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RESEARCH ARTICLE

Fat Metabolism-related lncRNA and Target Regulation and Application Studies in Chickens

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ABSTRACT

Lipids are essential for cellular and organismal life. These make up a significant portion of cell membranes, act as energy reserves and are also signal molecules. In both humans and more distant species like chickens, the liver and adipose tissue serve as the primary organs for energy synthesis and storage. It is now well accepted that Long noncoding RNAs (lncRNAs) contribute to many different types of biological processes, including lipid metabolism. The key elements influencing poultry slaughter performance and meat quality are the distribution of adipose tissue-specific components and the rate of fat deposition. Previous research revealed that the physicochemical properties and gene expression patterns of diverse adipose tissues varied. To evaluate the role of lncRNAs play in chicken abdominal and intramuscular adipogenesis, transcriptome analysis was carried out by Ribo-Zero RNA-Seq technology. The lncRNAs expression profiles were investigated during different development stages of the Gushi chicken i.e. 7, 14, 21 and 28 weeks. Among the screened lncRNA profiles, 77.44 % lncRNAs were identified by all three databases i.e. CNCI (Coding-Noncoding Index), CPC (coding potential calculator), and PFAM (protein family's database). The intergenic region was home to eighty percent of these long noncoding RNAs (lncRNA). Another goal of this study was to gain a better understanding of the role of lncRNAs in the abdominal and intramuscular adipogenesis in Gushi chickens, differentially expressed (DE) lncRNAs were further characterized. The isolated lncRNAs were compared at various stages of development of Gushi chicken. Four randomly selected DE-lncRNAs were validated using qRT-PCR and compared via RNA-seq sequencing. Several biological processes, such as cell adhesion, fatty acid production, and epigenetic gene regulation, were found to be enriched by DE-lncRNAs, as revealed by functional enrichment analysis. Altogether, our findings open out the known lncRNAs in Gushi chickens. This will provide useful resources for future research into the posttranscriptional regulators responsible for tissue-specific adipogenesis and for locating adipogenic lncRNAs in Gushi chickens.

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INTRODUCTION

Based on the organ / source, adiposes tissues differ in terms of their biochemical and physiological characteristics. Multiple studies have demonstrated that, in contrast to the characteristics of belly fat, IMF (intramuscular fat) has distinct biological characteristics, including hormonal responsiveness, fatty acid composition and glucose utilization, as well as the ability to provide energy to the body (Chu *et al.*, 2017; Han *et al.*, 2017). The accumulation of chronological fat and fat specific to tissues is hypothesized to be the result of numerous complex regulation processes. Many transcriptional factors (PPAR, CCAAT / enhancer binding proteins) and adipogenic genes are used to precisely control adipogenesis (Zhou *et al*., 2010; Sun *et al*., 2013).

PPAR and C/EBP have been shown to promote triglyceride (TG) storage and lipid accumulation by activating adipogenic-specific genes like fatty acid synthase (FASN), fatty acid binding protein 4 (FABP4), sterol regulatory element-binding proteins-1c (SREBP-1c), adiponectin, C1Q and collagen domain containing

(ADIPOQ) (Ferré *et al*., 2010; Iverson *et al*., 2013; Li *et al*., 2016; Mellouk *et al*., 2018; Zhang *et al*., 2020).

The term "long noncoding RNA" (lncRNA) refers to RNAs that are 200 nucleotides or longer but cannot encode a genetic code, meaning they cannot be used to make proteins (Sun *et al*., 2013, Chen *et al*., 2018). According to mounting evidence, Cancer, disease, animal development, and cell differentiation are just a few of the many biological processes in which lncRNAs play a key function. The role of lncRNAs appears to be multifaceted as summarized in Fig. 1 (Yang *et al*., 2014; Sui *et al*., 2019; Chen and Shen, 2020). There has been a significant rise in the uncovering of long noncoding RNAs in mammals that play a role in lipid metabolism and adipogenesis. SRA, also known as steroid receptor RNA activator, was discovered to be one of the early identified mediators of the adipogenic process (Zhang *et al*., 2020). Adipogenesis is promoted by PU.1 AS lncRNA (Antisense long noncoding RNA) binding to PU.1 mRNA to form mRNA/AS lncRNA duplexes in preadipocytes (Pang *et al*., 2013). Adipogenic differentiation induced noncoding RNA (ADINR) is responsible for regulating adipogenesis. It does this by increasing the amount of histone modification on the C/EBP promoter by its specific binding to PA1 (Liu *et al*., 2018; Xiao *et al*., 2021).

