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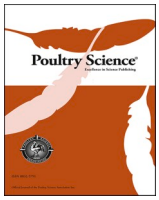

Transcriptome analysis of multiple tissues and identification of tissue-specific genes in Lueyang black-bone chicken

Pan Li [a,1], Yufei Yang [a,1], Bo Ning [a], Yingmin Tian [b], Ling Wang [a,c,d,e], Wenxian Zeng [a,c,d,e], Hongzhao Lu [a,c,d,e], Tao Zhang [a,c,d,e,*]

[a] School of Biological Science and Engineering, Shaanxi University of Technology, 723001 Hanzhong, China
[b] School of Mathematics and Computer Science, Shaanxi University of Technology, 723001 Hanzhong, China
[c] Engineering Research Center of quality improvement and safety control of Qinba special meat products, 723001 Hanzhong, China
[d] QinLing-Bashan Mountains Bioresources Comprehensive Development C. I. C, Shaanxi University of Technology, 723001 Hanzhong, China
[e] Qinba State Key Laboratory of Biological Resources and Ecological Environment, Shaanxi University of Technology, 723001 Hanzhong, China

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ABSTRACT

Systematically constructing a gene expression atlas of poultry tissues is critically important for advancing poultry research and production. In this study, the gene expression profiles of 9 major tissues of Lueyang black-bone chicken were successfully constructed by transcriptome sequencing technology. Through in-depth analysis of transcriptome data, a total of 10 housekeeping genes (HKGs) and 87 marker genes (MGs) were identified. Furthermore, by applying weighted gene co-expression network analysis (WGCNA), we delineated nine tissue-specific modules and 90 hub genes, offering novel insights into the regulatory networks underlying tissue-specific gene expression. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis showed that HKGs were predominantly involved in maintaining fundamental cellular functions, with significant enrichment in pathways related to oxidative phosphorylation, cell cycle regulation, and DNA replication. MGs were closely associated with tissue-specific physiological functions, providing valuable insights into the molecular mechanisms governing tissue functionality. Notably, through multidimensional validation, *EEF1A1* and *FTH1* were confirmed to exhibit cross-tissue expression stability, establishing them as ideal reference genes for multi-tissue qPCR experiments in chickens. Additionally, we successfully identified tissue marker genes, including *TNNT2, PIT54, SFTPC*, and PGM1, which are specific to the heart, liver, lung, and breast muscle, respectively. The results of this study have important scientific value in expanding reference gene selection and elucidating tissue-specific molecular mechanisms, and provide solid theoretical support and technical guidance for poultry breeding improvement and production practice optimization.

Introduction

Poultry, domesticated birds raised by humans, serve as a vital source of meat, eggs, and feathers, contributing significantly to human lifestyle in areas such as diet, clothing, entertainment, and the social economy (Lee, 2021; Phanprasit et al., 2024). Among poultry species, chickens are the most widely raised, which have been domesticated for more than 4000 years. Through selective breeding for traits such as weight (Sinpru et al., 2021), plumage color (Khumpeerawat et al., 2021) and egg-laying ability (Isa et al., 2020), people have developed a wide variety of distinctive local chicken breeds (Mancinelli et al., 2023). With the development of science and technology, particularly in genomics and artificial intelligence, it is expected to further strengthen the breeding, genetic improvement, disease prevention and scientific breeding practices of new chicken varieties (Krasikova et al., 2024; Z. Pan et al., 2023). The normal development, mutual coordination and healthy function of the various tissues and organs of poultry underpin their stable and efficient production. Therefore, it is crucial for poultry research and production to systematically construct gene expression profiles of various tissues of poultry. Understanding gene expression

* Corresponding author at: School of Biological Science and Engineering, Shaanxi University of Technology, 723001 Hanzhong, China.
E-mail addresses: liipan0321@163.com (P. Li), yangfeifeimm@163.com (Y. Yang), ningbo990217@163.com (B. Ning), tianym1994@163.com (Y. Tian), wangling619@163.com (L. Wang), zengwenxian2013@126.com (W. Zeng), lhz780823@snut.edu.cn (H. Lu), zl780823@163.com (T. Zhang).
[1] These authors have contributed equally to this work and share first authorship.

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regulation patterns, identifying tissue-specific genes and marker genes (**MGs**) are key steps in advancing poultry breeding and health management.

Transcriptome sequencing technology, which has matured significantly, is increasingly combined with machine learning and artificial intelligence. It has become a core strategy for investigating biological mechanisms such as the origins and evolution of animals, growth and development processes, and disease occurrence and development at the RNA level (Shokhirev et al., 2022; Zaitsev et al., 2022). Using transcriptome sequencing technology to analyze multi-tissue gene expression can obtain rich information on genome dynamic expression and gene regulation patterns, and provide more detailed insights into the genetic basis of breed formation. It has been widely applied in various species, including humans (D. Wang et al., 2019), mice (Isakova et al., 2020), cattle (Liu et al., 2022) and pigs (Teng et al., 2024). For example, T. Zhang et al. (2022) constructed a large-scale gene expression profile for the main tissues in beef cattle and identifying subgroups of genes with differential expression between beef and dairy cattle. Teng et al. (2024) explored the effects of multi-tissue genetic regulation in pigs by integrating RNA sequencing data with whole-genome information. In poultry, Jehl et al. (2020) analyzed long non-coding RNA expression profiles across chicken tissues using data from multiple public databases. Degalez et al. (2024) provided a gene atlas rich in long non-coding RNA for GRCg7b chicken assembly, characterizing their tissue-specific and sex-differential expression. In addition, RNA-seq technology has been used to screen out a range of reliable reference genes in a variety of chicken tissue and cell lines, including Leghorn Male Hepatoma cell line, chicken liver (Z. Chen et al., 2024), different segments of the chicken oviduct and the entire oviduct (Shu et al., 2024). Despite these advancements, poultry research lags behind that of humans, mice, and other livestock species. Several challenges in chicken research remain: (1) Widely used reference genes, such as *GAPDH*, exhibit inconsistent expression in multiple tissues of chicken, which affects the accuracy of the research results (Mozdziak et al., 2003; Yin et al., 2011). (2) Tissue marker genes, tissue-specific genes, and associated signaling pathways in different chicken tissues have not been systematically characterized. (3) Comprehensive analysis of the uniformity and differences in gene expression patterns among tissues, and inter-tissue dependencies is still underexplored.

In this study, Lueyang black-bone chickens, a local breed in Shaanxi Province, China, were sampled from 9 major tissues and organs for transcriptome sequencing. Multi-tissue transcriptional profiles were constructed to characterize the gene expression patterns of different tissues. As a traditional local breed with abundant genetic resources, the Lueyang black-bone chicken has not been subjected to hybridization with introduced breeds, retaining relatively ancient genetic information. This makes it an ideal model for poultry genetic research. Constructing a multi-tissue expression profile of Lueyang black-bone chicken, new reference genes, tissue-specific genes, and tissue marker genes applicable to chickens were identified and validated in other breeds. The findings of this study provide foundational data and a valuable reference for poultry research and production. Additionally, these results offer insights that can be utilized in the development of poultry-related disease models.

Materials and methods

Experimental animals and sample collection

All the experimental Lueyang black-bone chickens involved in this study were from Heihejia Agricultural Development Co., Ltd (Lueyang County, Hanzhong City, Shaanxi Province, China). All animal experimental procedures were approved and conducted in accordance with the guidelines of the Shaanxi Province Animal Care and Use Committee.

Three 3-month-old female Lueyang black-bone chickens (**LC**) were selected for sample collection (Table S1). Bursa of Fabricius (**BF**), Breast muscle (**BM**), Heart (**HE**), Kidney (**KI**), Liver (**LI**), Lung (**LU**), Ovary (**OV**), Skin (**SK**), Spleen (**SP**) were stored in ultra-low temperature refrigerator for sequencing and data verification. To validate the suitability of the selected housekeeping genes and to account for potential sex-related biological variations that might influence tissue-specific gene expression, the experimental verification incorporated both female and male chicken samples. One female and one male were selected from7-month-old Lueyang black-bone chickens, 1-month-old Liangfenghua broilers (**LFH**), 2-month-old Liangfenghua broilers, 4-month-old Liangfenghua broilers and 2-month-old Silkies **(SI)**. Additionally, to specifically evaluate the expression patterns of the screened genes in the male reproductive system, testicular tissue samples were included in the analysis. The experimental verification encompassed a total of 10 distinct tissue types: bursa of Fabricius, breast muscle, heart, kidney, liver, lung, ovary, skin, spleen and testis.

Histological analysis

Nine fresh tissue samples were fixed in 4% paraformaldehyde, embedded in paraffin, cut into 4 μm sections, and then stained with hematoxylin and eosin (**H&E**) according to routine protocols. The samples were observed under 4x and 20x magnification using an inverted microscope (Olympus BX53), and images were collected using computer-assisted microscopy.

RNA extraction, library construction and sequencing

Total RNA was extracted using a Trizol kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The quality of RNA was evaluated by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, California, USA), and the purity and integrity of RNA were checked by agarose gel electrophoresis without RNase. After total RNA was extracted, mRNA was enriched by Oligo(dT) beads. Then the enriched mRNA was fragmented into short fragments using fragmentation buffer and reversely transcribed into cDNA by using NEBNext Ultra RNA Library Prep Kit for Illumina (NEB #7530,New England Biolabs, Ipswich, MA, USA). The double-stranded cDNA was purified and then end-repaired. A base added, and ligated to Illumina sequencing adapters. The ligation reaction was purified using AMPure XP beads (1.0X) and amplified by polymerase chain reaction (**PCR**). The resulting cDNA library was sequenced using Illumina Novaseq6000 by Gene Denovo Biotechnology Co. (Guangzhou, China).

Sequencing data processing

Fastp(version 0.18.0) is used for quality control of raw data (S. Chen et al., 2018). clean reads were obtained by removing reads containing ploy-N (N≥10 %), low-quality reads containing more than 50 % of low-quality bases (q value ≤ 20) and those containing adapter. Reads was compared with the rRNA database using the comparison tool Bowtie2 (version 2.2.8) (Langmead et al., 2012). The reading of the rRNA map will then be removed. The remaining clean reads were further used for assembly and gene abundance calculations. HISAT2.2.4 was used to locate clean reads to the reference genome with the parameters set to default values (Kim et al., 2015). The reference genome data source is the Ensembl database (http://asia.ensembl.org/) and the reference genome information is Chicken (Red Jungle fowl) (GRCg6a). StringTie v1.3.1 is used to assemble the mapped read of each sample (Pertea et al., 2015). For each transcription region, TPM values were calculated using RSEM software to quantify its expression abundance and variation (Li et al., 2011). R package gmodels (http://www.rproject.org/) was used for principal component analysis (**PCA**) of samples to reveal the relationships among samples. The correlation between samples was calculated using R language and quantified by Pearson correlation coefficient. The higher the similarity between two samples, the closer the correlation coefficient is to 1.

