

RNA-sequence reveals differentially expressed genes affecting the crested trait of Wumeng crested chicken

Tiansong Wang,^{*,†} Yong Zhang,^{*,†,1} Yong Guo,^{*,†} Xiong Zhang,[‡] Hong Yang,[§] Xingzhou Tian,^{*,†}
Min Zhu,^{*,†} Zhili Guo,^{*,†} Shanshan Zeng,^{*,†} Keyin Luo,[#] and Qi He[¶]

^{*}Key Laboratory of Animal Genetics, Breeding and Reproduction in the Plateau Mountainous Region, Ministry of Education, Guizhou University, Guiyang, 550025, China; [†]Guizhou Provincial Key Laboratory of Animal Genetics, Breeding and Reproduction, Guizhou University, Guiyang, 550025, China; [‡]Institute of Animal Husbandry and Veterinary Science, Guizhou Academy of Agricultural Sciences, Guiyang, Guizhou Province 550005, China; [§]Bijie City Animal Husbandry Station, Bijie 551700, China; [#]Liupanshui City Agriculture and Rural Bureau, Liupanshui 553001, China; and [¶]Agriculture and Rural Bureau of Huaxi District, Guiyang City, Guizhou Province 550025, China

ABSTRACT Wumeng crested chicken has a cluster of slender feathers on its head, and the underlying skull region exhibits an obvious tumor-like protrusion. This is the typical skull structure of crested chickens. The associated regulatory genes are located on autosomes and are incompletely dominant. This trait is related to brain herniation, but the genetic mechanisms of its formation and development are unclear. In this study, RNA sequencing (**RNA-Seq**) analysis was conducted on 6 skull tissue samples from 3 Wumeng crested chickens with prominent skull protrusions and 3 without a prominent skull protrusion phenotype. A total of 46,376,934 to 43,729,046 clean reads were obtained, the percentage of uniquely mapped reads compared with the reference genome was between 89.73%–91.00%, and 39,795,458–41,836,502 unique reads were obtained. Among different genomic regions, the highest frequency of sequencing reads occurred in exon regions (85.44–88.28%). Additionally, a total of 423 new transcripts and 26,999

alternative splicings (**AS**) events were discovered in this sequencing analysis. This study identified 1,089 differentially expressed genes (**DEGs**), among which 485 were upregulated and 604 were downregulated. Gene Ontology (**GO**) and Kyoto Encyclopedia of Genes and Genomes (**KEGG**) pathway analyses indicated that the DEGs were enriched in terms related to signal transduction, cell development, cell differentiation, the lysosome, serine, and threonine metabolism, and the interaction of cytokines with cytokine receptors. Based on the comprehensive analysis of DEGs combined with reported quantitative trait loci (**QTLs**), the expression of *BMP2*, *EPHA3*, *EPHB1*, *HOXC6*, *SCN2B*, *BMP7*, and *HOXC10* was verified by real-time quantitative polymerase chain reaction (**qRT-PCR**). The qRT-PCR results were consistent with the RNA-Seq results, indicating that these 7 genes may be candidates genes regulating the crested trait.

Key words: Wumeng crested chicken, epigenetic mechanism, crested traits, RNA-Seq, differentially expressed gene

2021 Poultry Science 100:101357

<https://doi.org/10.1016/j.psj.2021.101357>

INTRODUCTION

Wumeng crested chicken (**Figure S1**) is a newly discovered germplasm resource from Liupanshui City, Guizhou Province, China in 2013. It shows the characteristics of crested chicken breeds. Its feathers are mostly hemp and yellow in color, with few black feathers, and the bill and feet are black. The central production area

of this chicken (east longitude 104°18'20"–105°42'50", north latitude 25°19'44"–26°55'33") has karst topography, the altitude is between 1,400 m and 1,900 m, the climate is a subtropical humid monsoon climate, and the annual average temperature is 13.5°C.

The crested phenotype refers to a tuft of fine feathers on the top of a bird's head, in which the feathers can reach the eyes (**Bartels, 2003**). Many domestic and foreign poultry species have this specific trait, such as Taihe black-bone chicken (**Zhang et al., 2016**), white crested Polish chicken (**Frahm and Rehkämper, 1998**), and Zhenjiang crested duck (**Wang et al., 2018; Yuan et al., 2019**). Studies have shown that the inheritance of the crested trait conforms to the classic Mendelian law of inheritance (**Fisher, 1934**). The F1 generation

© 2021 The Authors. Published by Elsevier Inc. on behalf of Poultry Science Association Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Received March 10, 2021.

Accepted June 22, 2021.

¹Corresponding author: gzuzy@139.com

offspring from a cross of crested Silkie and noncrested White Plymouth Rock chickens are all crested, the ratio of crested and noncrested individuals in the F2 generation is 3:1 (2324/789), and no differences are found as a result of the reciprocal cross, showing that the crested trait exhibits autosomal incomplete dominant inheritance (Wang et al., 2012c). Cerebral herniation in a chicken produces a spherical frontal protrusion. The external shape of the brain under the skull is significantly different from that in a normal chicken. The forebrain and hindbrain are obviously separated, and the cerebral hemispheres are squeezed into the spherical area of the skull (Frahm et al., 2001; Frahm and Rehkämper, 2004; Stange et al., 2018). Research on the inheritance and occurrence of Polish chicken brain herniation showed that this trait presents autosomal recessive inheritance and is closely related to the formation of crested heads and that homozygosity in birds with crested heads is related to brain herniation, leading to skull deformities (Yoshimura et al., 2012).

In 1928, genetic linkage maps of various domestic animals and eight linkage groups of chickens were first drawn by Serebrovsky and Petrov, in which the crested trait locus was included. Later, it was discovered that the crested, frizzle, and dominant white traits were linked and were located in linkage group II (Romanov et al., 2004). Subsequent studies confirmed that the dominant white feather trait regulatory gene *PMEL17* is located in the E22C19W28 linkage group, so it was inferred that the crested trait may also be located in the E22C19W28 linkage group (Kerje et al., 2004). Through linkage inheritance and genome-wide association analysis, it was proven that the crested trait was located in the LGE22C19W28_E50C23 linkage group and was completely associated with the *HOXC* cluster on the chromosome; the *HOXC8*-SSR, *HOXC8*-3end, and *Homeobox C11* (*HOXC11*) genes were further demonstrated to be highly related to the formation of the crested trait. Recent studies have found that *Homeobox C8* (*HOXC8*) exhibits ectopic expression in skull skin, so it is speculated that the crested trait is caused by a cis-acting regulatory mutation and that this mutation is the basis for the ectopic expression of *HOXC8* (Wang et al., 2012b). In addition, the *HOXC8* gene was genotyped with the flanking markers *HOXC8*-SSR, *HOXC8*-3end, and *HOXC11* related to coronal protrusions, and no correlation of the reported markers was found in Swiss crested chicken. The two exons of the *HOXC8* gene of crested chicken were sequenced, and no polymorphic sites were found in the coding region (Joller et al., 2018). In summary, the molecular genetic mechanism of the chicken crested trait is still unclear, and further research is urgently needed.

In this study, RNA-Seq and bioinformatics analyses were performed to screen key candidate genes that regulate the crested trait, to explore gene expression level changes in the whole genome during the development of crested chickens, and to study the developmental regulation of the crested trait at the genome level. In addition, qRT-PCR was used to verify some key candidate genes

at the mRNA level to provide a molecular basis for elucidating the formation mechanism of the crested trait.