Increasing research efforts are uncovering the role of long noncoding RNAs (lncRNAs) in the adipogenic growth process in humans as well as in animals (Guil and Esteller, 2012; Zhang *et al*., 2019). Nevertheless, to the best of our knowledge, the lncRNAs responsible for tissue-specific variations in adipogenesis in chickens remain unknown.

In China, the Gushi chicken is a well-known native breed for both its meat and its eggs, making it a popular choice for both breeding stock and commercial production. The average annual egg production for this breed is 180, and sexual maturity is attained at 20 weeks. Peak egg production occurred between 27 and 35 weeks of age. Moreover, due to the excellent quality of its meat, this species is commonly used for meat breeding purposes also. Due to the established link between abdominal fat accumulation and meat quality features, researchers have been interested in elucidating the role and various pathways employed by lncRNA in the development of these qualities. Throughout the course of this study, we analyzed lncRNAs expression in the abdomen adipose tissue of Gushi hens across various ages under experimental conditions. The findings of this study may be useful for guiding future investigations into poultry adipogenesis.

MATERIALS AND METHODS

Ethics Statement: The Animal Experimentation Management Regulations were adhered to in every aspect of the care that was provided for the animals involved in the current research study (Ministry of Science and Technology of China, 2004).

Sample Collection/Sampling: The Avian Farm of Henan Agriculture University provided the experimental Gushi chicken for all the experiments (Zhengzhou, Henan, China). Fifty female one-day-old Gushi chicks were reared and nurtured under same habitat. Water was constantly available to the birds, and they always had free access to feed. For this experiment, three healthy chicks were selected randomly at ages 7, 14, 21 and 28 weeks.

The selected chickens were put through euthanasia by giving a KCl (1-2 mg/kg) intravenous injection while they were under general anesthesia. After being collected, all the tissues utilized in the experiment were frozen in liquid nitrogen as soon as possible. These samples were stored at a temperature of -80ºC until the RNA extraction process.

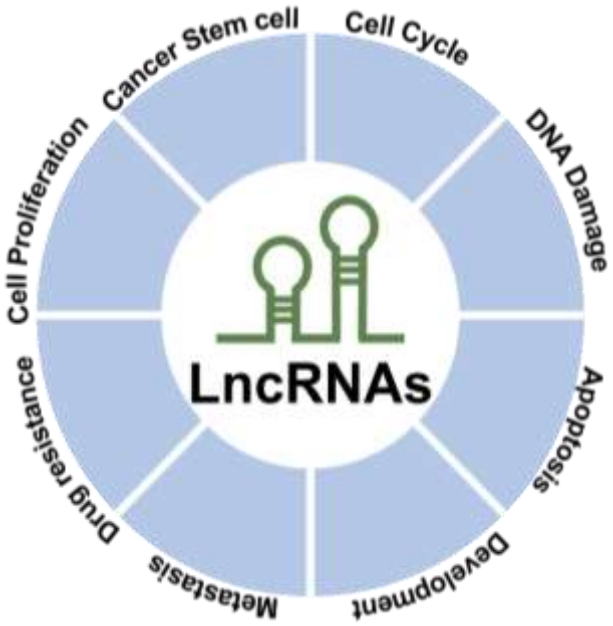

Fig. 1: Roles of lncRNA identification Lon noncoding RNAs are essential for various critical function including regulation of cell cycle, DNA damage, apoptosis, development, cancer cell metastasis, drug resistance, cell proliferation, cancer stem cell etc. (adapted from Chen and Shen, 2020).