Screening for housekeeping genes

Housekeeping genes (**HKGs**) are those that are constitutively expressed in all tissue/cell types (Zhu et al., 2008). We screened co-expressed genes, which are expressed in all tissues (TPM > 1). GO and KEGG enrichment analysis were performed. To investigate the expression changes of HKGs in the expression profile of Lueyang black-bone chicken, the coefficient of variation (**CV**) was used to evaluate the degree of variation of each gene (de Jonge et al., 2007). The calculation formula is CV = σ/μ. σ represents the standard deviation and μ represents the mean value. To identify HKGs exhibiting superior expression levels and stability compared to the commonly used reference genes *ACTB* and *GAPDH*, we established stringent screening criteria based on the transcriptomic data from multiple tissues in this study. Specifically, candidate HKGs were required to meet two key thresholds: TPM > 1000 and CV < 0.15 in each tissue. These criteria were derived from the TPM values of *ACTB* and *GAPDH* observed in the multi-tissue transcriptome.

In this study, the RefFinder online analysis platform (https://blooge.cn/RefFinder/, accessed on February 16, 2025) was utilized to assess the stability of five internal candidate genes. The platform serves as a comprehensive analytical tool, incorporating the comparison of △Ct, BestKeeper, NormFinder, and geNorm, four major algorithms, through a multidimensional calculation model to generate the stability ranking of reference genes (Xie et al., 2023).

Screening of tissue-specific genes and marker genes

Tissue-specific genes (**TSGs**) refers to genes that are highly expressed in one specific tissue type but not expressed or expressed at a very low level in other tissues (She et al., 2009). Based on this, we screened two types of TSGs. One is that genes are expressed only in one tissue, which we call particular TSGs. The screening criteria are genes with TPM < 1 in other tissues and TPM ≥ 1 in a specific tissue. The other category is highly expressed TSGs, which refers to genes that are highly expressed in certain tissues but less expressed in other tissues. Tissue-specific expression of genes means that they have tissue-related functions and can be used as marker genes for specific tissues (El Amrani et al., 2015). Therefore, we used high-expression TSGs as marker genes for specific tissues. We used strict criteria to screen for highly expressed TSGs (She et al., 2009). The following criteria were included: (1) the gene expression level (TPM) of the gene in one tissue was greater than 1000, and more than three times that of other tissues, (2) the TPM value of candidate gene target tissue was greater than 50 % of the average expression level in all other tissues, and (3) the expression level of the candidate gene was at the top 25 % of all genes in each tissue. Gene ontology (**GO**) and Kyoto Encyclopedia of Genes and Genomes (**KEGG**) enrichment analysis of particular TSGs and co-expressed genes was performed based on an online platform (https://www.omicshare.com/tools/).

Weighted gene co-expression network analysis

Genes with standardized TPM > 1 were used for weighted gene co-expression network analysis (**WGCNA**). Co-expression networks were constructed using WGCNA (v1.47) package in R (Langfelder et al., 2008). Cluster analysis adopts cluster function and average cluster method. The appropriate soft-thresholding power (β) was selected based on the criterion of approximate scale-free topology criterion. Then, the one-step method was used to construct the network and detect the modules. We set the parameter minModuleSize to 30 and mergeCutHeight to 0.25 (B. Wang et al., 2022). Pearson correlation was used to quantify the phenotypic data of different tissues of Lueyang black-bone chickens and gene modules. The modules with Pearson correlation coefficient (r) > 0.65 and $P < 1.0E\text{-}4$ were considered to be tissue specific modules. GO and KEGG enrichment analysis was performed for these modules. Then, the generated networks were visualized, and the degree value was calculated by Cytoscape (v3.9.1). The top 10 genes in each module based on degree were identified as hub genes.

Real-time quantitative PCR and PCR analysis of HKGs and marker genes

To verify the HKGs and MGs, we selected 3 HKGs and 9 MGs for RT-qPCR and PCR. Total RNA from heart, liver, spleen, lung, kidney, skin, ovary, testis, breast muscle and bursa of Fabricius were extracted by Trizol method. The RNA was reverse-transcribed into cDNA using EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix (Transgen Biotech, Beijing, China), and then RT-qPCR was performed in SLAN-96S (Shanghai Hongshi Medical Technology, Shanghai, China). The information of primers was listed in Table S2. The $2^{-\Delta\Delta Ct}$ method was used to transform the Ct values. T-test was used to evaluate the results. Three samples were used for RT-qPCR experiments, and three techniques were repeated for each sample. *ACTB* was used as an reference to normalize the gene expression level.

The PCR amplification system was 10 μL, including 5 μL 2×EasyTaq PCR Super MIX (Transgen Biotech, Beijing, China), 1 μL template, and 0.2 μL upstream and downstream quantitative primers. Fill with nuclease-free water to 10 μL. Amplification was performed in Biometra TAdvanced Twin (Jena, Germany). PCR amplification products were analyzed at 80V by electrophoresis on 2 % agarose gel in 1 × TAE buffer for 50 min. Agarose gels were observed under the Gel Imaging Analysis System (UVItec LTD., Cambridge, United Kingdom). The PCR assay involved in this study was repeated at least twice (n ≥ 2).

Data processing and statistical analysis

The data results in this study are at least three replicates, expressed as mean ± SD. The data was analyzed by GraphPad Prism 8 (GraphPad Software, San Diego, CA). Statistical evaluation was performed by using a Student's t test and ANOVA. A *P* value of less than 0.05 was considered statistically significant. Statistical significance is indicated by * or *P* value. * represents $P < 0.05$; ** represents $P < 0.01$, and *** indicates $P < 0.001$.

Results

Histological analysis and RNA-seq data quality control

The normal development of tissues and organs is fundamental to the proper functioning of an organism. In this study, the microstructure of 9 tissues from Lueyang black-bone chickens were analyzed. The bursa of Fabricius exhibited a well-defined structure, with a clear boundary between cortex and medulla (Fig. 1A). In the breast muscle, the alignment of muscle fibers was evident, with pink sarcoplasm and dark nucleus positioned in the center of the muscle fibers (Fig. 1B). In the heart, cardiomyocytes have a complete structure, orderly arrangement and well-defined boundaries (Fig. 1C). In the kidney, the tubular epithelial cells were arranged neatly, normal-sized and well-shaped glomeruli with clear boundaries (Fig. 1D). In the liver, hepatocytes and hepatic sinuses were arranged radially around the central vein, the lobulation of hepatic lobules was not distinct (Fig. 1E). In the lung, the tertiary bronchi were centrally located within each pulmonary lobule and connected to surrounding atria, which were connected to numerous pulmonary capillaries (Fig. 1F). The ovary contained follicles at different developmental stages (Fig. 1G). The structural layers of the skin were clear and intact (Fig. 1H). In the spleen, the pulpa splenica alba and the pulpa splenica rubra were distinctly demarcated, and the pulpa splenica alba contained well-defined germinal centers (Fig. 1I). These observations confirm that the tissues and organs of the Lueyang black-bone chickens in this study were complete, well-developed, and healthy, providing reliable experimental materials for subsequent analyses.

Given that this study focused on black-bone chickens, particular

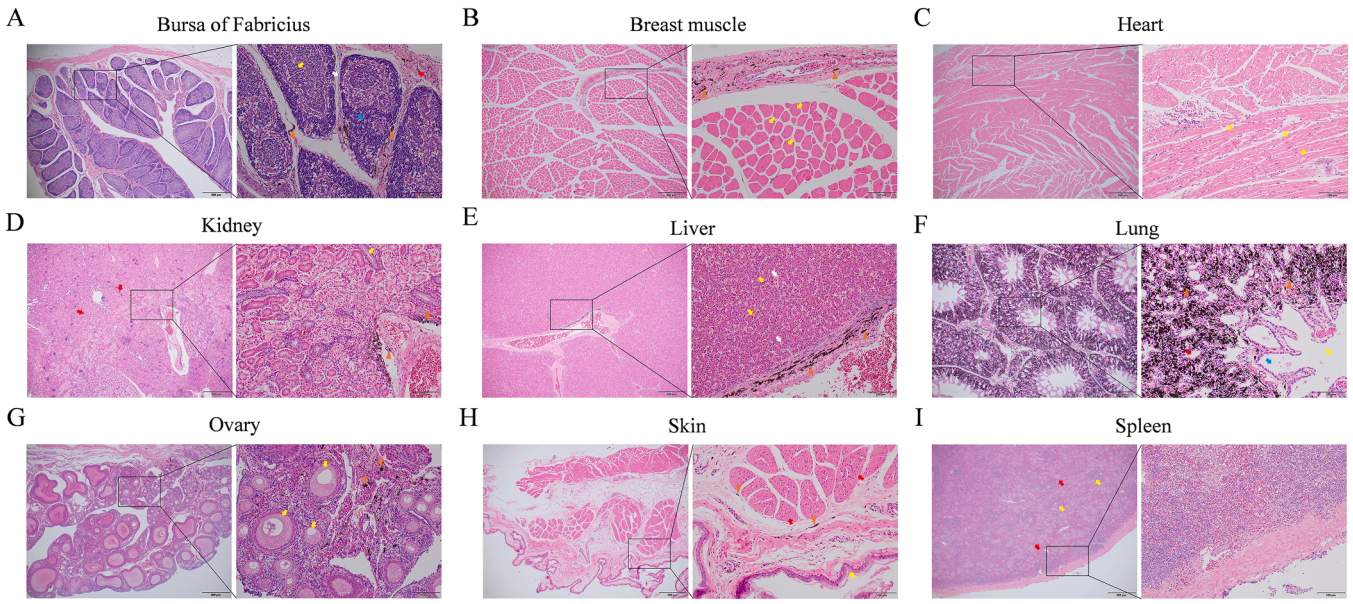

Fig. 1. Hematoxylin and eosin staining of nine tissues and organs from Lueyang black-bone chicken. The orange triangle indicates melanin particles. (A) Hematoxylin and eosin staining of bursae of Fabryssa. The yellow arrow points to the medulla. The blue arrow points to the cortex. The red arrow points to the submucosa. The white arrow points to lamina propria. (B) Hematoxylin and eosin staining of breast muscle. The yellow arrow points to the muscle fibers. (C) Hematoxylin and eosin staining of heart. The yellow arrow points to the heart muscle. (D) Hematoxylin and eosin staining of kidney. The yellow arrow points to the collecting tubule. The red arrow points to the glomerulus. (E) Hematoxylin and eosin staining of liver. Yellow arrows point to red blood cells. The white arrow points to the hepatic sinusoid. (F) Hematoxylin and eosin staining of lung. The yellow arrow points to the tertiary bronchi. The blue arrow points to the atria. Red arrows point to pulmonary capillaries. (G) Hematoxylin and eosin staining of ovary. The yellow arrow points to the follicles. (H) Hematoxylin and eosin staining of skin. Yellow arrow points to the epidermis. The red arrow points to the smooth muscle. (I) Hematoxylin and eosin staining of spleen. The yellow arrow points to the pulpa splenica alba. The red arrow points to the pulpa splenica rubra.

attention was paid to melanin distribution across various organs. Melanin was detected in the skin, breast muscle, lung, ovary, liver, kidney, and bursa of Fabricius, with the lung containing the highest density of melanin particles. Notably, no melanin was observed in the heart or spleen. Interestingly, melanin was primarily deposited in the loose connective tissues of these organs. The mechanism underlying melanin distribution across different organs and within specific organ regions remains to be elucidated.

