It plays a vital role in triglyceride synthesis, fatty acid transport, and fat metabolism [36,37]. *FABP5* is also a key gene in the PPAR signaling pathway, which promotes adipocyte growth and differentiation by regulating PPARδ signaling [38]. Ma et al.[39] found that the activity of preadipocytes in the induction of differentiation into adipocytes decreased when the *FABP5* gene was knocked out, and the expression of *PPARγ* and *C/EBPα* genes associated with adipocyte differentiation was downregulated. This result indicates

that *FABP5* is an important factor influencing the activity and differentiation of preadipocytes. Previous studies have shown that the *FABP5* gene is closely related to fat deposition in pigs, and its polymorphic locus has an important effect on intramuscular fat content in chickens [40]. In our study, dual-luciferase assays and qRT-PCR confirmed the targeting relationship between miR-3532-5p and *FABP5*. It is speculated that gga-miR-3532-5p participates in the process of intramuscular fat formation in chicken embryos by inhibiting the expression of the *FABP5* gene, which may be an important regulatory factor affecting the differences in meat quality and flavor between RC and CB.

In conclusion, our study successfully constructed a miRNA library of embryonic breast muscles from RC and CB chickens and identified miRNAs that are associated with chicken quality. GO and KEGG analyses were performed to investigate the functional roles of these miRNAs. We constructed an interaction network of these miRNAs and their putative targets that may affect chicken breast meat quality. Furthermore, our study presents evidence that miR-3532-5p target *FABP5*, thereby participating in the process of intramuscular fat formation in chicken embryos. Our results lay the foundation for future analysis of the regulatory mechanism of miR-3532-5p in chicken intramuscular fat deposition. The results of this analysis provide new information that may be applied to further studies of the molecular regulatory mechanisms involved in the different meat quality traits exhibited by chicken embryos.

Acknowledgements

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Conflict of Interest

No conflict of interest between all authors.

Author Contributions

In the process of writing the article, L. Zhang and S. Ren are responsible for the provision, integration and writing of the article data, H. Liao is responsible for the provision of research animals, J. Sun is the article reviewer.

References

1. Yang XJ, Han YX, Wang HL, Ren S, Zhang L, Qin YM, Qi MF, Zhang WZ, Liao HR, Sun J: Study on nutritional value and flavor of muscle from different breed of chicken. *China Poultry*, 40, 9-14, 2018. DOI: 10.16372/j.issn.1004-6364.2018.02.003

2. Wang Y, Hui X, Wang H, Kurban T, Hang C, Chen Y, Xing J, Wang J: Association of H-FABP gene polymorphisms with intramuscular fat content in Three-yellow chickens and Hetian-black chickens. *J Anim Sci Biotechnol*, 7:9, 2016. DOI: 10.1186/s40104-016-0067-y

3. Wu G, Shi X, Zhou J, Wang L, Li Y, Song C, Xiang A, Dong P, Sun S: Differential expression of meat quality and intramuscular fat deposition related genes in Hanjiang black pigs. *Acta Biochim Biophys Sin (Shanghai)*, 47, 145, 2015. DOI: 10.1093/abbs/gmu127

4. Bonny SPF, Hocquette JF, Pethick DW, Farmer LJ, Legrand I, Wierzbicki J, Allen P, Polkinghorne RJ, Gardner GE: The variation in the eating quality of beef from different sexes and breed classes cannot be completely explained by carcass measurements. *Animal*, 10, 987-995, 2016. DOI: 10.1017/S175173111500292X

5. Ambros V: The functions of animal microRNAs. *Nature*, 431, 350-355, 2004. DOI: 10.1038/nature02871

6. Mens MMJ, Ghanbari M: Cell cycle regulation of stem cells by MicroRNAs. *Stem Cell Rev Rep*, 14, 309-322, 2018. DOI: 10.1007/s12015-018-9808-y

7. Ge Y, Sun Y, Chen J: IGF-II is regulated by microRNA-125b in skeletal myogenesis. *J Cell Biol*, 192, 69-81, 2011. DOI: 10.1083/jcb.201007165

8. Lee EK, Lee MJ, Abdelmohsen K, Kim W, Kim MM, Srikantan S, Martindale JL, Hutchison ER, Kim HH, Marasa BS, Selimyan R, Egan JM, Smith SR, Fried SK, Gorospe M: miR-130 suppresses adipogenesis by inhibiting peroxisome proliferator-activated receptor gamma expression. *Mol Cell Biol*, 31, 626-638, 2010. DOI: 10.1128/MCB.00894-10

9. Guan L, Hu X, Liu L, Xing Y, Zhou Z, Liang X, Yang Q, Jin S, Bao J, Gao H, Du M, Li J, Zhang L: bta-miR-23a involves in adipogenesis of progenitor cells derived from fetal bovine skeletal muscle. *Sci Rep*, 7:43716, 2017. DOI: 10.1038/srep43716

10. Jebessa E, Ouyang H, Abdalla BA, Li Z, Abdullahi AY, Liu Q, Nie Q, Zhang X: Characterization of miRNA and their target gene during chicken embryo skeletal muscle development. *Oncotarget*, 9, 17309-17324, 2018. DOI: 10.18632/oncotarget.22457