MATERIALS AND METHODS

Experimental Animal and Skull Sample Collection

The skull tissue samples of 6 Wumeng crested chickens hens (50 days old) with ($n = 3$, the C group) and without ($n = 3$, the NC group) a protruding skull phenotype used in this experiment were obtained from a commercial chicken farm (Jinfengqi Natural Animal Husbandry Co., Ltd., Liupanshui, China). All animal procedures were performed under the Guidelines for Care and Use of Laboratory Animals of Guizhou University (Guiyang, China), and all experimental methods and management procedures were approved by the Experimental Animal Ethics Committee of Guizhou University (Guiyang, China).

Before the samples were collected, the chickens were subjected to electric shock, bleeding, and dissection. Within 20 min after slaughter, protruding skull tissues of the crested chickens and corresponding skull tissues from another group were collected. The samples were collected in RNase-free tubes, frozen immediately in liquid nitrogen, and stored at -80°C for RNA extraction.

RNA Extraction and Quality Inspection

According to the instructions, using Trizol reagent (Invitrogen, Carlsbad, CA) to extract total RNA from skull tissue. Use NanoPhotometer spectrophotometer (IMPLEN, CA) to detect RNA purity, Bioanalyzer 2100 system (Agilent Technologies, CA) using the middle RNA Nano 6000 analysis kit to evaluate RNA integrity.

Library Construction and Sequencing

A total of 1 μg RNA per sample was used. Sequencing libraries were generated using the NEBNext UltraTM RNA Library Prep Kit for Illumina (NEB, Ipswich, MA), and index codes were added to attribute the sequences to each sample. The clustering of the indexed samples was performed on a cBot Cluster Generation System using the TruSeq PE Cluster Kit v3-cBot-HS (Illumina, San Diego, CA). After cluster generation, the library preparations were sequenced on an Illumina HiSeq 2000 platform and 150 bp paired-end reads were generated (Novogene, China).

Raw Data Quality Control and Reference Genome Comparison

The image data of the sequenced fragments obtained from a high-throughput sequencer were converted into sequence numbers (reads) via CASAVA ver 1.8.2 (Illumina) base recognition to generate a file in FASTQ format. Raw data (raw reads) in FASTQ format were first

processed with in-house Perl scripts. In this step, clean data (clean reads) were obtained by removing reads containing adapters, reads containing poly-N and low-quality reads from raw data. At the same time, the Q20, Q30, sequencing error rate, and GC content of the clean data were calculated. All downstream analyses were based on clean data with high quality.

Read Mapping to the Reference Genome

The chicken reference genome and gene model annotation file were downloaded from NCBI (<https://www.ncbi.nlm.nih.gov/genome/?term=gallus+gallus>). HISAT2v2.0.5 was used to construct an index of the reference genome, and the clean paired-end reads were compared with the reference genome quickly and accurately. rMATS (4.0.2) software was used to analyze alternative splicing (AS), and the threshold for screening significant differences in AS was a false discovery rate of less than 0.05. StringTie (1.3.3b) was used to predict new transcription.

Differentially Expressed Gene Analysis

Gene abundances were analyzed with software FeatureCounts v1.5.0-p3 and expression levels were normalized by Fragments Per Kilobase of transcript per Million mapped reads. DESeq2 (1.16.1) software was used to analyze the differentially expressed gene (DEGs) between the chickens with or without the crested phenotype. DESeq2 provides statistical programs that use a model based on the negative binomial distribution to determine differential expression according to digital gene expression data. The Benjamini and Hochberg method was used to adjust the P -value. DEGs were filtered according to the standards of a $|\log_2\text{FoldChange}| \geq 0.0$ and a P -value ≤ 0.05 .

GO Enrichment and KEGG Pathway Analysis of DEGs

ClusterProfiler R (3.4.4) software was used to realize the GO and KEGG pathway enrichment analysis of the

DEGs, and the GO terms (<http://www.geneontology.org/>) and KEGG pathways (<http://www.genome.jp/kegg/>) that were significantly enriched with DEGs with a P -value of less than 0.01 as the threshold for significant enrichment.

Validation of RNA-Seq Results via qRT-PCR

To verify the accuracy of the sequencing results, 7 DEGs with large differences in expression levels that were related to the crested trait were selected for qRT-PCR verification by transcriptome sequencing analysis. The primer information for the 7 genes is shown in Table S1, and β -actin was used as a house-keeping gene. The 6 samples used are the same as RNA-Seq. According to the instructions of the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, MA), RNA was reverse transcribed into cDNA, and qRT-PCR was performed with 3 repeats on a CFX96TMTouch system (BIO-RAD, Hercules, CA). Twenty μL reaction system includes 10 μL SYBR Green Master Mix (2 \times) (No ROX) (MCE, NJ), 0.4 μL Forward Primer (10 $\mu\text{mol/L}$), 0.4 μL Reverse Primer (10 $\mu\text{mol/L}$), 2 μL cDNA and 7.2 μL RNase-Free Water. Reaction program: 95°C for 5 min; 95°C for 10 s, annealing for 30 s, 40 cycles; 95°C for 15 s; 60°C for 60 s; 95°C for 15 s.

Statistical Analysis

Relative gene expression levels were calculated via the $2^{-\Delta\Delta\text{Ct}}$ method, using R (V3.2) to estimate the correlations between the mRNA expression levels of 7 DEGs determined through qRT-PCR and RNA-Seq, and the results were presented in a histogram generated with GraphPad Prism software (version 8, GraphPad, La Jolla, CA).

Table 1. Reads quality statistics after filtering sequencing data.

| Sample name ¹ | Raw reads ² | Clean reads ³ | Clean bases ⁴ | Error rate ⁵ (%) | Q20 ⁶ (%) | Q30 ⁷ (%) | GC ⁸ (%) | Total mapped ⁹ (%) | Unique mapped ¹⁰ (%) |
|--------------------------|------------------------|--------------------------|--------------------------|-----------------------------|----------------------|----------------------|---------------------|-------------------------------|---------------------------------|
| C1 | 48175766 | 46376934 | 6.96G | 0.03 | 97.85 | 93.91 | 49.73 | 92.02 | 90.21 |
| C2 | 47854426 | 45438064 | 6.82G | 0.02 | 98.04 | 94.41 | 49.71 | 92.65 | 90.86 |
| C3 | 50051348 | 46182960 | 6.93G | 0.02 | 97.97 | 94.25 | 49.52 | 91.43 | 89.73 |
| NC1 | 46149130 | 43729046 | 6.56G | 0.02 | 97.99 | 94.36 | 49.96 | 92.84 | 91.00 |
| NC2 | 47178902 | 44932958 | 6.74G | 0.03 | 97.89 | 94.05 | 49.08 | 93.12 | 90.75 |
| NC3 | 46742410 | 43925624 | 6.59G | 0.03 | 97.81 | 93.96 | 49.18 | 92.92 | 90.81 |

¹C1, C2, C3 represent crested chickens; NC1, NC2, NC3 represent non-crested chickens.

²The number of reads in the original data.

³The number of reads after filtering the original data.

⁴The number of bases after filtering the original data.

⁵Overall data sequencing error rate.

⁶The percentage of bases with a Phred value greater than 20 to the total bases.