To ensure the reliability of transcriptome analysis of 9 tissue samples, quality control analysis of RNA-seq data was performed. A total of 116,416,120 raw reads were generated (Table S3). After removing the low-quality data, 36,803,926 to 48,175,558 clean reads were retained for each sample. The Q20 and Q30 values exceeded 97.45 % and 93.01 %, respectively, indicating high sequencing accuracy. The average GC content across all samples was 49.25 %, and the total mapped for each sample exceeded 84 %. The results demonstrate that the overall quality of the transcriptome sequencing data was high and suitable for subsequent analysis.

Analysis of gene expression abundance and correlation across different tissues

The types, quantities, similarities, and differences in gene expression play a pivotal role in determining the growth, development, functional performance, and unique characteristics of each organ. To evaluate the variation in gene expression among chicken tissues and organs and their biological logical clustering, we performed PCA, correlation analysis, and gene expression abundance analysis based on gene expression information. The results revealed high consistency in gene expression within the same tissue, with samples clustering closely together, indicating good reproducibility of each tissue. Gene expression profiles of eight tissues, including heart, liver, spleen, lung, kidney, bursa of Fabricius, ovary, and skin, were highly similar, while the gene expression profile of breast muscle exhibited significant differences from those of other tissues (Fig. 2A). Further confirmation using Pearson correlation coefficients showed strong correlations in gene expression patterns among the heart, liver, spleen, lung, kidney, bursa of Fabricius, ovary, and skin. In contrast, the breast muscle displayed a relatively independent gene expression pattern (Fig. 2B), consistent with the PCA results. This suggests that skin tissue shares a strong correlation with internal organs, whereas muscle tissue is less correlated. As shown in Fig. 2C, the abundance of gene expression also varied across different tissues. The ovary exhibited the highest gene expression abundance, while the liver had the lowest. These differences may be closely associated with the degree of tissue differentiation and their specific physiological functions.

Construction of multi-tissue co-expression profiles and screening of housekeeping genes

To identify genes and signaling pathways broadly involved in the growth and development of poultry tissues and the regulation of fundamental biological processes, we first screened co-expression genes across 9 tissues (TPM $>$ 1) and constructed a tissue co-expression gene profile (Fig. S1). A total of 7,612 co-expression genes were identified, followed by GO and KEGG enrichment analysis. GO enrichment analysis revealed that these co-expression genes were predominantly involved in cytoplasm, cellular metabolic process, translation, gene expression and cellular protein metabolic process. KEGG enrichment analysis indicated that the co-expression genes were enriched in pathways related to DNA replication, protein processing in the endoplasmic reticulum, citrate cycle (TCA cycle), carbon metabolism, and autophagy (Fig. 3A–B). These findings suggest that the co-expression genes across the 9 tissues primarily contribute to the maintenance of fundamental biological processes, consistent with findings in other organisms (X. Pan et al., 2022; T. Zhang et al., 2022).

Addressing the current challenges of instability and lack of species specificity in poultry housekeeping genes, we utilized CV and TPM

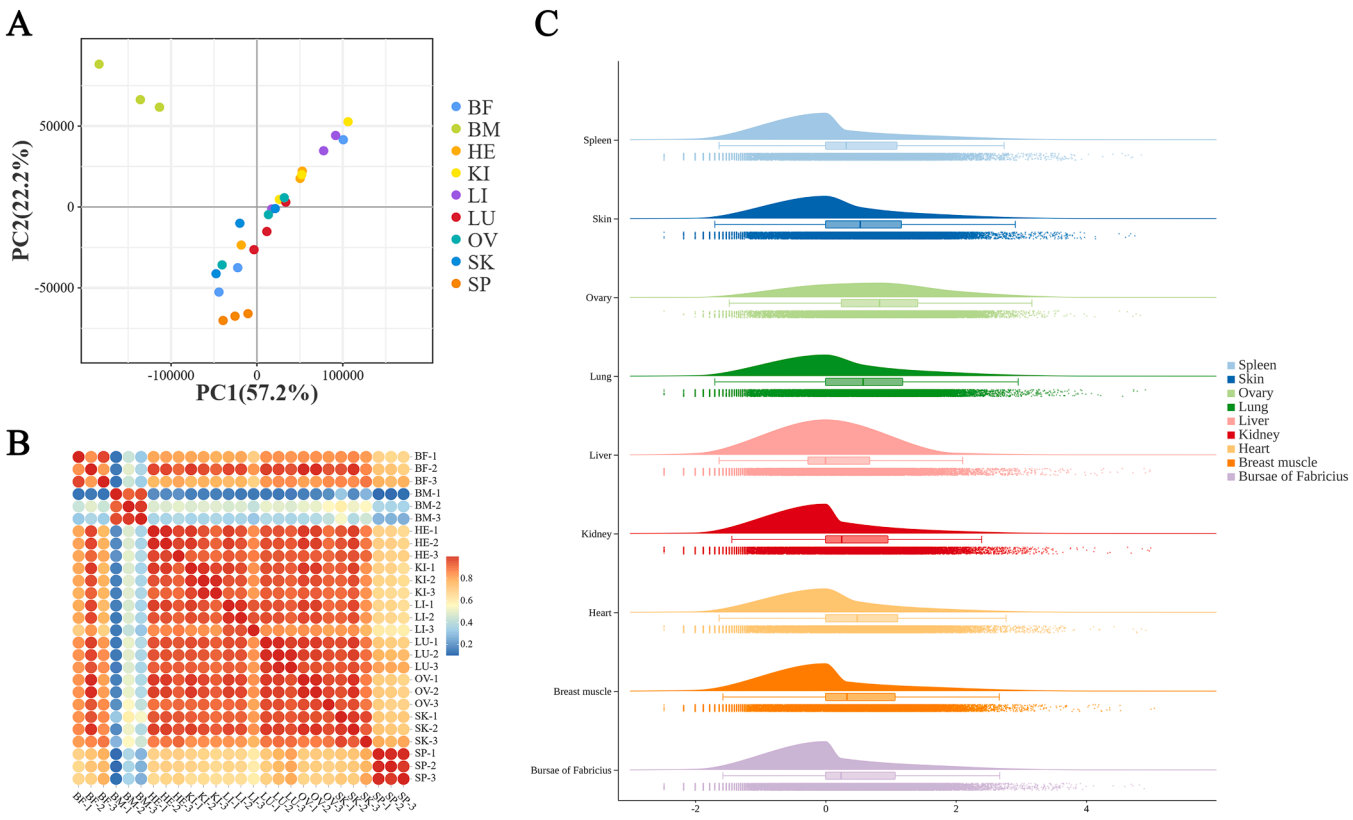

Fig. 2. Multi-tissue gene expression profile. (A) Principal component analysis. (B) Correlation heat map. Color intensity indicates correlation between tissues, with red indicating high correlation (1) and blue indicating low correlation (0). (C) Gene expression abundance map.

values to identify and verify housekeeping genes from the multi-tissue co-expression gene set. CV was employed to quantify gene variation across tissues, and the analysis demonstrated that the CV of co-expression genes was lower than that of the entire gene set (Fig. S2), indicating that co-expression genes had lower variation. Based on CV and expression levels, we selected 10 genes (*EEF1A1, RPS24, FTH1, RPLP1, SERF2, RPS10, UBA52, RPL37A, RPS8, RPL14*) as candidate housekeeping genes, characterized by stable and high expression across all tissues (Fig. 3C, Table S4). Interestingly, we found that *GAPDH*, a widely used housekeeping gene, exhibited unstable expression across multiple tissues in Lueyang black-bone chickens, while *ACTB* was relatively stable (Fig. 3C).

To validate the selected candidate housekeeping genes, three genes were chosen based on their CV and TPM values for further analysis. PCR and RT-qPCR were first performed in the 9 tissues used for transcriptome sequencing analysis in this study (Fig. 3D–G). The results showed that *ACTB* was uniformly and stably expressed across all tissues, whereas *GAPDH* was significantly different in different tissues. The housekeeping genes *EEF1A1* and *FTH1,* identified in this study, demonstrated stable and high expression levels across all tissues, consistent with the results for *ACTB* and superior to *GAPDH*. These findings confirm the accuracy and reliability of the transcriptomic data and provide a preliminary screening of new housekeeping genes. To further assess the universality and stability of the identified housekeeping genes, we analyzed tissue samples from various breeds and developmental stages (10 tissues including bursa of Fabricius, breast muscle, heart, kidney, liver, lung, ovary, skin, spleen and testis of 7-month-old Lueyang black-bone chickens, 1-month-old Liangfenghua broilers, 2-month-old Liangfenghua broilers, 4-month-old Liangfenghua broilers, 2-month-old Silkies) (Fig. 3H–K, Fig. S3). Through Ct value, CV value and PCR analysis, we confirmed that the identified housekeeping genes were consistently stable and highly expressed across domestic chicken samples. To comprehensively assess the expression stability of candidate reference genes, five widely recognized analytical methods: △Ct, geNorm, NormFinder, BestKeeper, and RefFinder were employed. The △Ct and NormFinder methods consistently identified *FTH1, EEF1A1*, and *ACTB* as the top three genes exhibiting the highest stability in expression. The geNorm analysis highlighted *ACTB* and *EEF1A1* as the most stable reference genes. The BestKeeper method ranked *FTH1, RPS24*, and *EEF1A1* as the top three most stable genes. RefFinder was utilized to provide a consolidated ranking of the candidate reference genes across different tissues, resulting in the following order of stability: *FTH1* > *EEF1A1* > *ACTB* > *RPS24* > *GAPDH* (Table S5).