Ribo-Zero RNA-Seq based Sequencing Analysis: Using the TRIzol reagent, total RNAs were in vitro extracted from three biological replicates of chicken preadipocytes and adipocytes (TaKaRa, Dalian, China). The isolation of total RNA was caried out by RNase-Free Dnase Set from QIAGEN, GmBH, Hilden, Germany, and the product was subjected to RNA purification using RNA Clean XP Kit from Beckman Coulter, Brea, CA, USA. To remove the rRNA, we employed the Ribo- Zero rRNA Removal Kit (Epicentre, Madison, Wisconsin, USA). Using an Illumina TruSeq RNA Sample Prep Kit, the libraries were made according to the manufacturer's guidelines. Using the 2100 Bioanalyzer, the RNA integrity number (RIN value >8) was confirmed (Agilent Technologies, Santa Clara, CA, USA). The Illumina HiSeq 2500 equipment was then used to sequence eight libraries. Fastp was used to generate the paired-end readings and assess the quality of the raw data. Clean reads were obtained after removing reads of multimers, reads of the adapter and low-quality reads using Ensemble that agreed quality control were plotted to the *Gallus gallus* genome-5.0 using Hisat2 (2.0.4) (Zhao *et al*., 2014; Kim *et al*., 2015; Zhang *et al*., 2020).

LncRNA Identification: LncRNAs were filtered based on the following criteria: (1) There were five different transcript classes namely "i", "j", "x", "u", and "o"; (2) Read counts below three were discarded, and transcripts

having an open reading frame (ORF) of 100 amino acids or less and 200 nucleotides or less in length were included; (3) Alignment of transcripts to protein domains was performed using the Swiss-Protein and Pfam databases; (4) Protein-coding mRNAs were screened for overlap and deleted transcripts that overlapped them; and (5) The Coding-Noncoding Index (CNCI), Coding protein families database (PfamScan), and Potential Calculator-2 (CPC2) were used to determine the transcripts' ability to code for proteins (Luo *et al*., 2014; Zhai *et al*., 2021).

Functional and variation analysis of DE-IncRNAs: Using Cuffldiff software, the expression amounts of lncRNAs were estimated by FPKM (Fragments Per Kilobase Million) (Trapnell *et al*., 2013). The criterion of p value 0.05 and fold change (FC) 2 was used to screen differentially expressed lncRNAs (DE-lncRNAs). Genomic analysis was 581avid581ed to identify the cis target genes of lncRNAs, which were frequently within 100 kb of the lncRNA. LncTar programme predicted the lncRNA's trans target genes (Li *et al*., 2015). To learn more about lncRNAs' roles, we performed co-expression analysis between the differentially expressed genes and the DE lncRNAs (Li *et al*., 2019). A significant link was defined as one with a p-value of 0.05 or less and a Pearson correlation coefficient of higher than 0.95. Networks of lncRNA and mRNA were viewed using Cytoscape 3.4.036 (Lopes *et al.*, 2010). For this study, we used the DAVID (http://david.abcc.ncifcrf.gov/) web-based application to examine pathways for functional enhancement in the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO).

Quantification and characterization of total RNA vis RT-qPCR: Using RNAiso, the total RNA was isolated (Takara, Dalian, China). Takara's PrimeScript RT reagent Kit was used to create cDNA in accordance with the manufacturer's instructions. Primer3plus programme is responsible for creating the RT-qPCR primers (Zhao *et al*., 2014; Zhang *et al*., 2020). In order to conduct RT-qPCR, Takara's SYBR Premix Ex Taq II kit was utilized. The reference gene GAPDH was chosen, and the 2Ct technique was used to determine the gene expression level.

Statistical Analysis: The results were examined using SPSS 22.0. (SPSS Inc., Chicago, IL, USA). Two groups were evaluated using two-tailed t tests in the current study, and the findings were presented as mean SEM. P values of 0.05, 0.01, and 0.001 were denoted, respectively, by one asterisk (*), two asterisks (*), and three asterisks (*).

RESULTS

Characteristics and Identification of lncRNAs: In the current work, eight cDNA libraries were built to identify lncRNAs linked to adipogenic differentiation at four developmental stages in Gushi chickens. Each library had the GC content ranging from 47.36 to 51.48%. Based on the results of the comparative study, between 90.01 and 96.42 percent of the reads were mapped to the reference genome. The transcripts sequenced from each library were combined using StringTie merging software, and the combined transcripts were subsequently analyzed and annotated. Among the confirmed lncRNAs, 77.44% of the lncRNAs were confirmed by three databases (CPC, CNCI, and PFAM). Moreover, it was observed that 80% of lncRNA were in intergenic region, 11% were sense intronic, 8% lncRNA reads were antisense lncRNAs, and 1% lncRNAs were sense overlapping reads as described in Fig. 2.