⁷The percentage of bases with a Phred value greater than 30 to the total bases.

⁸The percentage of G and C in the four bases in clean reads.

⁹The number and percentage of reads aligned to the genome.

¹⁰The number and percentage of reads aligned to the unique position of the reference genome.

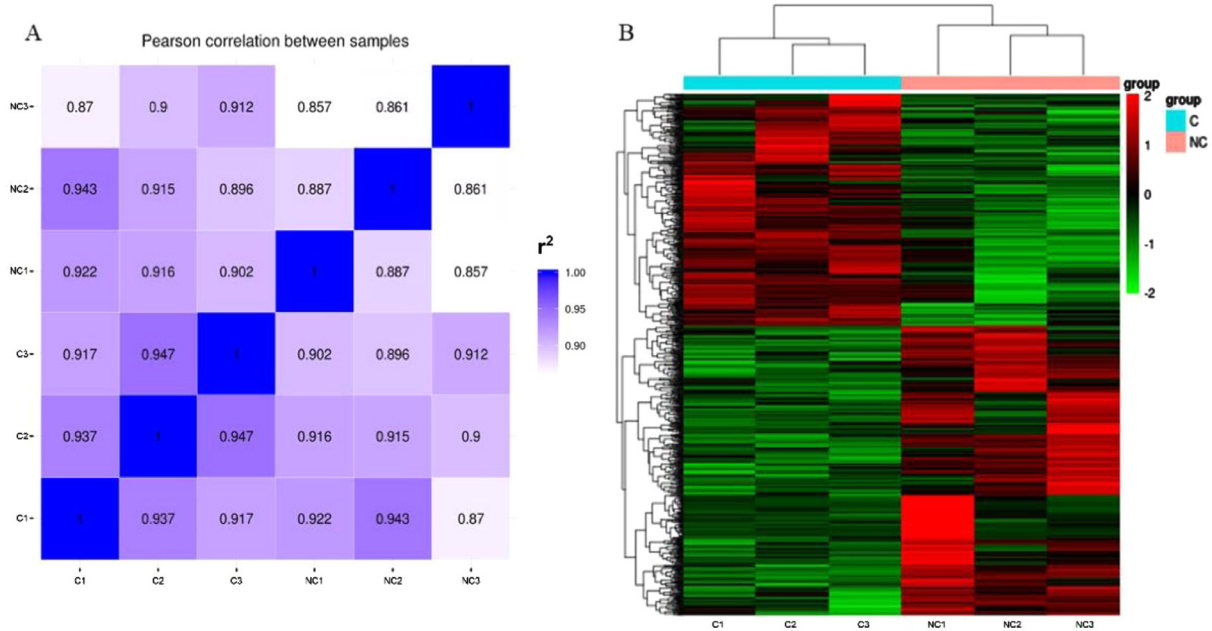


Figure 1. (A) Heat map of the correlations between samples. The horizontal and vertical coordinates are the squared values of the correlation coefficients of each sample. (B) Differentially expressed gene clustering heat map. The abscissa is the sample name, and the ordinate is the normalized value of the differential gene FPKM. The redder the color is, the higher the expression level, and the greener the color is, the lower the expression level.

RESULTS

Screening and Analysis of RNA-Seq Data From the Skull Tissue of Crested Wumeng Crested Chickens

RNA-Seq analysis was performed on 6 skull tissue samples from Wumeng crested chickens with or without the crested phenotype (Table 1). A total of 286.2 million raw reads were obtained, with an average of 476,900 raw reads per sample. The RNA-Seq sequencing error rate was 0.02 to 0.03%. After quality inspection and screening, 270.6 million clean reads were finally obtained, the number of clean bases was 40.60 G, the number of clean bases from each sample ranged from 6.56 to 6.96 G, the Q20 values ranged from 97.81 to 98.04%, and the Q30 values ranged from 93.91 to 94.41%. Guanine (G) and cytosine (C) bases accounted for 49.08 to 49.96% of the total bases in the clean reads. When the clean reads were aligned to the chicken reference genome, the percentage of total mapped clean reads was 91.43 to 93.12%, and the percentage of the reads (uniquely mapped) that were aligned to unique positions in the reference genome was between 89.73 and 91.00%. In addition, when the 6 samples were grouped, the Pearson correlation coefficient (r^2) between the groups was found to be greater than 0.85 (Figure 1A), and the samples in the 2 groups were clustered together (Figure 1B); these results indicated that the 3 biological samples in each group showed similar high performance, that the experimental sampling procedures and experimental conditions met the sequencing requirements and the obtained could be used for the subsequent biological information analysis.

After comparing the clean reads of the 6 sequenced samples with the chicken reference genome, it was found that the number of reads that aligned to exon regions of the genome accounted for the highest proportion of the clean reads (85.44–88.28%), followed by intergenic regions (6.79–7.53%) and intron regions (4.36–7.23%) (Table S2). RNA-Seq revealed 423 new transcripts from Wumeng crested chicken and 26,999 AS events (Table S3). Five types of AS events were observed, including skipped exons (SEs), mutually exclusive exons (MXEs), retained introns (RIs), alternative 5' splice sites (A5SSs), and alternative 3' splice sites (A3SSs), among which the main AS events identified were SEs and A3SSs. A total of 118 ASs showed significant differences, which were mainly SEs and RIs, and differences in upregulation were observed.

DEG Screening

After RPKM saturation and RNA-Seq correlation analyses, DESeq2 software (1.16.1) was used to analyze expression differences between the 2 comparison groups, and the differential gene expression profile between the C and NC groups was detected via the RPKM method. A total of 1,089 DEGs were identified in the C and NC groups. Compared with the NC group, there were 485 upregulated genes and 604 downregulated genes identified in the skull tissue of the chickens in the C group. Figure 2 shows the volcano map of differential gene expression between the C and NC groups. According to the combined results of RNA-Seq and gene function analyses, the top 10 upregulated and top 10 downregulated DEGs with the