Analysis of tissue-specific expression patterns and screening of marker genes

The growth, differentiation and specific functions of tissues are governed by specific genes. Screening and functional enrichment of tissue specific genes contributes to the precise regulation of tissue growth and development. In this study, we identified and defined tissue specific expression genes as those exclusively expressed in a single tissue, referred to as particular tissue specific genes. Based on multi-tissue transcriptomic data, a total of 4,850 particular TSGs were identified across the 9 tissues (Fig. 4A). The number of particular TSGs varied from 115 to 3,113 in different tissues, with the ovary showing the highest number. To investigate the biological roles of these particular TSGs, GO and KEGG enrichment analyses were performed (Fig. S4).The particular TSGs of bursa of Fabricius were significantly enriched in chemokine signaling pathway and JAK-STAT signaling pathway. Particular TSGs of breast muscle were significantly enriched in regulation of actin cytoskeleton and AMPK signaling pathway. Particular TSGs of the heart were enriched in cardiac muscle contraction and cAMP signaling pathway. Particular TSGs of the kidney were enriched in pathways such as mineral absorption and arginine and proline metabolism. Particular TSGs in the liver were mainly involved in the metabolism of nutrients, including

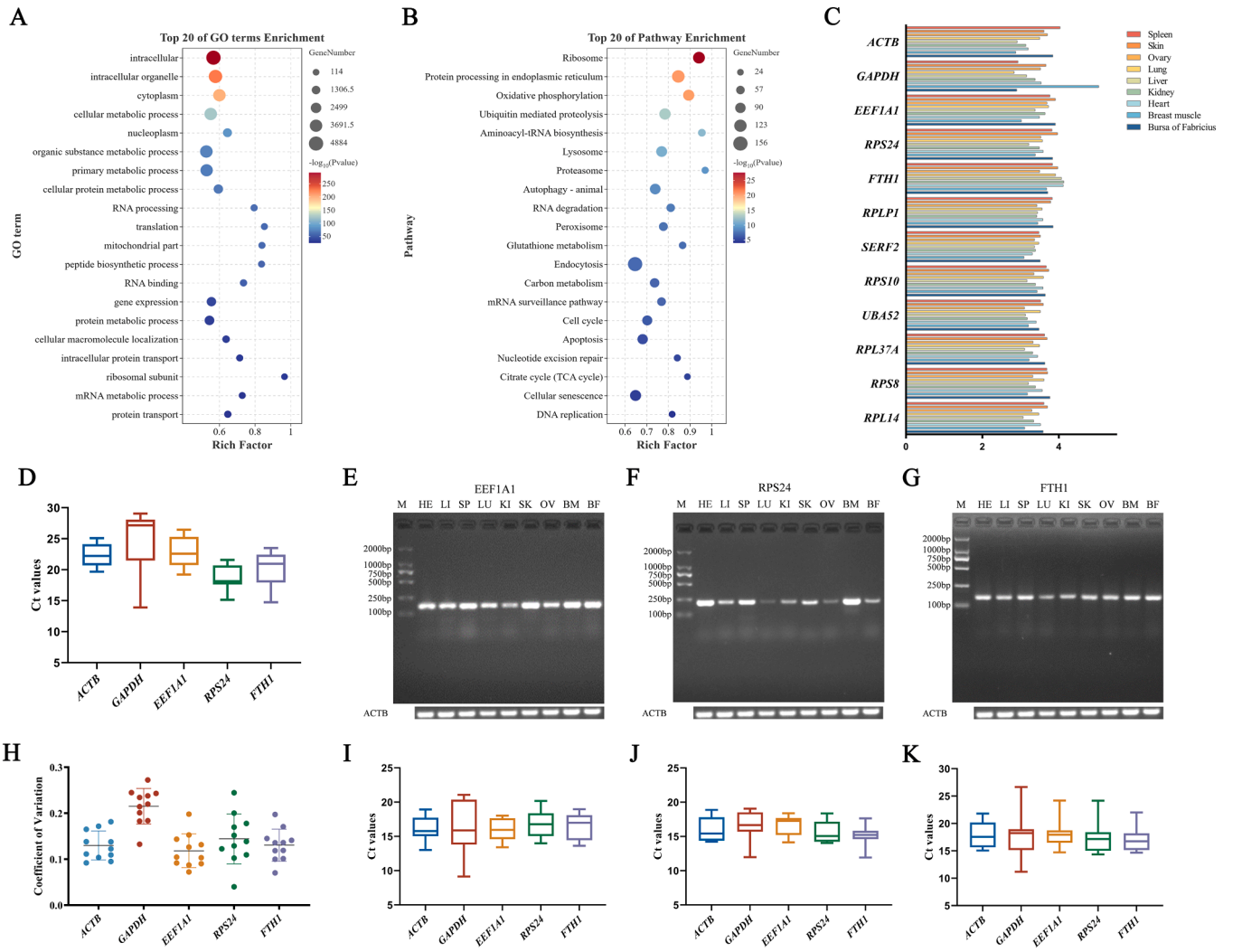

Fig. 3. Housekeeping genes analysis of Lueyang black-bone chickens. (A) GO enrichment analysis of co-expression genes. (B) KEGG enrichment analysis of co-expression genes. (C) Expression of 12 HKGs in 9 tissues. (D) Ct values of HKGs in 3-month-old Lueyang black-bone chickens (E-G) PCR of HKGs (*EEF1A1, RPS24, FTH1*) in 3-month-old Lueyang black-bone chickens. (H) Coefficient of variation of HKGs in different chickens. (I-K) Ct values of HKGs in 7-month-old Lueyang black-bone chickens, 4-month-old Liangfenghua broilers and 2-month-old Silkies.

tryptophan metabolism and histidine metabolism. Lung- particular TSGs were significantly enriched in IL-17 signaling pathway and Fc gamma R-mediated phagocytosis. Ovarian particular TSGs were mainly involved in ovarian steroidogenesis and GnRH secretion. Particular TSGs of the skin were significantly enriched in linoleic acid metabolism and vascular smooth muscle contraction. Particular TSGS in the spleen were significantly enriched in terms of immunity and inflammation, including primary immunodeficiency and B cell receptor signaling pathway. These enrichments result closely align with the biological functions of the respective tissues.

To further expand the research and analysis of the molecular mechanism of tissue characteristics, tissue marker genes were defined as genes highly expressed in a specific tissue and expressed at lower levels in other tissues. Using stringent criteria, we identified 87 candidate tissue marker genes (Fig. 4B). The candidate marker genes in bursa of Fabricius, breast muscle, heart, kidney, liver, lung, ovary, skin, and spleen were 7, 18, 10, 11, 22, 8, 3, 4, and 4, respectively (Table S6). The selected marker genes were enriched by GO and KEGG (Table S7). Candidate marker genes of bursa of Fabricius, such as *HMGB1* and *VPREB3*, were significantly enriched in base excision repair and necroptosis. Candidate marker genes in breast muscle (such as *GPI* and *PGM1*) were mainly involved in energy metabolism, including glycolysis/gluconeogenesis, carbon metabolism, etc. Cardiac candidate marker genes (such as *MYL2* and *TNNT2*) were significantly enriched in cardiac muscle contraction and a Pelin signaling pathway. Kidney candidate marker genes (such as *PCK1* and *SULTIC3*) were mainly involved in sulfur metabolism, mineral absorption, etc. Candidate marker genes of the liver, such as *FGB* and *PIT54*, were involved in complement and coagulation cascades and platelet activation. Lung candidate marker genes (such as *SFTPA1* and *SFTPC*) were mainly involved in phagosome and pertussis. Ovarian candidate marker genes (such as *CCNB1*, etc.) were significantly enriched in signaling pathways such as oocyte meiosis and cell cycle. The skin candidate marker gene *ACTG2* was significantly enriched in vascular smooth muscle contraction. Candidate marker genes in the spleen, such as *CXCL12* and *CCL26*, were mainly involved in cytokine-cytokine receptor interaction.

To validate the reliability of these marker genes identified through RNA-seq, nine candidate marker genes (*TNNT2, PIT54, JCHAIN, SFTPC, SULT1C3, RBP7, CHGB, PGM1, VPREB3*) were randomly selected for PCR and RT-qPCR verification. Validation was conducted on multiple tissues from Lueyang black-bone chickens, Liangfenghua broilers, and Silkies (Fig. 4C–D, Fig. S5-S7). Results confirmed that these marker genes were specifically expressed in their respective tissues in Lueyang black-bone chickens, consistent with the transcriptomic data and

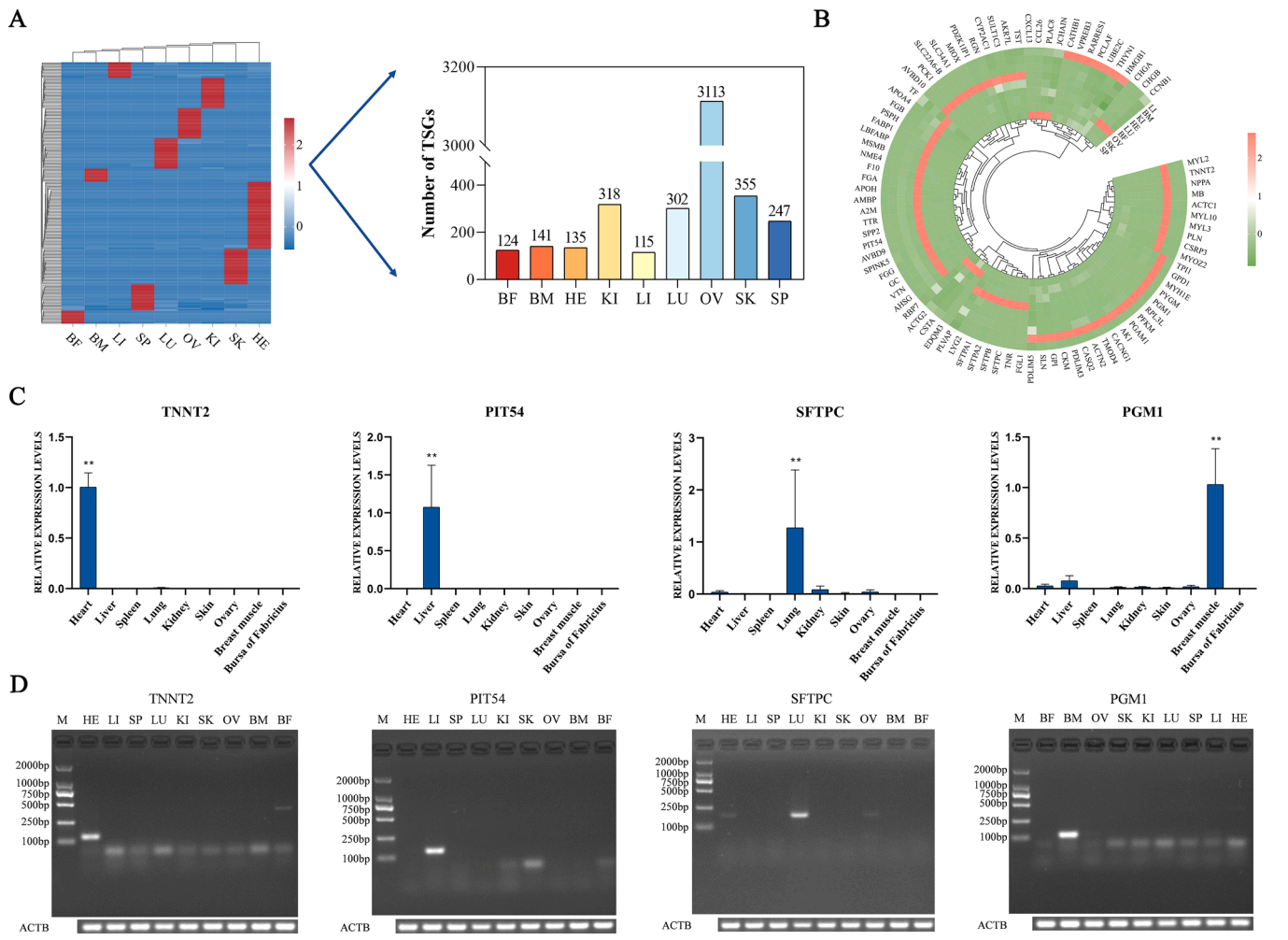

Fig. 4. Analysis of tissue specific genes in various tissues of Lueyang black-bone chickens. (A) Expression of candidate marker genes in each tissue. (B) Heatmap and number of particular TSGs in 9 tissues. (C-D) Verification of marker genes in 3-month-old Lueyang black-bone chickens.