Genomic Analysis and Characterization of IncRNAs: To fully investigate the characteristics of lncRNAs, we compared the genomic properties of novel lncRNAs to those of existing lncRNAs and mRNAs. New lncRNAs were less common than mRNAs but had higher expression levels (FPKM) than existing lncRNAs. In comparison to mRNAs, the new lncRNAs have a lower coding potential score, while other known lncRNAs have a similar coding potential score. In line with lncRNAs, new lncRNAs exhibited a reduced exon-to-mRNA ratio as the bovine adipocytes developed. Chicken new lncRNA lengths were also shorter than mRNA lengths.

Differentially Expressed (DE) lncRNAs during developmental stages: Expression of DE-lncRNAs were analyzed during different developmental stages of Gushi chicken, namely, 7w (7-weeks), 14w (14-weeks), 21w (21-weeks), and 28w (28-weeks). The analysis of lncRNAs depicted that 65 lncRNAs were DE-lncRNAs between w7 vs w14 group, 84 lncRNAs were DE-lncRNAs between w7 vs w21 group, 136 lncRNAs were DE-lncRNAs between w7 vs w28 group, 56 lncRNAs were DE-lncRNAs between w14 vs w28 group, and 65 lncRNAs were DE-lncRNAs between w21 vs w28 group. The results have been summarized using Venn diagram (Fig. 3) to compare the DE-lncRNAs in different groups. Finally, Venn diagram analysis described that only 1 lncRNA was common among the five comparison groups.

The qRT-PCR Verification of DE-lncRNA: Randomly selected 4 DE-lncRNAs were confirmed using qRT-PCR. The relative expression of the DE-lncRNA was confirmed by qRT-PCR and expression pattern was confirmed by FPKM from RNA-Seq. The results verified that expression readings of these DE-lncRNAs were consistent with relative expression during different developmental stages of Gushi chickens (Fig. 4).

Identification and Functional Analysis of Chicken DE-lncRNAs: To further explicate the likely significance of the DE-lncRNAs in the abdominal adipogenesis in Gushi chickens, their predicted cis- and trans-regulatory target genes and functional enrichment analysis were carried out. The cis- and trans-regulatory target genes of the DE-lncRNAs were projected, and functional enhancement analysis was performed to further clarify the probable role of the DE-lncRNAs in the abdominal adipogenesis in Gushi chickens. Different biological processes were enriched by cis-regulated target genes including cell adhesion and epigenetic gene regulation. Moreover, trans-regulated target genes enrichment includes biological processes like fatty acid biosynthesis and fatty acid metabolism.

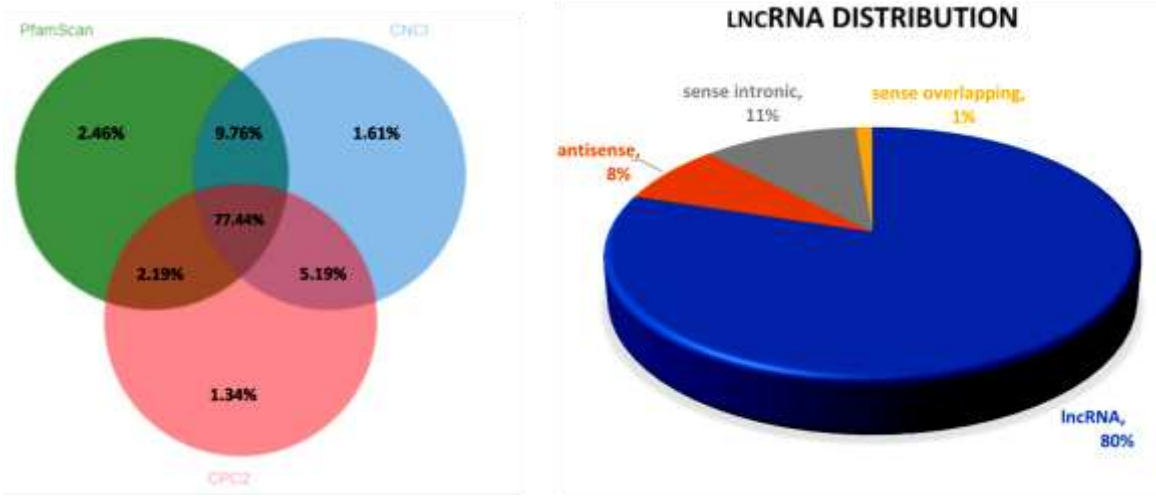

Fig. 2: lncRNA identification lncRNA identification using PFAM, CNCI, and CPC lncRNA distribution using transcript classification.