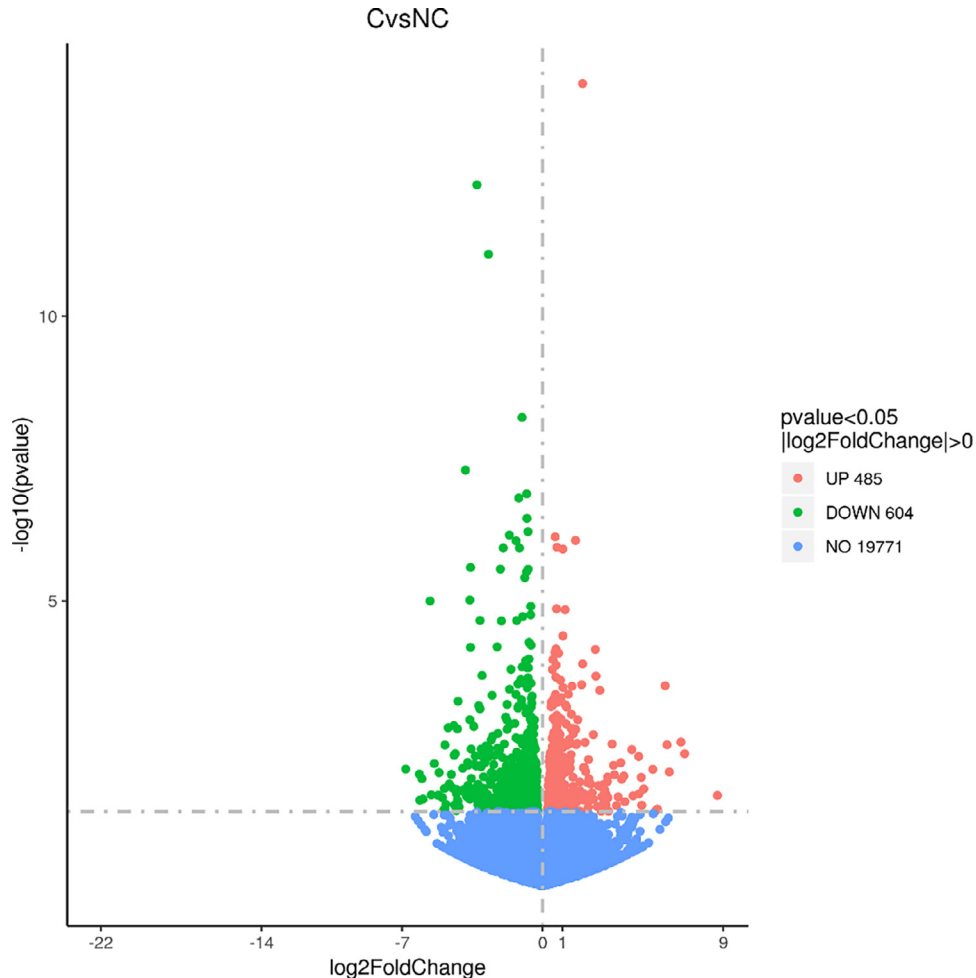


Figure 2. Volcano map of differential gene expression. The abscissa is the log₂FoldChange value, the ordinate is the -log₁₀p value and the blue dotted line represents the threshold of the differential gene screening criteria.

highest absolute fold difference values between the skull tissues of the C and NC groups are shown in Table S4. According to the RNA-Seq results for the skull tissue, the Homeobox C6 (*HOXC6*) and Homeobox C10 (*HOXC10*) genes related to the crested trait were found among the upregulated DEGs.

GO Enrichment Analysis of DEGs

In this study, 1,089 DEGs were enriched in 1,771 GO terms, and 42 GO terms were significantly enriched (P -value <0.01). There are 1,325, 222, and 224 GO terms were enriched in biological process (BP), cellular component (CC), and molecular functions (MFs) respectively. Among them, there are 36, 4, and 2 GO terms that are significantly enriched in BP, CC, and MF respectively (P -value <0.01). The GO terms showing significant differences that were related to the crested trait included signal transduction, cell development, cell differentiation, regulation of developmental processes, positive regulation of developmental processes, intracellular signal transduction, etc. The candidate genes for the crested trait included bone morphogenetic protein 2 (*BMP2*), ephrin receptor A3 (*EPHA3*), and ephrin receptor B1 (*EPHB1*). The results of DEG GO

enrichment analysis in skull tissue of Wumeng crested chickens with or without the crested trait are shown in Figure 3.

Analysis of the KEGG Pathways of the DEGs

The KEGG pathway enrichment results showed that the DEGs were enriched in 132 KEGG pathways, among which 4 KEGG pathways were significantly enriched (P -value <0.01), including the lysosome, glycosaminoglycan degradation, glycine, serine and threonine metabolism, and glycosaminoglycan biosynthesis-keratan sulfate pathways.

The upregulated DEGs were enriched in 98 KEGG pathways, and 6 KEGG pathways were significantly enriched (P -value <0.01), including the lysosome, cytokine-cytokine receptor interaction, glycosaminoglycan degradation, cell adhesion molecules, phagosome, and glycosaminoglycan biosynthesis-keratan sulfate pathways. The downregulated DEGs were enriched in 110 KEGG pathways, and 3 KEGG pathways were significantly enriched (P -value <0.01), including the glycine, serine, and threonine metabolism, nitrogen metabolism, and calcium signaling pathways. This study indicated that the KEGG gga04060 pathway may be involved in the regulation of the crested trait. There are 17 DEGs in

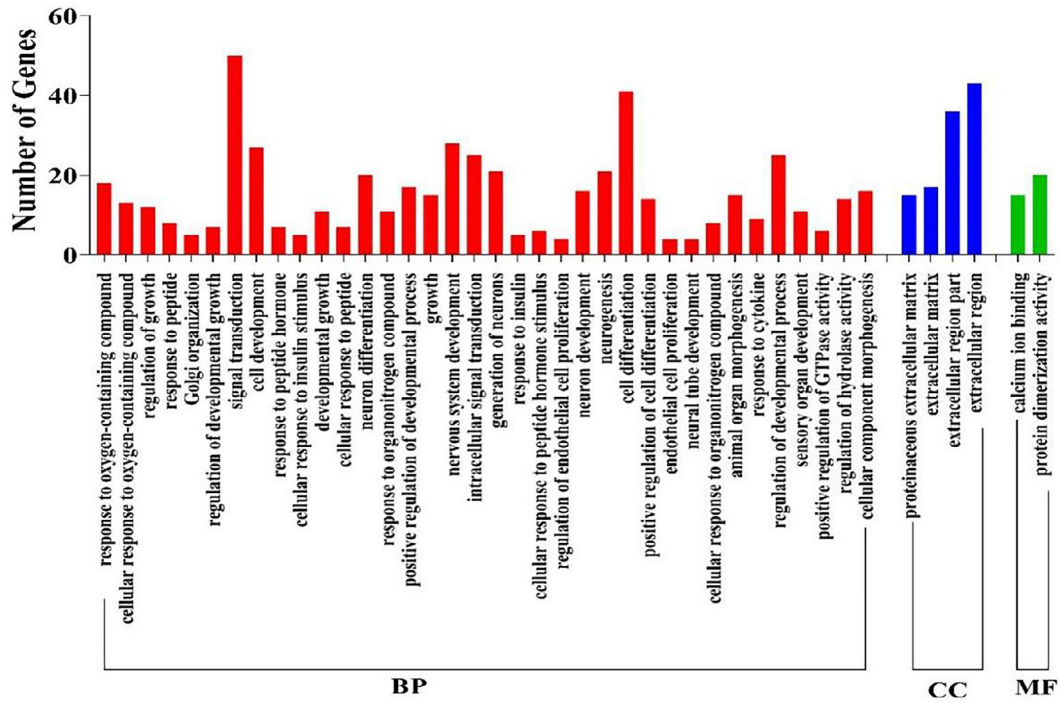


Figure 3. GO enrichment analysis histogram. The abscissa is the GO term, and the ordinate is the number of enriched genes for the GO term. Abbreviation: GO, Gene Ontology.

this pathway that may play an important role in crested phenotypic formation (Table S5).

Crested Trait-Related Candidate Genes

Based on the results of the differential gene expression, GO, KEGG pathway, QTL database, and gene function analyses, 7 genes were considered potential candidate genes for regulating the crested trait, including *BMP2*, *EPHA3*, *EPHB1*, *HOXC6*, sodium voltage-gated channel $\beta 2$ subunit (*SCN2B*), bone morphogenetic protein 7 (*BMP7*), and *HOXC10* (Table 2).

