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Research Article

Genome-wide and RNA-Seq Highlight Genetic Characteristics of Rumpless Signals in Piao Chicken

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Abstract

Piao chicken is a unique rumpless chicken breed in China. Understanding the genetic relationship between rumpless chicken and other specific selection target breeds in the process of differentiation of the rumpless signal genes is helpful to reveal the genetic basis of rumplessnesss. In this study, transparent staining was performed on the bones of 16 tailed chickens and 16 Piao chickens to observe the dynamic bone formation process of embryos at different developmental stages. Microarray data of 988 chickens, resequencing results of 30 Piao chickens and 30 tailed chickens and 10 transcriptome samples were used for analysis. The results showed that tailbone development had tail buds before 4 to 6 days of incubation, and the difference was not significant between tailed chicken and Piao chicken, and the tail buds gradually degenerated in the later stage of Piao chicken. Most genes showed obvious inhibited expression in 8 d of embyro. We also found that exon skip was the most common type, and the PSI values of the *ADGRL3* and *PROM1* genes were differentially expressed between tailed chicken and Piao chicken of embryonic development constantly, which may be a special role that has not been recognized before. The network attributes of Piao chicken is tighter and the linkage between genes is stronger, especially the wnt signaling pathway and notch signaling pathway were mentioned.

Keywords: Piao chicken, Rumpless chicken, Dynamic transcriptional characteristics, Co-expression network, Signaling pathway

Introduction

There are hundreds of domestic chicken breeds worldwide, including indigenous, commercial, and cockfighting chicken, were developed by artificial selection for different purposes [1]. The rich genetic diversity and extensive genetic basis of these breeds provided excellent materials [2,3] in the sustainable utilization and conservation of these genetic resources [4,5]. “Rumpless” is when a genetic mutation causes an animal to lose a tail and have a stunted tail bud [6]. The rumpless chicken has the loss of the tail omnium and the tail vertebrae, which the first two bones behind the ischium have the shape of vertebrae, and the last one is irregularly shaped [7]. This difference in the tip of the tail shows how different species terminate their spines [8]. Previous studies have reported that the mutation region of rumplessness in embryo of rumpless Araucana chicken at different stages is located the 2.14 Mb chromosome region of chromosome 2, and two unique genes *IRX1* and *IRX2* are central for developmental prepatterning. Although there were no mutations in their coding region sequences, the protein expression levels

of *IRX1* and *IRX2* were significantly different between tailed and rumpless chicken [3]. Wang et al.[9] revealed that *IRX4*, *IL18*, *HSPB2* and *CRYAB* genes are implicated in tail development, and showed that rumplessness might be selected along with high-yield traits in Piao chicken. The three regions with the strongest repeatable selection signal are CHR2:85.52-86.07 Mb, CHR4:75.42-75.48 Mb, and CHR24:6.14-6.25 Mb. Hongshan chicken have rumpless, however, the number of caudal vertebrae in rumpless Hongshan chickens was normal, so rumplessness in Hongshan chicken was not related to the development of the caudal vertebrae. rumplessness in Hongshan was due to abnormal development of tail feather rather than abnormal development of caudal vertebrae [10]. Wang's reorting allowed us to reduce the search area to 71.8-72 Mb on the Z chromosome, which appeared a strong candidate pseudogene *LOC431648* involving in wnt/β-catenin signaling pathway to regulate feather development in chicken [11]. By measuring two tail length variables (central and maximum) of Japanese indigenous chickens, it was revealed that the shape of the tail feathers varies with the growth stage [12]. The research team previously identified 17 selected regions related to rumplessness by comparing the genomes of tailed chicken and rumpless chicken, which are mainly distributed on chromosomes 2, 10 and 15, and are mainly involved in neural development, skeletal development, ganglia development and other processes. At the same time, the QTLs in the candidate regions were found to be related to egg weight, carcass weight, pecking feather, tibia character, spleen weight, body weight and growth traits. It is noteworthy that *IRX4* (CHR2:86065381-86068294) and *IRX2* (2:8,6624614-86631727) genes were annotated near the interval 86100001-86140000 and 86190001-86230000 on 2 chromosome [13].