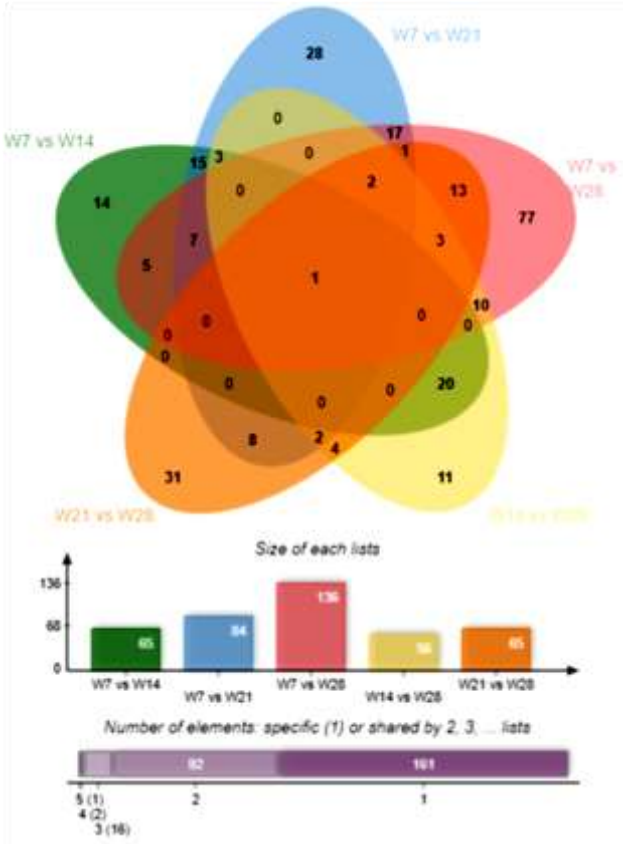

Fig. 3: Venn diagram of lncRNAs among 5 comparison groups in various developmental stages of Gushi chicken abdominal adipose tissue.

DISCUSSION

The metabolic energy of chickens and their ability to maintain homeostasis are significantly impacted by the presence of adipose tissue (Rudas and Scanes, 1983; Zhang *et al*., 2019; Zhang *et al*., 2020). Abdominal fat is often excised as a byproduct in the poultry industry. However, accumulation of these fat deposits in abdomen increases the feed cost and reduces palatability. On the contrary, IMF content improves taste, juiciness, and tenderness (Zhou *et al*., 2010) but hormonal responsiveness has negative effects on carcass quality and slaughter effectiveness (Jiang *et al*., 2017). It is believed that preadipocyte proliferation and differentiation have a big impact on adipogenesis (Zhang *et al*., 2019).

Many biological activities, including gene expression, cellular division, differentiation, and death, are controlled by long noncoding RNAs. In addition, recent research has revealed that lncRNAs play a crucial part in triglyceride (fat) synthesis in animals. The temporal expression properties of several lncRNAs in animals and their roles in the control of certain biological activities are well established. The identification of DE-lncRNAs in this study showed their specificity during different developmental stages of Gushi chickens. Cell proliferation mechanisms were associated with isolated DE-lncRNAs in the w7 vs w14 comparison group. Lipogenesis associated mechanisms were associated with DE-lncRNAs isolated in the w7 vs w21 comparison group. Moreover, fatty acid synthesis and fatty acid metabolism mechanisms were associated with DE-lncRNAs isolated in the w21 vs w28 comparison group. It can be concluded that fat pads in Gushi chicken are filled with fat after the age of 21 weeks which lines up with the way Gushi chickens tend to look when they're full of fat. After reaching adulthood, Gushi chicken devotes most of its energy from the feed to producing eggs, leaving very little energy for fat storage. Therefore, the deposition of fat before w28 may help during peak egg production.