Validation of DEGs

To verify the results of RNA-Seq, 7 DEGs related to the crested trait showing large differences in expression levels according to RNA-Seq, including *BMP2*, *EPHA3*, *EPHB1*, *HOXC6*, *SCN2B*, *BMP7*, and *HOXC10*, were selected for qRT-PCR verification. The correlation

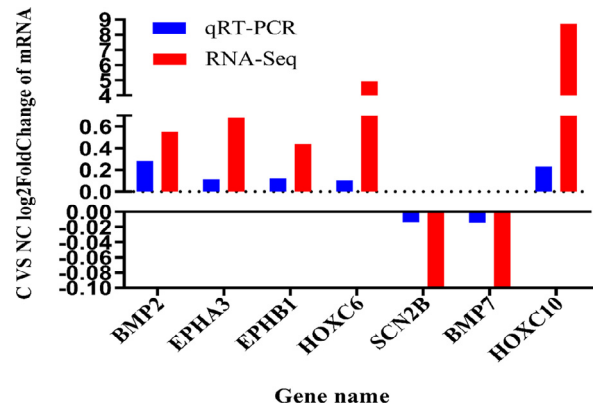


Figure 4. Correlations of mRNA expression levels of 7 DEGs between C and NC using RNA-Seq and qRT-PCR. The x- and y-axis correspond to the log₂ (ratio of C/NC) measured from RNA-Seq and qRT-PCR, respectively.

between the observed mRNA expression levels of the DEGs and the RNA-Seq results indicated that they were consistent (Figure 4), verifying the accuracy and

Table 2. Candidate gene information for crested traits.

| Gene name | Log2FoldChange ¹ | P-value ² | Gene_chr ³ | Gene length ⁴ | Gene function description |
|-----------|-----------------------------|----------------------|-----------------------|--------------------------|---|
| HOXC10 | 8.710552772 | 0.02581501 | NC_008465.4 | 1642 | homeobox C10 |
| HOXC6 | 4.940210301 | 0.03840981 | NC_008465.4 | 1058 | homeobox C6%2C transcript variant X1 |
| BMP7 | -0.917693596 | 0.01453960 | NC_006107.5 | 4421 | bone morphogenetic protein 7 |
| BMP2 | 0.55069126 | 0.03525094 | NC_006090.5 | 5947 | bone morphogenetic protein 2%2C transcript variant X8 |
| SCN2B | -1.582833165 | 0.03056920 | NC_006111.5 | 2655 | sodium voltage-gated channel beta subunit 2 |
| EPHA3 | 0.682289723 | 0.00178093 | NC_006088.5 | 6867 | EPH receptor A3%2C transcript variant X1 |
| EPHB1 | 0.440134458 | 0.00860424 | NC_006096.5 | 4688 | EPH receptor B1%2C transcript variant X2 |

¹The ratio of gene expression levels between the treatment group and the control group is processed by the shrinkage model of the different analysis software, and finally, the logarithm is taken to the base of 2.

²Significance test value.

³The name of the chromosome where the gene is located.

⁴Gene length, the sum of all exon non-overlapping regions from the beginning to the end of the gene.

repeatability of the RNA-Seq results and gene expression data.

DISCUSSION

The development of high-throughput sequencing technology (NGS) has changed transcriptomics. The method of RNA analysis through complementary DNA sequencing is known as RNA-Seq (Wang et al., 2009). Compared with previous microarray-based methods and Sanger sequencing, RNA-Seq achieves higher coverage, a deeper resolution of dynamic characteristics, and a more effective comparison of gene expression patterns (Finotello and Di Camillo, 2015; Kukurba and Montgomery, 2015). Through RNA-Seq analysis and the verification of the results, this study screened 1089 DEGs related to the crested phenotype. The qRT-PCR verification results for the *BMP2*, *EPHA3*, *EPHB1*, *HOXC6*, *SCN2B*, *BMP7*, and *HOXC10* DEGs were consistent with the RNA-Seq results, and these genes are located within QTLs, indicating that they participate in the regulation of the crested trait and have relatively strong genetic effect on the crested phenotype.

BMP2 is a member of the transforming growth factor β (TGF- β) superfamily and is a specific osteogenic gene. It is involved in multiple biological processes, such as bone formation, fat deposition, cancer occurrence, and growth and development. Most of the research on *BMP2* focuses on bone metabolism (Wu et al., 2015). Studies by Kovermann et al. (2019) have shown that in vitro culture medium containing *BMP2* can induce human synovial stem cells to form cartilage. In combined culture medium containing TGF- β 1 and *BMP2*, the expression of aggrecan (*ACAN*) was shown to be significantly upregulated and promoted cartilage formation. In a skull defect model, extracellular calcium ions can enhance the phosphorylation of the Smad signaling pathway by *BMP2* and enable osteocalcin, osteogenic specific transcription factor (Runt-related Transcription Factor 2, *Runx2*), and osteogenic-related transcription factor (Osterix, *Osx*) expression to be upregulated to promote bone regeneration in vivo (Aquino-Martínez et al., 2017). The typical phenotype of crested chickens includes the protrusion of the skull, and the expression of the *BMP2* gene in the protruding skull tissue of crested chickens is upregulated, indicating that *BMP2* has a positive regulatory effect on bone formation, which is consistent with the above research results. Based on the above analysis, it is speculated that *BMP2* is an important candidate gene regulating the chicken crested trait.

BMP7 is also a member of the transforming growth factor β (TGF- β) superfamily. It plays roles in chicken skin pigmentation (Hou et al., 2020), early signal transmission in the induction of epidermal organs (Harris et al., 2004), the statoacoustic ganglion (SAG), and developmental processes such as growth and neuron survival in vitro. When laying hens are sexually mature, calcium is necessary for eggshell formation, and the medulla oblongata can serve as a source of calcium for

laying hens, mediated by *BMP7* (Gloux et al., 2019). Studies have shown that *BMP7* does not directly induce osteogenic differentiation. Instead, it first promotes chondrocyte differentiation and produces morphogens and then regulates osteogenic differentiation in an auto-crine manner (Gerstenfeld et al., 2002). Mou et al. (2011) found that the regionalized production of retinoic acid uniformly upregulated the expression of *BMP7*, thereby altering the neck skin phenotype but not the body skin phenotype. This finding indicates that *BMP7* is closely related to the crested trait.