11. Zhang M, Li DH, Li F, Sun JW, Jiang RR, Li ZJ, Han RL, Li GX, Liu XJ, Kang XT, Sun GR: Integrated analysis of MiRNA and genes associated with meat quality reveals that Gga-MiR-140-5p affects intramuscular fat deposition in chickens. *Cell Physiol Biochem*, 46, 2421-2433, 2018. DOI: 10.1159/000489649

12. Ma X, Wei D, Cheng G, Li S, Wang L, Wang Y, Wang X, Zhang S, Wang H, Zan L: Bta-miR-130a/b regulates preadipocyte differentiation by targeting PPARG and CYP2U1 in beef cattle. *Mol Cell Probes*, 42, 10-17, 2018. DOI: 10.1016/j.mcp.2018.10.002

13. Li F, Li D, Zhang M, Sun J, Li W, Jiang R, Han R, Wang Y, Tian Y, Kang X, Sun G: miRNA-223 targets the GPAM gene and regulates the differentiation of intramuscular adipocytes. *Gene*, 685, 106-113, 2019. DOI: 10.1016/j.gene.2018.10.054

14. Cheng X, Chen X, Wang P, Chen T, Sun J, Xi Q, Zhang Y: Exploration of targets regulated by miR-125b in porcine adipocytes. *In Vitro Cell Dev Biol Anim*, 56, 103-111, 2020. DOI: 10.1007/s11626-019-00427-3

15. Li G, Fu S, Chen Y, Jin W, Zhai B, Li Y, Sun G, Han R, Wang Y, Tian Y, Li H, Kang X: MicroRNA-15a regulates the differentiation of intramuscular preadipocytes by targeting ACAA1, ACOX1 and SCP2 in chickens. *Int J Mol Sci*, 20:4063, 2019. DOI: 10.3390/ijms20164063

16. Zhang P, Du J, Wang L, Niu L, Zhao Y, Tang G, Jiang Y, Shuai S, Bai L, Li X, Wang J, Zhang S, Zhu L: MicroRNA-143a-3p modulates preadipocyte proliferation and differentiation by targeting MAPK7. *Biomed Pharmacother*, 108, 531-539, 2018. DOI: 10.1016/j.biopha.2018.09.080

17. Velleman SG: Muscle development in the embryo and hatchling. *Poult Sci*, 86, 1050-1054, 2007. DOI: 10.1093/ps/86.5.1050

18. Chartrin P, Bernadet MD, Guy G, Mourot J, Hocquette JF, Rideau N, Duclos MJ, Baéza E: Do age and feeding levels have comparable effects on fat deposition in breast muscle of mule ducks? *Animal*, 1, 113-123, 2007. DOI: 10.1017/S1751731107658029

19. Furuhashi M, Hotamisligil GS: Fatty acid-binding proteins: Role in metabolic diseases and potential as drug targets. *Nat Rev Drug Discov*, 7, 489-503, 2008. DOI: 10.1038/nrd2589

20. O'Sullivan SE, Kaczocha M: FABP5 as a novel molecular target in prostate cancer. *Drug Discov Today*, 25, 2056-2061, 2020. DOI: 10.1016/j.drudis.2020.09.018

21. Li P, Yu M, Zhou C, Qi H, Wen X, Hou X, Li M, Gao X: FABP5 is a critical regulator of methionine- and estrogen-induced SREBP-1c gene expression in bovine mammary epithelial cells. *J Cell Physiol*, 234, 537-

549, 2019. DOI: 10.1002/jcp.26762

22. Li B, Qiao L, An L, Wang W, Liu J, Ren Y, Pan Y, Jing J, Liu W: Transcriptome analysis of adipose tissues from two fat-tailed sheep breeds reveals key genes involved in fat deposition. *BMC Genomics*, 19:338, 2018. DOI: 10.1186/s12864-018-4747-1

23. Shibue K, Yamane S, Harada N, Hamasaki A, Suzuki K, Joo E, Iwasaki K, Nasteska D, Harada T, Hayashi Y, Adachi Y, Owada Y, Takayanagi R, Inagaki N: Fatty acid-binding protein 5 regulates diet-induced obesity via GIP secretion from enteroendocrine K cells in response to fat ingestion. *Am J Physiol Endocrinol Metab*, 308, E583-591, 2015. DOI: 10.1152/ajpendo.00543.2014

24. Wang X, Shao F, Yu J, Jiang H, Gong D, Gu Z: MicroRNA-122 targets genes related to liver metabolism in chickens. *Comp Biochem Physiol B Biochem Mol Biol*, 184, 29-35, 2015. DOI: 10.1016/j.cbpb.2015.02.002

25. Vucicevic M, Stevanov-Pavlovic M, Stevanovic J, Bosnjak J, Gajic B, Aleksic N, Stanimirovic Z: Sex determination in 58 bird species and evaluation of CHD gene as a universal molecular marker in bird sexing. *Zoo Biol*, 32, 269-276, 2013. DOI: 10.1002/zoo.21010