There is mounting proof that lncRNAs significantly affect adipogenesis. Insight into the part of lncRNA in the regulation of adipogenesis facilitates research into lipid accumulation and the formation of adipose tissue. In this study, we thoroughly examined the genomic lncRNA atlas during chicken adipogenic development. Intricate mechanisms are involved in regulating lncRNA expression. MicroRNAs (miRNAs) have been found to have their inhibitory effect on their target genes redirected by cytoplasmic lncRNA, which acts as a molecular sponge (Yang *et al*., 2020). Together, messenger RNA (mRNA), long non-coding RNA (lncRNA) and microRNA (miRNA) regulate gene expression at the transcription and posttranscriptional levels. Therefore, these act synergistically to control phenotypic features. The majority of lncRNAs, according to the analysis of the prediction study, were found to be in the cytoplasm.

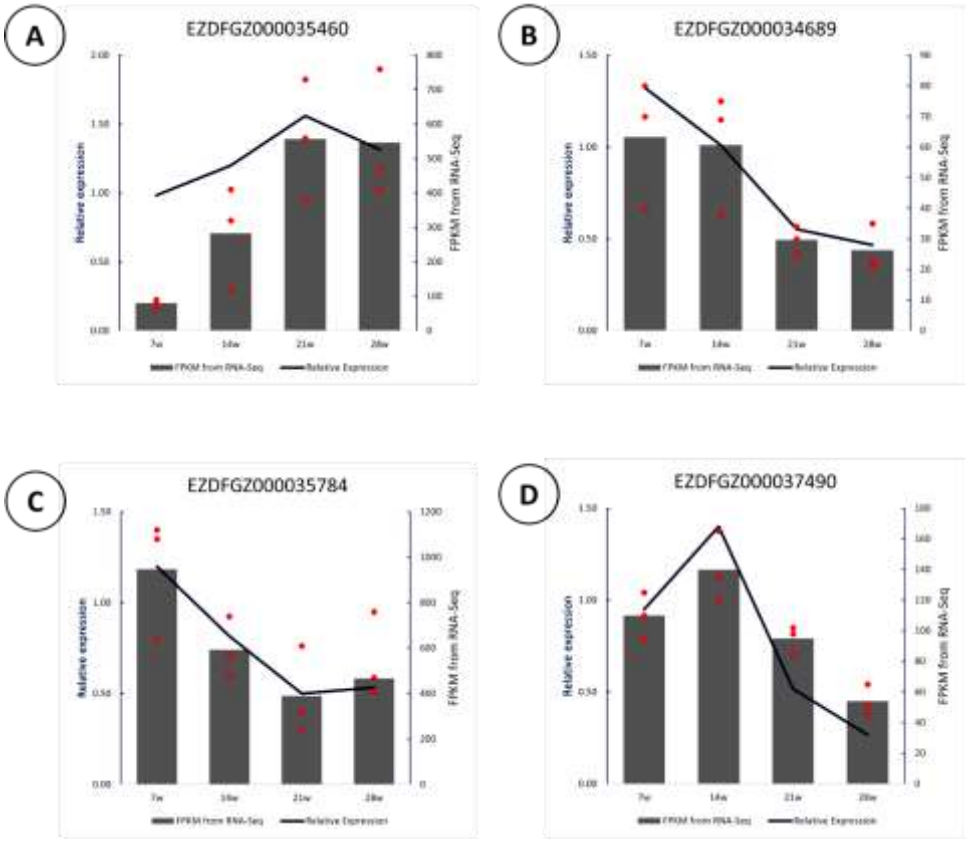

Fig. 4: The qRT-PCR verification of DE-lncRNA. Line graphs represent the qRT-PCR results (relative expression) and bar graphs represent the sequencing FPKM results

Conclusions: Altogether, we analyzed 272 DE-lncRNAs that exhibited traits comparable to those of known lncRNAs, including fewer exons, shorter lengths, and lower expression levels when evaluated to those of protein-coding genes. We discovered that whereas certain lncRNAs revealed comparable gene expression patterns throughout the adipogenic development in various adipose tissue-derived preadipocytes, others demonstrated that intramuscular and abdominal preadipocytes have unique patterns of gene expression. Moreover, analysis of DE-lncRNA during various developmental stages revealed that these lncRNA are associated with regulation of cell adhesion, fatty acid production, and posttranslational gene regulation in Gushi chickens.

Authors contribution: Designed and Performed the experiments: WX. Analyzed the data and wrote the manuscript: SL

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