Homeobox genes are clusters of highly conserved gene sequences in the *Hox* gene family (Robert et al., 1989). *Hox* genes can regulate the normal development of individual morphology during the development of animal embryos (Morgan, 1997), including effects on the morphology of head (Krumlauf, 2016), body (Morin-Kensicki et al., 2002), and vertebrae (Martín-Del-Campo et al., 2019) among other organs. The *Hox* gene family consists of 39 genes divided into 4 clusters (*HOXA*, *HOXB*, *HOXC*, and *HOXD*) (Cillo et al., 2001) located on 4 different chromosomes; the *HOXC6* and *HOXC10* genes are members of the *HOXC* cluster. The related research on *HOXC6* and *HOXC10* has been conducted mainly in the context of cancer (Dang et al., 2020), cell proliferation and migration (Kim et al., 2019; Ma et al., 2020), neural development (Wu et al., 2008), and adipose tissue browning (Ng et al., 2017). There is no research indicating that *HOXC6* and *HOXC10* are related to the formation of crested phenotype. In March 2004, the first draft of the chicken genome was published, in which the physical map of LGE22C19W28 was approximately 70 kb. In the Gallus_gallus-2.1 component, LGE22C19W28 and LGE50C23 were merged into LGE22C19W28_E50C23 (Sequence and comparative analysis of the chicken, 2004; Groenen et al., 2000; Wallis et al., 2004). Using an Illumina 60K chicken single nucleotide polymorphism (SNP) microsphere chip to analyze the SNPs of a Beijing oil chicken F2 population and broiler commercial strains, three SNPs were detected on the chicken GGA2 and GGA28 chromosomes and in the LGE22C19W28_E50C23 (LGE22) linkage group. QTLs are significantly related to head traits. Three QTLs were identified by linkage analysis. In the 5 cM/0.51 Mb region of LGE22, 1 microRNA and 7 genes (*MIR1668*, *LARP4*, *COX14*, *ACCN2*, *HDAC7*, *TWIST3*, *SLC48A1*, *NEUROD4*) were found; this region is similar to that containing *HOXC8* and closely connected to it, and it is speculated that it is an important area affecting the crested trait (Sun et al., 2015). A genome-wide association study and linkage analysis of crested chickens and crested ducks proved that the crested trait is associated with the LGE22C19W28_E50C23 linkage group and is related to the *HOXC* cluster on the chromosome; *HOXC6* and *HOXC10* are also included in the *HOXC* cluster. According to the collinearity of the *Hox* gene activation sequence (Mallo, 2018) and the adjacent positions of *HOXC6* and *HOXC10* in the *HOXC8* and *HOXC11* candidate star genes of the chicken crested trait combined

with the RNA-Seq analysis of the skull tissue of Wumeng crested chicken, this study is the first to indicate that the *HOXC6* and *HOXC10* genes participate in the development and regulation of the chicken crested trait.

Candidate genes for the duck crested trait include *HOXC8* and the receptor tyrosine kinase family branch ligand “*Ephrin*” A and B subfamily *EphA* (*EphA1*~*EphA10*), and *EphB* (*EphB1*~*EphB6*) genes (Nikolov et al., 2013). *Eph* and *Ephrin* play important roles in animal nerve development, information transmission between tissues and cells, the immune system, bone, and tumor development, and metastasis (Nikolov et al., 2014; Kania and Klein, 2016; Lodola et al., 2017; Wang et al., 2020). Studies have shown that the *EphA2*, *EphA3*, and *EphB2* genes are highly expressed in the intracranial fat body of crested white ducks, and their expression levels are lower in the cerebellum and brain tissues near the fat body in crested white ducks. It is speculated that the expression of these genes is related to the formation of the intracranial fat body of the crested duck and the formation of the duck crest (Zhang et al., 2020). There is a missense mutation that results in an amino acid substitution (Arg758Cys) in the active site of the *EphB2* kinase domain that is significantly related to the crested head phenotype. There is also a G to A missense mutation located at 636 bp in the 6th exon of the *EphB2* gene, which leads to a switch from glycine to arginine (Gly636Arg). When genotyping and association analyses of 50 crowned and 75 crownless pigeons were performed, it was found that the mutation from G to A was very strongly related to the crested phenotype (Shapiro et al., 2013; Vickrey et al., 2015). Studies have shown that the same locus can control the crested traits of many varieties; there are also alternative alleles at this locus and additional modified loci that can control the development of crested traits. Combined with the regulatory role of *EphA2* and *EphB1* in duck and pigeon crested traits and the finding of this study that *EphA2* and *EphB1* were significantly enriched in GO terms such as neuron differentiation, nervous system development, and neuron production, *EphA2*, and *EphB1* can be considered to participate in the regulation of chicken crest formation.

The sodium voltage-gated channel is a protein complex composed of a pore-forming α subunit and two related β subunits (Cortada et al., 2019). *SCN2B* is one of the β subunit-encoding genes, and the main biological functions of its product are to form sodium channels, regulate the activity of sodium ion transporters, play a necessary role in normal action potential generation, and control the excitability of neurons and cardiomyocytes (Chen et al., 2002). *SCN2B* has been proven to be a candidate gene for a variety of neurological diseases (Jones et al., 1996), such as Brugada syndrome (Riuró et al., 2013), Draviet syndrome (Gong et al., 2019), and epilepsy (Wang et al., 2012a). At present, there is no research showing that *SCN2B* is related to crested phenotype formation, and it is necessary to study the correlation between the *SCN2B* gene and the development of

this phenotype. The results of this study showed that *SCN2B* is differentially expressed in the skull tissue of chickens with or without the crested phenotype with a high fold difference. Additionally, the microsatellite marker *SCN8A* is closely linked to the crested trait and is located in the crested trait linkage group E22C19W28 (Gao et al., 2005). Given that *SCN8A* and *SCN2B* are both sodium voltage-gated channels, it is speculated that *SCN2B* is involved in the formation of the crested phenotype.

RNA-Seq revealed a total of 1,089 DEGs between N and NC and indicated that some genes with known functions, such as *SCN2B*, *HOXC6*, and *HOXC10*, show expression differences in skull tissue, but the relationship with the crested trait needs to be further studied. These RNA-Seq results provide strong evidence of the significant differences in gene expression levels in the whole genome during the growth of Wumeng crested chicken chickens. This study provides an approach for studying Wumeng crested chickens and the molecular mechanism of crested trait development.

CONCLUSIONS

This study was based on RNA-Seq to study the skull tissue transcriptome of 6 Wumeng crested chickens, and candidate genes for crest traits were screened and identified. The results showed that there were 1,089 DEGs in the skull tissue of Wumeng crested chickens. The description of the differentially expressed known and new genes revealed that they may be related to the crested trait and other physiological functions. Comprehensive differential gene expression, GO and KEGG pathway enrichment, QTL database, gene biological function, and qRT-PCR verification analyses showed that *BMP2*, *EPHA3*, *EPHB1*, *HOXC6*, *SCN2B*, *BMP7*, and *HOXC10* may be candidates genes regulating the crested trait.

ACKNOWLEDGMENTS

This research was funded by the Science and Technology Plan Project of Guizhou Province, China ([2017] 2533-3). Authors are thankful to Jinfengqi Natural Animal Husbandry Co., Ltd. China for providing the experimental chickens.

DISCLOSURES

The authors declare no conflict of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at [doi:10.1016/j.psj.2021.101357](https://doi.org/10.1016/j.psj.2021.101357).