demonstrating its accuracy and reliability. Additional analysis across different breeds and developmental stages revealed variability in the tissue specificity of some marker genes. For example, *TNNT2, PIT54, SFTPC* and *PGM1* could be expressed specifically in specific tissues, while *CHGB, VPREB3* and *JCHAIN* were expressed in multiple tissues. *SULT1C3* showed specificity for liver and kidney. The skin marker gene *RBP7* exhibited high expression in both skin and the heart of 7-month-old female Lueyang black-bone chickens. In summary, this study successfully identified tissue marker genes for the heart (*TNNT2*), liver (*PIT54*), lung (*SFTPC*), and breast muscle (*PGM1*). The transcriptome data obtained in this study can be used for the screening and identification of tissue specific expression genes, tissue marker genes, and the study of tissue growth, differentiation and specific functions.

Analysis of multi-tissue co-expression gene network in Lueyang black-bone chicken

To investigate the biological relationships and potential functions of core driver genes within tissues, we employed weighted gene co-expression network analysis to analyze the screened genes (TPM > 1). When the scale-free topology fit index (R^2) reached 0.9, the optimal soft-thresholding power (β) was determined to be 13 (Fig. S8), indicating this value could effectively construct a scale-free network. We obtained 19 co-expression modules based on the multi-tissue transcript date of Lueyang black-bone chickens (Fig. 5A). The number of genes in each module ranges from 57 to 5,041 (Table S8). A hierarchical clustering algorithm was applied to generate the module eigengene cluster dendrogram (Fig. S9). To identify tissue specific modules, we assessed the correlation between the 19 modules and 9 tissues. Based on the criteria of correlation coefficient (r > 0.65) and *P* value (*P* < 1.0E-4), we identified 9 tissue-specific modules (Fig. 5B, Table S9). Bursa of Fabricius, breast muscle, heart, kidney, liver, lung, ovary, skin and spleen correspond to respectively midnightblue (r = 0.79, *P* = 1.18E-06), lightgreen (r = 0.95, *P* = 2.37E-14), red (r = 0.84, *P* = 3.54E-08), brown (r = 0.93, *P* = 2.46E-12), brown4 (r = 0.96, *P* = 4.26E-15), magenta (r = 0.94, *P* = 7.30E-13), darkslateblue (r = 0.90, *P* = 1.74E-10), ivory (r = 0.95, *P* = 2.75E-14) and cyan (r = 0.96, *P* = 5.43E-16).

To explore the functions of genes in these tissue-specific modules, GO and KEGG enrichment analyses were performed. The results demonstrated that the functional enrichment of genes in each module aligned with the function of the corresponding tissues (Fig. S10). For example, the cyan module was related to the spleen, the genes in this module were significantly enriched in pathways related to immunity and inflammation. The brown module, corresponding to the kidney, included genes involved in energy metabolism and amino acid metabolism. The hub genes in each module are further explored through the gene regulatory network. The top 10 genes with the highest connectivity in each module were designated as hub genes (Fig. 5C).We finally obtained 90 hub genes across the 9 tissues. For example, the hub gene *ACTC1* of the red module was significantly enriched in pathways such as cardiac muscle contraction and adrenergic signaling in cardiomyocytes. The hub gene *MASP2* of the Brown4 module was significantly enriched in the

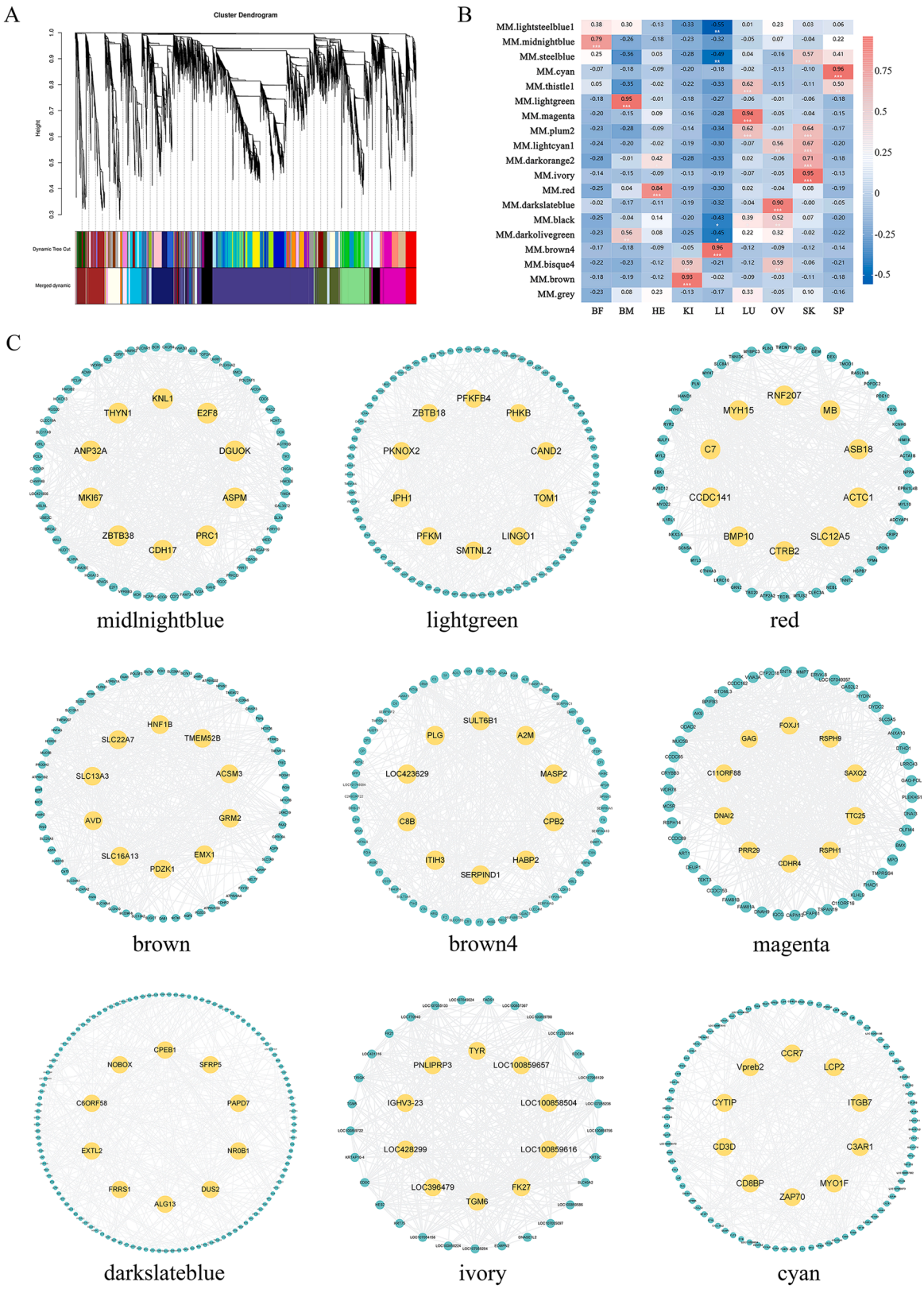

Fig. 5. Clustered network graph of the transcriptome in Lueyang black-bone chickens' tissues. (A) Gene clustering tree and module division. The upper part shows the clustering tree obtained from gene clustering, with the ordinate representing the clustering distance between genes. The lower part shows the modules derived from cutting the cluster tree, with each module represented by a distinct color. (B) Heatmap showing the relationship between 19 modules and 9 tissues. Red indicates high correlation. The box shows the Pearson correlation coefficient and its associated P-values (* means $P < 0.05$, ** means $P < 0.01$, *** means $P < 0.001$). (C) Gene co-expression networks of tissue-specific modules. Yellow indicates hub genes and green indicates non-hub genes.

complement and coagulation cascade signaling pathways. The hub genes of Cyan module is mainly involved in primary immunodeficiency, T cell receptor signaling pathway, etc.

Notably, we observed that some of the hub genes screened were tissue-specific genes. For instance, the cardiac hub genes *SLC12A5* and *CTRB2* were specifically expressed in heart tissue. Similarly, all kidney hub genes, with the exception of *ACSM3*, were found to be kidney-specific. In the liver, the hub gene *A2M* was uniquely expressed in hepatic tissue, while the lung hub genes *PRR29* and *GAG* were specific to pulmonary tissue. Additionally, the ovarian hub genes *ALG13, C6ORF58*, and *NOBOX* were exclusively associated with ovarian tissue, and the skin hub genes *TYR, PNLIPRP3, IGHV3-23, TGM6*, and *FK27* were specifically expressed in cutaneous tissue. These findings suggest that these genes may play unique regulatory roles in their respective tissues.

This study identified tissue-specific co-expression modules and the corresponding hub genes for each module. These hub genes exhibit strong connections with other genes within their respective modules and play critical roles in driving tissue-specific functions. The findings provide valuable insights into the molecular mechanisms underlying tissue specialization.