In the selection process of other breeds, is there any overlap between mutant genes in their genomes and those of rumpless chickens? What are the transcriptional characteristics of these overlapping genes between chicken and rumpless chickens? So far, the genetic mechanism of rumplessness in Chinese Piao chicken not been clarified [14]. The purpose of this research was to observe the key period of bone development of tailed chicken and Piao chicken by staining the embryonic bones and making transparent specimens to observe the overall skeleton histomorphology and the position relationship between bones and muscles. Based on the microarray data from public database GEO, the differentiation relationship of tail length traits among different selected breeds was compared [15]. Furthermore, we found the transcriptional expression characteristics of rumplessness in embryo development, which left the selective differentiation signal during the long process of chicken breed selection. The dynamic changes of gene expression regulation network and the functional classification of differential gene sets were compared between tailed chicken and rumpless chicken during development.

Material and Methods

Ethical Statement

All experimental procedures were approved by the Animal Protection and Use Committee of Inner Mongolia Agricultural University and strictly followed animal welfare and ethical guidelines ([2020]085).

Sample Collection and SNP Calling

The downloaded microarray data is used for analysis SNP and population structure analysis. Neighbor-joining tree was contructed using PHYLIP software (vision 3.69) [16] based on quality-controlled data *(Fig. 1)*. A total of 988 samples came from report of Zhang et al.[15] were regrouped and screened in this study, which were divided into local, commercial, game and wild types according to different uses. We calculated the pairwise F_{ST} in 4 and the top 5% loci were selected and the genes in the genome were mapped. Data on the genomes of tailed and rumpless chicken came from Hu's doctoral dissertation [13], including whole-genome re-sequencing results of 30 Piao chicken and 30 tailed [13]. In this experiment, compared the influence of rumplessnesss on the formation of different breeds by overlapping analysis between candidate genes of Hu [13] and differentiated genes of different breeds.

Egg Hatching

The egg incubator tank filled with water, temperature and humidity of incubators were adjusted 2 h in advance before placing fertilized eggs (Camellia chicken and Piao chicken). The temperature for chick hatching period and incubation eggs was 37.5-39.5ºC. The relative humidity during egg incubation was 65-70%, while during the hatching period was 55-60%. The round blunt end of the fertilized egg facing up. The egg incubator was ventilated

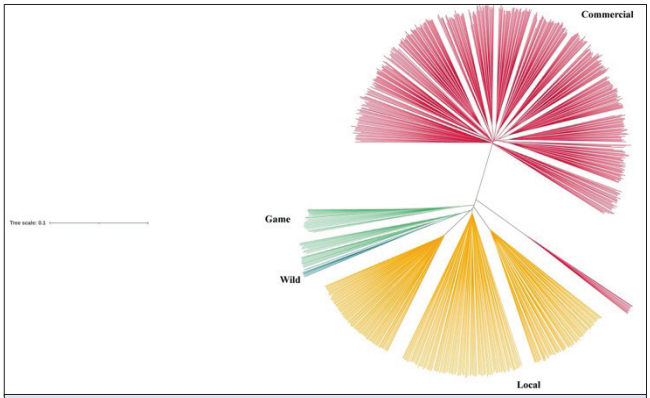

Fig 1. Neighbor-joining tree of G, W, L, and C chicken

for 10 min every day, and the eggs were turned 15 times every day during egg incubation [17].