REFERENCE

- Aquino-Martínez, R., N. Artigas, B. Gámez, J. L. Rosa, and F. Ventura. 2017. Extracellular calcium promotes bone formation from bone marrow mesenchymal stem cells by amplifying the effects of BMP-2 on SMAD signalling. *PLoS One* 12:e0178158.
- Bartels, T. 2003. Variations in the morphology, distribution, and arrangement of feathers in domesticated birds. *J. Exp. Zool. B Mol. Dev. Evol.* 298:91–108.
- Chen, C., V. Bharucha, Y. Chen, R. E. Westenbroek, A. Brown, J. D. Malhotra, D. Jones, C. Avery, P. J. Gillespie 3rd, K. A. Kazen-Gillespie, K. Kazarinova-Noyes, P. Shrager, T. L. Saunders, R. L. Macdonald, B. R. Ransom, T. Scheuer, W. A. Catterall, and L. L. Isom. 2002. Reduced sodium channel density, altered voltage dependence of inactivation, and increased susceptibility to seizures in mice lacking sodium channel beta 2-subunits. *Proc. Natl. Acad. Sci. U. S. A.* 99:17072–17077.
- Cillo, C., M. Cantile, A. Faiella, and E. Boncinelli. 2001. Homeobox genes in normal and malignant cells. *J. Cell. Physiol.* 188:161–169.
- Cortada, E., R. Brugada, and M. Verges. 2019. Trafficking and function of the voltage-gated sodium channel $\beta 2$ subunit. *Biomolecules* 9:604.
- Dang, Y., J. Chen, W. Feng, C. Qiao, W. Han, Y. Nie, K. Wu, D. Fan, and L. Xia. 2020. Interleukin 1 β -mediated HOXC10 overexpression promotes hepatocellular carcinoma metastasis by upregulating PDPK1 and VASP. *Theranostics* 10:3833–3848.
- Finotello, F., and B. Di Camillo. 2015. Measuring differential gene expression with RNA-seq: challenges and strategies for data analysis. *Brief Funct. Genomics* 14:130–142.
- Fisher, R. A. 1934. Crest and hernia in fowls due to a single gene without dominance. *Science* 80:288–289.
- Frahm, H. D., and G. Rehkämper. 1998. Allometric comparison of the brain and brain structures in the white crested polish chicken with uncrested domestic chicken breeds. *Brain Behav. Evol.* 52:292–307.
- Frahm, H. D., and G. Rehkämper. 2004. Brain size, brain composition and intracranial fat bodies in a population of free-living crested ducks ('Hochbrutflugenten'). *Br. Poult. Sci.* 45:590–597.
- Frahm, H. D., G. Rehkämper, and C. W. Werner. 2001. Brain alterations in crested versus non-crested breeds of domestic ducks (*Anas platyrhynchos* f.d.). *Poult. Sci.* 80:1249–1257.
- Gao, Y., X. X. Hu, X. M. Deng, J. D. Feng, and N. Li. 2005. Linkage mapping of the SCN8A gene to chicken linkage group E22C19W28. *Anim. Genet.* 36:284.
- Gerstenfeld, L. C., J. Cruceta, C. M. Shea, K. Sampath, G. L. Barnes, and T. A. Einhorn. 2002. Chondrocytes provide morphogenic signals that selectively induce osteogenic differentiation of mesenchymal stem cells. *J. Bone Miner. Res.* 17:221–230.
- Gloux, A., M. J. Duclos, A. Brionne, M. Bourin, Y. Nys, and S. Réhault-Godbert. 2019. Integrative analysis of transcriptomic data related to the liver of laying hens: from physiological basics to newly identified functions. *BMC Genomics* 20:821.
- Gong, J. E., H. M. Liao, H. Y. Long, X. M. Li, L. L. Long, L. Zhou, W. P. Gu, S. H. Lu, Q. Qu, L. M. Yang, B. Xiao, and J. Qu. 2019. SCN1B and SCN2B gene variants analysis in dravet syndrome patients: analysis of 22 cases. *Medicine (Baltimore)*. 98:e14974.
- Groenen, M. A., H. H. Cheng, N. Bumstead, B. F. Benkel, W. E. Briles, T. Burke, D. W. Burt, L. B. Crittenden, J. Dodgson, J. Hillel, S. Lamont, A. P. de Leon, M. Soller, H. Takahashi, and A. Vignal. 2000. A consensus linkage map of the chicken genome. *Genome Res.* 10:137–147.
- Harris, M. P., B. L. Linkhart, and J. F. Fallon. 2004. Bmp7 mediates early signaling events during induction of chick epidermal organs. *Dev. Dyn.* 231:22–32.
- Hou, H., X. Wang, C. Zhang, Y. Tu, W. Lv, X. Cai, Z. Xu, J. Yao, and C. Yang. 2020. Genomic analysis of GBS data reveals genes associated with facial pigmentation in Xinyang blue-shelled layers. *Arch. Anim. Breed* 63:483–491.
- Joller, S., P. Ammann, C. Flury, and C. Drögemüller. 2018. Evaluation of HOXC8 in crested Swiss chicken. *Anim. Genet.* 49:334–336.
- Jones, J. M., M. H. Meisler, and L. L. Isom. 1996. Scn2b, a voltage-gated sodium channel beta2 gene on mouse chromosome 9. *Genomics* 34:258–259.
- Kania, A., and R. Klein. 2016. Mechanisms of ephrin-Eph signalling in development, physiology and disease. *Nat. Rev. Mol. Cell Biol.* 17:240–256.
- Kerje, S., P. Sharma, U. Gunnarsson, H. Kim, S. Bagchi, R. Fredriksson, K. Schütz, P. Jensen, G. von Heijne, R. Okimoto, and L. Andersson. 2004. The Dominant white, Dun and Smoky color variants in chicken are associated with insertion/deletion polymorphisms in the PMEL17 gene. *Genetics* 168:1507–1518.
- Kim, J., D. H. Bae, J. H. Kim, K. S. Song, Y. S. Kim, and S. Y. Kim. 2019. HOXC10 overexpression promotes cell proliferation and migration in gastric cancer. *Oncol. Rep.* 42:202–212.
- Kovermann, N. J., V. Basoli, E. Della Bella, M. Alini, C. Lischer, H. Schmal, E. J. Kubosch, and M. J. Stoddart. 2019. BMP2 and TGF- β cooperate differently during synovial-derived stem-cell chondrogenesis in a dexamethasone-dependent manner. *Cells* 8:636.
- Krumlauf, R. 2016. Hox genes and the hindbrain: a study in segments. *Curr. Top. Dev. Biol.* 116:581–596.
- Kukurba, K. R., and S. B. Montgomery. 2015. RNA sequencing and analysis. *Cold Spring Harb. Protoc.* 2015:951–969.
- Lodola, A., C. Giorgio, M. Incerti, I. Zanotti, and M. Tognolini. 2017. Targeting Eph/ephrin system in cancer therapy. *Eur. J. Med. Chem.* 142:152–162.
- Ma, K., C. Zhao, K. Guo, Z. Fu, C. Che, B. Dong, C. Pang, S. Zhang, W. Liu, Z. Yang, R. Liang, and L. Wang. 2020. Low HOXC10 expression in liver cancer regulates proliferation via a mechanism involving miR-221 and the MAPK signaling pathway. *Oncol. Lett.* 20:127.
- Mallo, M. 2018. Reassessing the role of Hox genes during vertebrate development and evolution. *Trends Genet.* 34:209–217.
- Martín-Del-Campo, R., I. Sifuentes-Romero, and A. García-Gasca. 2019. Hox genes in reptile development, epigenetic regulation, and teratogenesis. *Cytogenet. Genome Res.* 157:34–45.
- Morgan, B. A. 1997. Hox genes and embryonic development. *Poult. Sci.* 76:96–104.
- Morin-Kensicki, E. M., E. Melancon, and J. S. Eisen. 2002. Segmental relationship between somites and vertebral column in zebrafish. *Development* 129:3851–3860.
- Mou, C., F. Pitel, D. Gourichon, F. Vignoles, A. Tzika, P. Tato, L. Yu, D. W. Burt, B. Bed'hom, M. Tixier-Boichard, K. J. Painter, and D. J. Headon. 2011. Cryptic patterning of avian skin confers a developmental facility for loss of neck feathering. *PLoS Biol.* 9:e1001028.
- Ng, Y., S. X. Tan, S. Y. Chia, H. Y. Tan, S. Y. Gun, L. Sun, W. Hong, and W. Han. 2017. HOXC10 suppresses browning of white adipose tissues. *Exp. Mol. Med.* 49:e292.
- Nikolov, D. B., K. Xu, and J. P. Himanen. 2013. Eph/ephrin recognition and the role of Eph/ephrin clusters in signaling initiation. *Biochim. Biophys. Acta* 1834:2160–2165.
- Nikolov, D. B., K. Xu, and J. P. Himanen. 2014. Homotypic receptor-receptor interactions regulating Eph signaling. *Cell Adh. Migr.* 8:360–365.
- Riuró, H., P. Beltran-Alvarez, A. Tarradas, E. Selga, O. Campuzano, M. Vergés, S. Pagans, A. Iglesias, J. Brugada, P. Brugada, F. M. Vázquez, G. J. Pérez, F. S. Scornik, and R. Brugada. 2013. A missense mutation in the sodium channel $\beta 2$ subunit reveals SCN2B as a new candidate gene for Brugada syndrome. *Hum. Mutat.* 34:961–966.
- Robert, B., D. Sassoon, B. Jacq, W. Gehring, and M. Buckingham. 1989. Hox-7, a mouse homeobox gene with a novel pattern of expression during embryogenesis. *EMBO J.* 8:91–100.
- Romanov, M. N., A. A. Sazanov, and A. F. Smirnov. 2004. First century of chicken gene study and mapping – a look back and forward. *Worlds Poult. Sci. J.* 60:19–41.
- Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution. (2004). *Nature* 432:695–716.
- Shapiro, M. D., Z. Kronenberg, C. Li, E. T. Domyan, H. Pan, M. Campbell, H. Tan, C. D. Huff, H. Hu, A. I. Vickrey, S. C. Nielsen, S. A. Stringham, H. Hu, E. Willerslev, M. T. Gilbert, M. Yandell, G. Zhang, and J. Wang. 2013. Genomic diversity and evolution of the head crest in the rock pigeon. *Science* 339:1063–1067.
- Stange, M., D. Núñez-León, M. R. Sánchez-Villagra, P. Jensen, and L. A. B. Wilson. 2018. Morphological variation under domestication: how variable are chickens? *R. Soc. Open Sci.* 5:180993.