Discussion

Chickens serve as an ideal model for studying poultry germplasm resource development and disease pathogenesis. As a significant agricultural animal, they provide critical meat and egg products for human consumption. Understanding the gene expression characteristics and regulatory networks in poultry tissues is essential for elucidating the biological mechanisms underlying health and disease. The Lueyang black-bone chicken, a unique dual-purpose breed from China valued for both medical and food applications, offers a pure genetic background and rich genetic diversity, making it an excellent resource for poultry genetic research. This study produced a comprehensive expression atlas

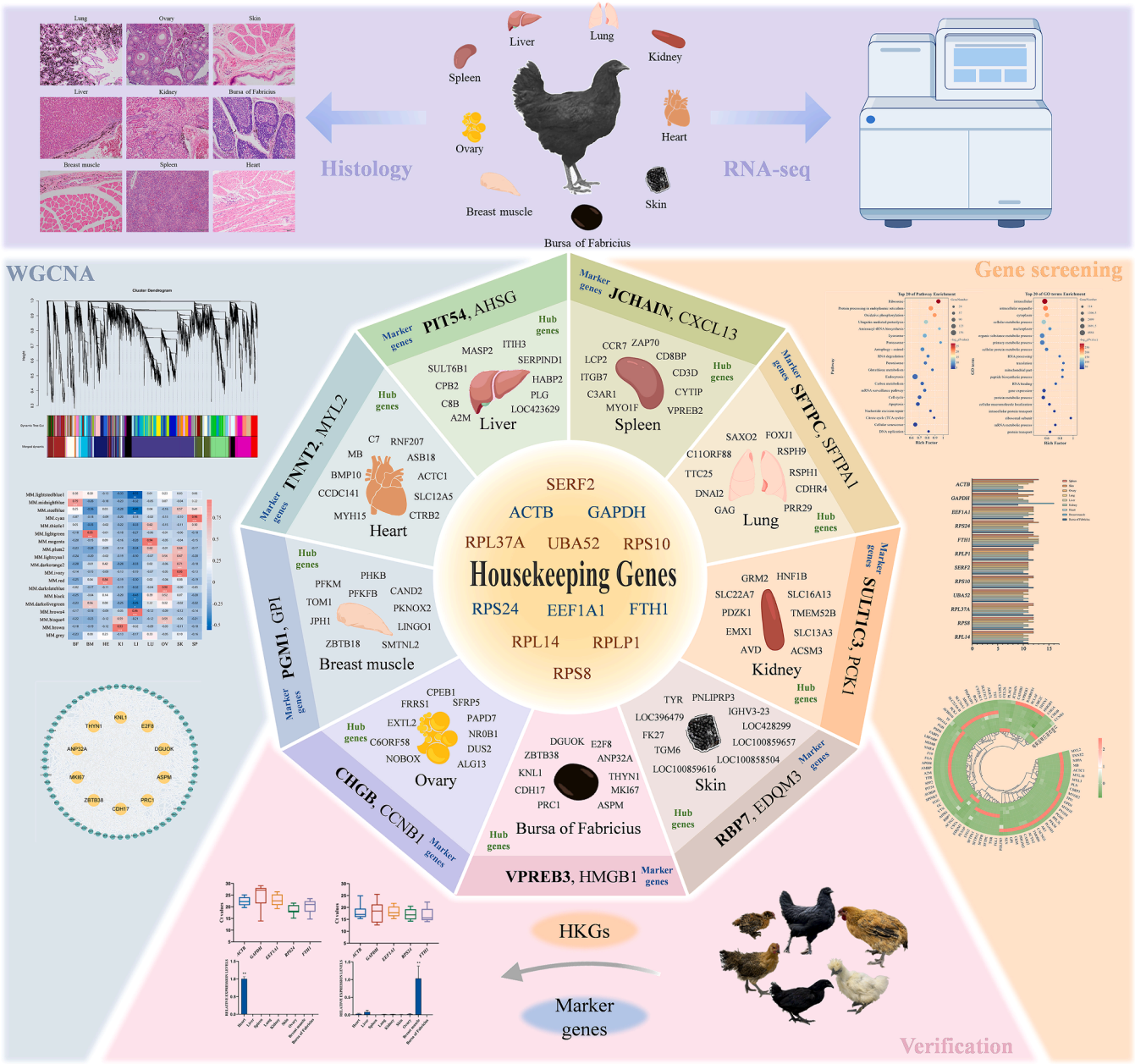

Fig. 6. The housekeeping genes, tissue marker genes, hub genes and related signaling pathways of Lueyang black-bone chicken.

across nine major tissues of Lueyang black-bone chickens, identifying reference genes, tissue marker genes, and associated signaling pathways (Fig. 6). These findings provide foundational data to support research on genetic traits, breeding, disease prevention, and poultry production.

Housekeeping genes are stably and ubiquitously expressed across all tissues and are indispensable for maintaining basic cellular physiological functions (Eisenberg et al., 2013). Currently, the identification of stable housekeeping genes through RNA-seq data analysis has been widely investigated across various species, including pigs (X. Pan et al., 2022), cattle (T. Zhang et al., 2022), humans (Corchete et al., 2020) and chickens (Z. Chen et al., 2024; Hasanpur et al., 2022). In this study, we identified 10 stably and highly expressed HKGs (*EEF1A1, RPS24, FTH1, RPLP1, SERF2, RPS10, UBA52, RPL37A, RPS8, RPL14*) which are involved in fundamental biological processes, including oxidative phosphorylation, TCA cycling, and apoptosis. The functions of these genes align with findings from other studies (X. Pan et al., 2022; Si et al., 2023). This study identified two reference genes, *EEF1A1* and *FTH1*, whose expression patterns demonstrated superior stability compared to traditional reference genes (*ACTB* and *GAPDH*). *EEF1A1*, a gene integral to protein synthesis, facilitates the binding of aminoacyl-tRNA to ribosomes and plays a critical role in peptide chain elongation (Zhang et al., 2024). Previous research has also established its utility as a reliable reference gene in studies of bursa (Mogilicherla et al., 2022) and chicken embryo ovaries development (Y. Wang et al., 2023). *FTH1* encodes a major iron-storage protein essential for maintaining iron balance and metabolic homeostasis (J. Cui et al., 2024). It is important to note that the validation of these genes was conducted on a limited number of chicken breeds, tissues, and organs. Therefore, future studies should expand the sample size to further confirm their applicability. These findings indicate that *EEF1A1* and *FTH1* are reliable for the normalization of gene expression in chicken multi-tissue studies, providing researchers with additional options for reference genes.

Tissue marker genes are closely linked to cell differentiation and tissue development, playing pivotal roles in understanding biological processes and tissue-specific functions (X. L. Cui et al., 2020; El Amrani et al., 2015). In this study, 87 tissue marker genes were identified, and functional enrichment analysis revealed their strong association with tissue-specific physiological functions. For example, *VPREB3*, a marker gene for the bursa of Fabricius, encodes a protein involved in B-cell development (Felizola et al., 2015; Z. Zhang et al., 2022). Rosnet et al. (2004) found that *VPREB3* was specifically expressed in the bursa of Fabricius, which further supported that *VPREB3* could be used as a marker gene for the bursa of Fabricius. *PGM1*, a marker gene for breast muscle, regulates glucose metabolism and has been reported to be closely related to muscle development (Bae et al., 2014). Huang et al. (2023) also found that *PGM1* was highly expressed in the breast muscle of chickens. This supports that *PGM1* can be used as a marker gene in chicken breast muscle. The cardiac marker gene *TNNT2* encodes cardiac troponin T, a protein exclusively expressed in heart muscle (Pettinato et al., 2020). The kidney marker gene *SLC34A1* was reported to encode the sodium phosphate co-transporter NPT 2a, which is located in the apical membrane of proximal tubular cells (Brunkhorst et al., 2024). The liver marker gene, *PIT54*, is found only in birds (Wicher et al., 2006). It is worth noting that Ahn et al. (2019) found that the expression of *PIT54* was significantly reduced in chicken liver after fasting. In this study, we found that *SFTPC* is specifically highly expressed in chicken lungs. Some studies have found that *SFTPC* is a marker gene of alveolar epithelial type 2 cells, which is an important part of alveoli (Sun et al., 2021). This further supports that *SFTPC* can be used as a marker gene for chicken lungs. In our study, *CHGB* was identified as a marker gene for the ovaries. However, in humans, the *CHGB* gene has been proposed as a candidate gene for schizophrenia susceptibility, which may be due to species differences (Iijima et al., 2004). *JCHAIN* is specifically highly expressed in chicken spleen and can be used as a candidate marker gene in spleen tissue. *RBP7* has been screened as a marker gene in chicken skin, however, it has been reported that *RBP7* is specifically expressed in chicken adipose tissue (Ahn et al., 2015). This may be due to the limited range of tissues and organs involved in this study, and more detailed studies are needed in the future.

Weighted gene co-expression network analysis is an effective approach for exploring gene relationships based on transcriptional expression. WGCNA identified 19 co-expression gene modules from 9 tissues, with 9 tissue-specific modules identified. Hub genes in each module were screened and further analyzed. Studies have reported that *THYN1*, a hub gene in the bursa of Fabricius, regulates the transcription factor Pax5, which is essential for B-cell development (Kitaura et al., 2019). Hub genes in breast muscle (such as *PYGM, PHKB*, etc.) have been reported to be closely related to glycogen metabolic diseases (Kishnani et al., 2019; Tarnopolsky, 2018). In the heart, hub genes include *RNF207, MYH15* and so on. Studies have found that *RNF207* is an important regulator of action potential duration (Roder et al., 2014). The hub genes of kidney include *HNF1B, SLC22A7* and so on. Among them, *HNF1B* mutation is the most common single gene cause of congenital kidney malformation and is required to directly reprogram fibroblasts to induce tubular epithelial cells (Clissold et al., 2015; Grand et al., 2023). The liver hub gene *ITIH3* is a potential biomarker for non-alcoholic steatohepatitis (Talari et al., 2024). The hub gene *TTC25* in the lung has been shown to be closely related to ciliary movement (Wallmeier et al., 2016). The ovarian hub gene *NOBOX* is an oocyte-specific homeobox gene. Some studies have found that mutations in *NOBOX* can lead to premature ovarian failure (Qin et al., 2007). Skin hub gene *TYR* is related to feather color differentiation of black-bone chickens (L. Wang et al., 2024), which is related to the experimental materials selected in this study. Spleen hub genes (such as *CCR7, LCP2*, etc.) are involved in immune regulation. *LCP2* variants have been found to impair neutrophil function and T-cell and B-cell antigen receptor signaling (Edwards et al., 2023). These hub genes are tightly connected to other genes in their respective modules and are vital for maintaining tissue-specific functions. In addition, we also noticed that some hub genes are tissue-specific genes. This study provides valuable insights into the associations between genes in Lueyang black-bone chickens, further research is needed to elucidate the underlying mechanisms of HKGs, MGs, and hub genes.

Conclusion

In this study, we established the gene expression profiles for multiple tissues of the Lueyang black-bone chicken and characterized the biological functions of different tissues based on these expression profiles. From the multi-tissue gene co-expression and specific expression data, we identified 10 housekeeping genes in chickens, and validated 2 reference genes, along with tissue marker genes such as *TNNT2, PIT54, SFTPC*, and *PGM1* for the heart, liver, lung, and breast muscle, respectively. Additionally, using weighted gene co-expression network analysis, we identified 19 co-expression modules from multi-tissue transcriptomic data, and subsequently discovered 9 tissue-specific modules and their associated hub genes. These findings significantly contribute to the expanding knowledge of the poultry genome and provide valuable data for further research on chicken growth and development, disease prevention, genetic breeding, and production regulation in poultry.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2025.104986.

References

Ahn, J., Shin, S., Suh, Y., Park, J.Y., Hwang, S., Lee, K., 2015. Identification of the avian RBP7 gene as a new adipose-specific gene and RBP7 promoter-driven GFP expression in adipose tissue of transgenic quail. PLoS One 10, e0124768.