26. Friedländer MR, Mackowiak SD, Li N, Chen W, Rajewsky N: miRDeep2 accurately identifies known and hundreds of novel microRNA genes in seven animal clades. *Nucleic Acids Res*, 40, 37-52, 2012. DOI: 10.1093/nar/gkr688

27. Wen M, Shen Y, Shi S, Tang T: miREvo: An integrative microRNA evolutionary analysis platform for next-generation sequencing experiments. *BMC Bioinformatics*, 13:140, 2012. DOI: 10.1186/1471-2105-13-140

28. Ouyang H, He X, Li G, Xu H, Jia X, Nie Q, Zhang X: Deep sequencing analysis of miRNA expression in breast muscle of fast-growing and slow-growing broilers. *Int J Mol Sci*, 16, 16242-16262, 2015. DOI: 10.3390/ijms160716242

29. Fu S, Zhao Y, Li Y, Li G, Chen Y, Li Z, Sun G, Li H, Kang X, Yan F: Characterization of miRNA transcriptome profiles related to breast muscle development and intramuscular fat deposition in chickens. *J Cell Biochem*, 119, 7063-7079, 2018. DOI: 10.1002/jcb.27024

30. Kajimoto K, Naraba H, Iwai N: MicroRNA and 3T3-L1 pre-adipocyte differentiation. *RNA*, 12, 1626-1632, 2006. DOI: 10.1261/rna.7228806

31. Wang Q, Li YC, Wang J, Kong J, Qi Y, Quigg RJ, Li X: miR-17-92 cluster accelerates adipocyte differentiation by negatively regulating tumor-suppressor Rb2/p130. *Proc Natl Acad Sci U S A*, 105, 2889-2894, 2008. DOI: 10.1073/pnas.0800178105

32. Chen C, Deng Y, Hu X, Ren H, Zhu J, Fu S, Xie J, Peng Y: miR-128-3p regulates 3T3-L1 adipogenesis and lipolysis by targeting Pparg and Sertad2. *J Physiol Biochem*, 74, 381-393, 2018. DOI: 10.1007/s13105-018-0625-1

33. Shi XE, Li YF, Jia L, Ji HL, Song ZY, Cheng J, Wu GF, Song CC, Zhang QL, Zhu JY, Yang GS: MicroRNA-199a-5p affects porcine preadipocyte proliferation and differentiation. *Int J Mol Sci*, 15, 8526-8538, 2014. DOI: 10.3390/ijms15058526

34. Liu L, Xiao Q, Gilbert ER, Cui Z, Zhao X, Wang Y, Yin H, Li D, Zhang H, Zhu Q: Whole-transcriptome analysis of atrophic ovaries in broody chickens reveals regulatory pathways associated with proliferation and apoptosis. *Sci Rep*, 8:7231, 2018. DOI: 10.1038/s41598-018-25103-6

35. Wang Y, Brahmakshatriya V, Zhu H, Lupiani B, Reddy SM, Yoon BJ, Gunaratne PH, Kim JH, Chen R, Wang J, Zhou H: Identification of differentially expressed miRNAs in chicken lung and trachea with avian influenza virus infection by a deep sequencing approach. *BMC Genomics*, 10:512, 2009. DOI: 10.1186/1471-2164-10-512

36. Dallaglio K, Marconi A, Truzzi F, Lotti R, Palazzo E, Petrachi T, Saltari A, Coppini M, Pincelli C: E-FABP induces differentiation in normal human keratinocytes and modulates the differentiation process in psoriatic keratinocytes in vitro. *Exp Dermatol*, 22, 255-261, 2013. DOI: 10.1111/exd.12111

37. Descalzi Cancedda F, Dozin B, Zerega B, Cermelli S, Cancedda R: Ex-FABP: A fatty acid binding lipocalin developmentally regulated in chicken endochondral bone formation and myogenesis. *Biochim Biophys Acta*, 1482, 127-135, 2000. DOI: 10.1016/s0167-4838(00)00159-x

38. Yu CW, Liang X, Lipsky S, Karaaslan C, Kozakewich H, Hotamisligil GS, Bischoff J, Cataltepe S: Dual role of fatty acid-binding protein 5 on endothelial cell fate: a potential link between lipid metabolism and angiogenic responses. *Angiogenesis*, 19, 95-106, 2016. DOI: 10.1007/s10456-015-9491-4

39. Ma X, Ren X, Han P, Hu S, Wang J, Yin J: SiRNA against Fabp5 induces 3T3-L1 cells apoptosis during adipocytic induction. *Mol Biol Rep*, 37, 4003-4011, 2010. DOI: 10.1007/s11033-010-0059-5

40. Estellé J, Pérez-Enciso M, Mercadé A, Varona L, Alves E, Sánchez A, Folch JM: Characterization of the porcine FABP5 gene and its association with the FAT1 QTL in an Iberian by Landrace cross. *Anim Genet*, 37, 589-591, 2006. DOI: 10.1111/j.1365-2052.2006.01535.x