- Sun, Y., R. Liu, G. Zhao, M. Zheng, Y. Sun, X. Yu, P. Li, and J. Wen. 2015. Genome-wide linkage analysis identifies loci for physical appearance traits in chickens. *G3 (Bethesda)* 5:2037–2041.
- Vickrey, A. I., E. T. Domyan, M. P. Horvath, and M. D. Shapiro. 2015. Convergent evolution of head crests in two domesticated columbids is associated with different missense mutations in *EphB2*. *Mol. Biol. Evol.* 32:2657–2664.
- Wallis, J. W., J. Aerts, M. A. Groenen, R. P. Crooijmans, D. Layman, T. A. Graves, D. E. Scheer, C. Kremitzki, M. J. Fedele, N. K. Mudd, M. Cardenas, J. Higginbotham, J. Carter, R. McGrane, T. Gaige, K. Mead, J. Walker, D. Albracht, J. Davito, S. P. Yang, S. Leong, A. Chinwalla, M. Sekhon, K. Wylie, J. Dodgson, M. N. Romanov, H. Cheng, P. J. de Jong, K. Osoegawa, M. Nefedov, H. Zhang, J. D. McPherson, M. Krzywinski, J. Schein, L. Hillier, E. R. Mardis, R. K. Wilson, and W. C. Warren. 2004. A physical map of the chicken genome. *Nature* 432:761–764.
- Wang, J., X. Zheng, Q. Peng, X. Zhang, and Z. Qin. 2020. Eph receptors: the bridge linking host and virus. *Cell. Mol. Life Sci.* 77:2355–2365.
- Wang, J. W., X. Y. Shi, H. Kurahashi, S. K. Hwang, A. Ishii, N. Higurashi, S. Kaneko, and S. Hirose. 2012a. Prevalence of *SCN1A* mutations in children with suspected Dravet syndrome and intractable childhood epilepsy. *Epilepsy Res.* 102:195–200.
- Wang, S., Y. Zhang, X. Yuan, R. Pan, W. Yao, L. Zhong, Q. Song, S. Zheng, Z. Wang, Q. Xu, G. Chang, and G. Chen. 2018. Identification of differentially expressed microRNAs during preadipocyte differentiation in Chinese crested duck. *Gene* 661:126–132.
- Wang, Y., Y. Gao, F. Imsland, X. Gu, C. Feng, R. Liu, C. Song, M. Tixier-Boichard, D. Gourichon, and Q. Li. 2012b. The crest phenotype in chicken is associated with ectopic expression of *HOXC8* in cranial skin. *PLoS One* 7:e34012.
- Wang, Y., Y. Gao, F. Imsland, X. Gu, C. Feng, R. Liu, C. Song, M. Tixier-Boichard, D. Gourichon, Q. Li, K. Chen, H. Li, L. Andersson, X. Hu, and N. Li. 2012c. The crest phenotype in chicken is associated with ectopic expression of *HOXC8* in cranial skin. *PLoS One* 7:e34012.
- Wang, Z., M. Gerstein, and M. Snyder. 2009. RNA-Seq: a revolutionary tool for transcriptomics. *Nat. Rev. Genet.* 10:57–63.
- Wu, Y., G. Wang, S. A. Scott, and M. R. Capecchi. 2008. *Hoxc10* and *Hoxd10* regulate mouse columnar, divisional and motor pool identity of lumbar motoneurons. *Development* 135:171–182.
- Wu, Y., L. Xia, Y. Zhou, Y. Xu, and X. Jiang. 2015. Icariin induces osteogenic differentiation of bone mesenchymal stem cells in a MAPK-dependent manner. *Cell Prolif.* 48:375–384.
- Yoshimura, K., K. Kinoshita, M. Mizutani, Y. Matsuda, and N. Saito. 2012. Inheritance and developmental pattern of cerebral hernia in the crested Polish chicken. *J. Exp. Zool. B Mol. Dev. Evol.* 318:613–620.
- Yuan, X., S. Zheng, Y. Zhang, Q. Guo, S. Wang, Y. Bi, W. Dai, X. Shen, T. Gu, R. Pan, Q. Song, Z. Wang, Y. Zhang, Q. Xu, G. Chang, and G. Chen. 2019. Embryonic morphology observation and *HOXC8* gene expression in crest cushions of Chinese Crested duck. *Gene* 688:98–106.
- Zhang, H., T. Ge, S. Peng, S. Zhong, and Z. Zhou. 2016. Microstructure features of proventriculus and ultrastructure of the gastric gland cells in Chinese Taihe Black-bone silky fowl (*Gallus gallus domesticus* Brisson). *Anat. Histol. Embryol.* 45:1–8.
- Zhang, Y., Q. Guo, Y. Bian, Z. Wang, Q. Xu, G. Chang, and G. Chen. 2020. Whole genome re-sequencing of crested traits and expression analysis of key candidate genes in duck. *Gene* 729:144282.