Ahn, J., Woodfint, R.M., Lee, J., Wu, H., Ma, J., Suh, Y., Hwang, S., Cressman, M., Lee, K., 2019. Comparative identification, nutritional, and physiological regulation of chicken liver-enriched genes. Poult. Sci. 98, 3007–3013.

Bae, E., Kim, H.E., Koh, E., Kim, K.S., 2014. Phosphoglucomutase1 is necessary for sustained cell growth under repetitive glucose depletion. FEBS Lett. 588, 3074–3080.

Brunkhorst, M., Brunkhorst, L., Martens, H., Papizh, S., Besouw, M., Grasemann, C., Turan, S., Sikora, P., Chromek, M., Cornelissen, E., Fila, M., Lilien, M., Allgrove, J., Neuhaus, T.J., Eltan, M., Espinosa, L., Schnabel, D., Gokce, I., González-Rodríguez, J. D., Khandelwal, P., Keijzer-Veen, M.G., Lechner, F., Szczepańska, M., Zaniew, M., Bacchetta, J., Emma, F., Haffner, D., 2024. Presentation and outcome in carriers of pathogenic variants in SLC34A1 and SLC34A3 encoding sodium-phosphate transporter NPT 2a and 2c. Kidney Int.

Chen, S., Zhou, Y., Chen, Y., Gu, J., 2018. fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics 34, i884–i890.

Chen, Z., Hua, G., Shu, X., Zhuang, W., Zhang, J., Zhu, R., Zheng, X., Chen, J., 2024. Screening of reliable reference genes for the normalization of RT-qPCR in chicken liver tissues and LMH cells. Sci. Rep. 14, 17828.

Clissold, R.L., Hamilton, A.J., Hattersley, A.T., Ellard, S., Bingham, C., 2015. HNF1B-associated renal and extra-renal disease-an expanding clinical spectrum. Nat. Rev. Nephrol. 11, 102–112.

Corchete, L.A., Rojas, E.A., Alonso-López, D., De Las Rivas, J., Gutiérrez, N.C., Burguillo, F.J., 2020. Systematic comparison and assessment of RNA-seq procedures for gene expression quantitative analysis. Sci. Rep. 10, 19737.

Cui, J., Chen, Y., Yang, Q., Zhao, P., Yang, M., Wang, X., Mang, G., Yan, X., Wang, D., Tong, Z., Wang, P., Kong, Y., Wang, N., Wang, D., Dong, N., Liu, M., E, M., Zhang, M., Yu, B., 2024. Protosappanin A protects DOX-induced myocardial injury and cardiac dysfunction by targeting ACSL4/FTH1 axis-dependent ferroptosis. Adv. Sci. (Weinh) 11, e2310227.

Cui, X.L., Nie, J., Ku, J., Dougherty, U., West-Szymanski, D.C., Collin, F., Ellison, C.K., Sieh, L., Ning, Y., Deng, Z., Zhao, C.W.T., Bergamaschi, A., Pekow, J., Wei, J., Beadell, A.V., Zhang, Z., Sharma, G., Talwar, R., Arensdorf, P., Karpus, J., Goel, A., Bissonnette, M., Zhang, W., Levy, S., He, C., 2020. A human tissue map of 5-hydroxymethylcytosines exhibits tissue specificity through gene and enhancer modulation. Nat. Commun. 11, 6161.

de Jonge, H.J., Fehrmann, R.S., de Bont, E.S., Hofstra, R.M., Gerbens, F., Kamps, W.A., de Vries, E.G., van der Zee, A.G., te Meerman, G.J., ter Elst, A., 2007. Evidence based selection of housekeeping genes. PLoS One 2, e898.

Degalez, F., Charles, M., Foissac, S., Zhou, H., Guan, D., Fang, L., Klopp, C., Allain, C., Lagoutte, L., Lecerf, F., Acloque, H., Giuffra, E., Pitel, F., Lagarrigue, S., 2024. Enriched atlas of lncRNA and protein-coding genes for the GRCg7b chicken assembly and its functional annotation across 47 tissues. Sci. Rep. 14, 6588.

Edwards, E.S.J., Ojaimi, S., Ngui, J., Seo, G.H., Kim, J., Chunilal, S., Yablonski, D., O'Hehir, R.E., van Zelm, M.C., 2023. Combined immunodeficiency and impaired PI3K signaling in a patient with biallelic LCP2 variants. J. Allergy Clin. Immunol. 152, 807–813.e807.

Eisenberg, E., Levanon, E.Y., 2013. Human housekeeping genes, revisited. Trends Genet. 29, 569–574.

El Amrani, K., Stachelscheid, H., Lekschas, F., Kurtz, A., Andrade-Navarro, M.A., 2015. MGFM: a novel tool for detection of tissue and cell specific marker genes from microarray gene expression data. BMC Genom. 16, 645.

Felizola, S.J., Katsu, K., Ise, K., Nakamura, Y., Arai, Y., Satoh, F., Sasano, H., 2015. Pre-B lymphocyte protein 3 (VPREB3) expression in the adrenal cortex: precedent for non-immunological roles in normal and neoplastic human tissues. Endocr. Pathol. 26, 119–128.

Grand, K., Stoltz, M., Rizzo, L., Röck, R., Kaminski, M.M., Salinas, G., Getwan, M., Naert, T., Pichler, R., Lienkamp, S.S., 2023. HNF1B Alters an evolutionarily conserved nephrogenic program of target genes. J. Am. Soc. Nephrol. 34, 412–432.

Hasanpur, K., Hosseinzadeh, S., Mirzaaghayi, A., Alijani, S., 2022. Investigation of chicken housekeeping genes using next-generation sequencing data. Front. Genet. 13, 827538.

Huang, Z., Cai, Z., Zhang, J., Gu, Y., Wang, J., Yang, J., Lv, G., Yang, C., Zhang, Y., Ji, C., Jiang, S., 2023. Integrating proteomics and metabolomics to elucidate the molecular network regulating of inosine monophosphate-specific deposition in Jingyuan chicken. Poult. Sci. 102, 103118.

Iijima, Y., Inada, T., Ohtsuki, T., Senoo, H., Nakatani, M., Arinami, T., 2004. Association between chromogranin b gene polymorphisms and schizophrenia in the Japanese population. Biol. Psychiatry 56, 10–17.

Isa, A.M., Sun, Y., Shi, L., Jiang, L., Li, Y., Fan, J., Wang, P., Ni, A., Huang, Z., Ma, H., Li, D., Chen, J., 2020. Hybrids generated by crossing elite laying chickens exhibited heterosis for clutch and egg quality traits. Poult. Sci. 99, 6332–6340.

Isakova, A., Fehlmann, T., Keller, A., Quake, S.R., 2020. A mouse tissue atlas of small noncoding RNA. Proc. Natl. Acad. Sci. U. S. A. 117, 25634–25645.

Jehl, F., Muret, K., Bernard, M., Boutin, M., Lagoutte, L., Désert, C., Dehais, P., Esquerré, D., Acloque, H., Giuffra, E., Djebali, S., Foissac, S., Derrien, T., Pitel, F., Zerjal, T., Klopp, C., Lagarrigue, S., 2020. An integrative atlas of chicken long non-coding genes and their annotations across 25 tissues. Sci. Rep. 10, 20457.

Khumpeerawat, P., Duangjinda, M., Phasuk, Y., 2021. Factors affecting gene expression associated with the skin color of black-bone chicken in Thailand. Poult. Sci. 100, 101440.

Kim, D., Langmead, B., Salzberg, S.L., 2015. HISAT: a fast spliced aligner with low memory requirements. Nat. Methods 12, 357–360.

Kishnani, P.S., Goldstein, J., Austin, S.L., Arn, P., Bachrach, B., Bali, D.S., Chung, W.K., El-Gharbawy, A., Brown, L.M., Kahler, S., Pendyal, S., Ross, K.M., Tsilianidis, L., Weinstein, D.A., Watson, M.S., 2019. Diagnosis and management of glycogen storage diseases type VI and IX: a clinical practice resource of the American College of Medical Genetics and Genomics (ACMG). Genet. Med. 21, 772–789.

Kitaura, F., Yuno, M., Fujita, T., Wakana, S., Ueda, J., Yamagata, K., Fujii, H., 2019. Normal B cell development and Pax5 expression in Thy28/ThyN1-deficient mice. PLoS One 14, e0220199.

Krasikova, A., Kulikova, T., Schelkunov, M., Makarova, N., Fedotova, A., Plotnikov, V., Berngardt, V., Maslova, A., Fedorov, A., 2024. The first chicken oocyte nucleus whole transcriptomic profile defines the spectrum of maternal mRNA and non-coding RNA genes transcribed by the lampbrush chromosomes. Nucl. Acids Res.

Langfelder, P., Horvath, S., 2008. WGCNA: an R package for weighted correlation network analysis. BMC Bioinform. 9, 559.

Langmead, B., Salzberg, S.L., 2012. Fast gapped-read alignment with Bowtie 2. Nat. Methods 9, 357–359.

Lee, J.H., 2021. Special issue: poultry genetics, breeding and biotechnology. Genes (Basel) 12.

Li, B., Dewey, C.N., 2011. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinform. 12, 323.

Liu, S., Gao, Y., Canela-Xandri, O., Wang, S., Yu, Y., Cai, W., Li, B., Xiang, R., Chamberlain, A.J., Pairo-Castineira, E., D'Mellow, K., Rawlik, K., Xia, C., Yao, Y., Navarro, P., Rocha, D., Li, X., Yan, Z., Li, C., Rosen, B.D., Van Tassell, C.P., Vanraden, P.M., Zhang, S., Ma, L., Cole, J.B., Liu, G.E., Tenesa, A., Fang, L., 2022. A multi-tissue atlas of regulatory variants in cattle. Nat. Genet. 54, 1438–1447.

Mancinelli, A.C., Menchetti, L., Birolo, M., Bittante, G., Chiattelli, D., Castellini, C., 2023. Crossbreeding to improve local chicken breeds: predicting growth performance of the crosses using the Gompertz model and estimated heterosis. Poult. Sci. 102, 102783.

Mogilicherla, K., Athe, R.P., Chatterjee, R.N., Bhattacharya, T.K., 2022. Identification of suitable reference genes for normalization of quantitative real-time PCR-based gene expression in chicken (Gallus gallus). Anim. Genet. 53, 881–887.

Mozdziak, P.E., Dibner, J.J., McCoy, D.W., 2003. Glyceraldehyde-3-phosphate dehydrogenase expression varies with age and nutrition status. Nutrition 19, 438–440.

Pan, X., Cai, J., Wang, Y., Xu, D., Jiang, Y., Gong, W., Tian, Y., Shen, Q., Zhang, Z., Yuan, X., Li, J., 2022. Expression profile of housekeeping genes and tissue-specific genes in multiple tissues of pigs. Animals (Basel) 12.

Pan, Z., Wang, Y., Wang, M., Wang, Y., Zhu, X., Gu, S., Zhong, C., An, L., Shan, M., Damas, J., Halstead, M.M., Guan, D., Trakooljul, N., Wimmers, K., Bi, Y., Wu, S., Delany, M.E., Bai, X., Cheng, H.H., Sun, C., Yang, N., Hu, X., Lewin, H.A., Fang, L., Zhou, H., 2023. An atlas of regulatory elements in chicken: a resource for chicken genetics and genomics. Sci. Adv. 9, eade1204.

Pertea, M., Pertea, G.M., Antonescu, C.M., Chang, T.C., Mendell, J.T., Salzberg, S.L., 2015. StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. Nat. Biotechnol. 33, 290–295.

Pettinato, A.M., Ladha, F.A., Mellert, D.J., Legere, N., Cohn, R., Romano, R., Thakar, K., Chen, Y.S., Hinson, J.T., 2020. Development of a cardiac Sarcomere functional genomics platform to enable scalable interrogation of Human TNNT2 variants. Circulation 142, 2262–2275.

Phanprasit, W., Laohaudomchok, W., Konthonbut, P., Noomnual, S., Rissanen, S., Jussila, K., Ikäheimo, T.M., Jaakkola, J.J.K., Näyhä, S., 2024. Self-assessed threshold for cold temperatures and thermal insulation of clothing among poultry workers. Sci. Rep. 14, 20406.

Qin, Y., Choi, Y., Zhao, H., Simpson, J.L., Chen, Z.J., Rajkovic, A., 2007. NOBOX homeobox mutation causes premature ovarian failure. Am. J. Hum. Genet. 81, 576–581.

Roder, K., Werdich, A.A., Li, W., Liu, M., Kim, T.Y., Organ-Darling, L.E., Moshal, K.S., Hwang, J.M., Lu, Y., Choi, B.R., MacRae, C.A., Koren, G., 2014. RING finger protein RNF207, a novel regulator of cardiac excitation. J. Biol. Chem. 289, 33730–33740.

Rosnet, O., Blanco-Betancourt, C., Grivel, K., Richter, K., Schiff, C., 2004. Binding of free immunoglobulin light chains to VpreB3 inhibits their maturation and secretion in chicken B cells. J. Biol. Chem. 279, 10228–10236.

She, X., Rohl, C.A., Castle, J.C., Kulkarni, A.V., Johnson, J.M., Chen, R., 2009. Definition, conservation and epigenetics of housekeeping and tissue-enriched genes. BMC Genom. 10, 269.

Shokhirev, M.N., Johnson, A.A., 2022. An integrative machine-learning meta-analysis of high-throughput omics data identifies age-specific hallmarks of Alzheimer's disease. Ageing Res. Rev. 81, 101721.

Shu, X., Hua, G., Zheng, X., Chen, Z., Zhang, J., Zhuang, W., Chen, J., 2024. Screening of reliable reference genes for the normalization of RT-qPCR in chicken oviduct tract. Poult. Sci. 103, 103980.
Si, J., Dai, D., Li, K., Fang, L., Zhang, Y., 2023. A multi-tissue gene expression atlas of Water Buffalo (Bubalus bubalis) reveals transcriptome conservation between buffalo and cattle. Genes (Basel) 14.
Sinpru, P., Bunnom, R., Poompramun, C., Kaewsatuan, P., Sornsan, S., Kubota, S., Molee, W., Molee, A., 2021. Association of growth hormone and insulin-like growth factor I genotype with body weight, dominance of body weight, and mRNA expression in Korat slow-growing chickens. Anim. Biosci. 34, 1886–1894.
Sun, Y.L., Hurley, K., Villacorta-Martin, C., Huang, J., Hinds, A., Gopalan, K., Caballero, I.S., Russo, S.J., Kitzmiller, J.A., Whitsett, J.A., Beers, M.F., Kotton, D.N., 2021. Heterogeneity in Human induced pluripotent stem cell-derived alveolar epithelial type II cells revealed with ABCA3/SFTPC reporters. Am. J. Respir. Cell Mol. Biol. 65, 442–460.
Talari, N.K., Mattam, U., Kaminska, D., Sotomayor-Rodriguez, I., Rahman, A.P., Péterfy, M., Pajukanta, P., Pihlajamäki, J., Chella Krishnan, K., 2024. Hepatokine ITIH3 protects against hepatic steatosis by downregulating mitochondrial bioenergetics and de novo lipogenesis. iScience 27, 109709.
Tarnopolsky, M.A., 2018. Myopathies related to glycogen metabolism disorders. Neurotherapeutics 15, 915–927.
Teng, J., Gao, Y., Yin, H., Bai, Z., Liu, S., Zeng, H., Bai, L., Cai, Z., Zhao, B., Li, X., Xu, Z., Lin, Q., Pan, Z., Yang, W., Yu, X., Guan, D., Hou, Y., Keel, B.N., Rohrer, G.A., Lindholm-Perry, A.K., Oliver, W.T., Ballester, M., Crespo-Piazuelo, D., Quintanilla, R., Canela-Xandri, O., Rawlik, K., Xia, C., Yao, Y., Zhao, Q., Yao, W., Yang, L., Li, H., Zhang, H., Liao, W., Chen, T., Karlskov-Mortensen, P., Fredholm, M., Amills, M., Clop, A., Giuffra, E., Wu, J., Cai, X., Diao, S., Pan, X., Wei, C., Li, J., Cheng, H., Wang, S., Su, G., Sahana, G., Lund, M.S., Dekkers, J.C.M., Kramer, L., Tuggle, C.K., Corbett, R., Groenen, M.A.M., Madsen, O., Gòdia, M., Rocha, D., Charles, M., Li, C.J., Pausch, H., Hu, X., Frantz, L., Luo, Y., Lin, L., Zhou, Z., Zhang, Z., Chen, Z., Cui, L., Xiang, R., Shen, X., Li, P., Huang, R., Tang, G., Li, M., Zhao, Y., Yi, G., Tang, Z., Jiang, J., Zhao, F., Yuan, X., Liu, X., Chen, Y., Xu, X., Zhao, S., Zhao, P., Haley, C., Zhou, H., Wang, Q., Pan, Y., Ding, X., Ma, L., Li, J., Navarro, P., Zhang, Q., Li, B., Tenesa, A., Li, K., Liu, G.E., Zhang, Z., Fang, L., 2024. A compendium of genetic regulatory effects across pig tissues. Nat. Genet. 56, 112–123.
Wallmeier, J., Shiratori, H., Dougherty, G.W., Edelbusch, C., Hjeij, R., Loges, N.T., Menchen, T., Olbrich, H., Pennekamp, P., Raidt, J., Werner, C., Minegishi, K., Shinohara, K., Asai, Y., Takaoka, K., Lee, C., Griese, M., Memari, Y., Durbin, R., Kolb-Kokocinski, A., Sauer, S., Wallingford, J.B., Hamada, H., Omran, H., 2016. TTC25 Deficiency results in defects of the outer dynein arm docking machinery and primary ciliary dyskinesia with left-right body asymmetry randomization. Am. J. Hum. Genet. 99, 460–469.
Wang, B., Fu, C., Wei, Y., Xu, B., Yang, R., Li, C., Qiu, M., Yin, Y., Qin, D., 2022. Ferroptosis-related biomarkers for Alzheimer's disease: identification by bioinformatic analysis in hippocampus. Front. Cell. Neurosci. 16, 1023947.
Wang, D., Eraslan, B., Wieland, T., Hallström, B., Hopf, T., Zolg, D.P., Zecha, J., Asplund, A., Li, L.H., Meng, C., Frejno, M., Schmidt, T., Schnatbaum, K., Wilhelm, M., Ponten, F., Uhlen, M., Gagneur, J., Hahne, H., Kuster, B., 2019. A deep proteome and transcriptome abundance atlas of 29 healthy human tissues. Mol. Syst. Biol. 15, e8503.
Wang, L., Xue, Z., Tian, Y., Zeng, W., Zhang, T., Lu, H., 2024. A single-cell transcriptome atlas of Lueyang black-bone chicken skin. Poult. Sci. 103, 103513.
Wang, Y., Zhang, Y.Q., Wu, Z.W., Fang, T., Wang, F., Zhao, H., Du, Z.Q., Yang, C.X., 2023. Selection of reference genes for RT-qPCR analysis in developing chicken embryonic ovary. Mol. Biol. Rep. 50, 3379–3387.
Wicher, K.B., Fries, E., 2006. Haptoglobin, a hemoglobin-binding plasma protein, is present in bony fish and mammals but not in frog and chicken. Proc. Natl. Acad. Sci. U. S. A. 103, 4168–4173.
Xie, F., Wang, J., Zhang, B., 2023. RefFinder: a web-based tool for comprehensively analyzing and identifying reference genes. Funct. Integr. Genom. 23, 125.
Yin, R., Liu, X., Liu, C., Ding, Z., Zhang, X., Tian, F., Liu, W., Yu, J., Li, L., Hrabé de Angelis, M., Stoeger, T., 2011. Systematic selection of housekeeping genes for gene expression normalization in chicken embryo fibroblasts infected with Newcastle disease virus. Biochem. Biophys. Res. Commun. 413, 537–540.
Zaitsev, A., Chelushkin, M., Dyikanov, D., Cheremushkin, I., Shpak, B., Nomie, K., Zyrin, V., Nuzhdina, E., Lozinsky, Y., Zotova, A., Degryse, S., Kotlov, N., Baisangurov, A., Shatsky, V., Afenteva, D., Kuznetsov, A., Paul, S.R., Davies, D.L., Reeves, P.M., Lanuti, M., Goldberg, M.F., Tazearslan, C., Chasse, M., Wang, I., Abdou, M., Aslanian, S.M., Andrewes, S., Hsieh, J.J., Ramachandran, A., Lyu, Y., Galkin, I., Svekolkin, V., Cerchietti, L., Poznansky, M.C., Ataullakhanov, R., Fowler, N., Bagaev, A., 2022. Precise reconstruction of the TME using bulk RNA-seq and a machine learning algorithm trained on artificial transcriptomes. Cancer Cell 40, 879–894.e816.
Zhang, T., Wang, T., Niu, Q., Xu, L., Chen, Y., Gao, X., Gao, H., Zhang, L., Liu, G.E., Li, J., Xu, L., 2022. Transcriptional atlas analysis from multiple tissues reveals the expression specificity patterns in beef cattle. BMC Biol. 20, 79.
Zhang, W., Wang, J., Shan, C., 2024. The eEF1A protein in cancer: clinical significance, oncogenic mechanisms, and targeted therapeutic strategies. Pharmacol. Res. 204, 107195.
Zhang, Z., Cai, J., Hao, S., Li, C., Chen, J., Li, T., Feng, X., 2022. Transcriptomic analysis of spleen B cell revealed the molecular basis of bursopentin on B cell differentiation. Vet. Res. 53, 109.
Zhu, J., He, F., Hu, S., Yu, J., 2008. On the nature of human housekeeping genes. Trends Genet. 24, 481–484.