Sample Collected of Chicken Embryos

We carefully removed the egg from the incubator, rounded end up, tip down, and placed it on an egg seat. Use tweezers to poke a small hole in the eggshell and remove the eggshell and chorionic membrane. When eggs are 1-3 d, they cannot be sampled because embryos have not been formed so samples are taken from 4 d. The embryo is bled to death by carefully cutting the blood vessels around the embryo (pre-embryo) or umbilical cord using small scissors. The embryos are removed with a small spoon and placed in a petri dish. The egg yolk is rinsed off with saline and the amniotic membrane is removed to reveal the embryo. Embryos are small before 7 d and can be directly put into formalin fixative. After 7 d, chicken embryos were and internal organs were removed and rinsed with distilled water to make soft tissue and vertebrae of chicken embryos more transparent. After 10 d, better penetrate the liquid and observe the shape of the coccyx, we removed the internal organs of the chicken embryos after death, removed the skin, and gently cut or pricked the muscles around the coccyx with a knife.

The chicken embryos were clamped on both sides with wire mesh, fixed with formalin (5-6 d, 4% formalin/12 h fixed; 7-8 d,10% formalin/12 h fixed; 9-21 d, 10% formalin/24 h fixed), washed with running water, dehydrated by ethanol solution with concentration ≥70%.

Pre-transparent Treatment

The completely dehydrated chicken embryos were put into 1-10% KOH transparent until the chicken embryo muscle tissue transparent. The transparent time was determined by the size of the chicken embryo (4 d, 1% KOH, 8 min 10 s; 5 d, 1% KOH, 22 min 36 s; 6 d, 1% KOH, 51 min 36 s; 7 d, 2% KOH, 2 h; 8 d, 3% KOH, 24 h; 9 d, 4% KOH, 24 h; 10 d, 5% KOH, 12 h).

Bone Staining Method

Cartilage staining solution (Alcian blue, 0.15% alcian blue ethanol (70%) solution: glacial acetic 70% ethanol =1 : 1 : 18, mixed evenly); Toluidine blue configuration (distilled water: toluidine blue staining solution =100 : 0.05, mixed evenly). Hard bone staining solution: Preparation of cyruxin teleost staining solution: 200 mL solution of 0.01 g alizarin red mixed with of 1% potassium hydroxide to make a dark purple stain.

Methods for cartilage staining [18]: embryos of chicken were immersed in ali xin blue cartilage solution for two days or immersed in toluidine blue cartilage solution for two days. Methods for decolorizing: remove excess color from the soft tissues other than the bones, the embryos of chicken were placed in absolute ethanol, 50% ethanol, and water for one day each. Hard bone staining: embryos of chicken completed the above procedure were immersed in the configured alizarin red bony staining solution for one day.

Decoloration and Transparency Steps

In this study, 25%, 50%, 75%, 100% gradient glycerol and 1% potassium hydroxide mixtures were used to remove excess staining solution (25%, 50%, 75%, 100% gradient glycerol refers to glycerol and potassium hydroxide volume ratio of glycerol: potassium hydroxide 1:3; Glycerol: potassium hydroxide is 1:1; Glycerol: potassium hydroxide 3:1; Glycerol: potassium hydroxide is 1:0). Next, 25%, 50%, 75% and 100% gradient glycerol were successively soaked in each gradient for 1 d, during which the specimen was constantly shaken to make the liquid exchange more thorough and achieve the desired transparent effect [18].

RNA-seq and Alternative Splice Processing

Please refer to Hu's paper for RNA-seq of gene expression in different embryonic stage of PB and RB [13]. In short, caudal bone samples from days 8, 11, 14, 16, and 21 of the PB and RB embryo were sequenced. The differentially expressed genes were screened by RNA-SEQ analysis (|Log2FoldChange| ≥1, P value <0.05, Q value <0.05).

In this study, rMATS 4.1.2 software [19] was used to identify five types of alternative splicing, namely skipped exon (SE), alternative 5'splice site (A5SS), alternative 3'splice site (A3SS), mutually exclusive exons (MXE) and retained intron (RI). PSI values (lncLevels) are used to filter differential alternative splicing (P value <0.05, Q value <0.05). When a gene has multiple splices, select the one with the most significant difference. The closer PSI value is to 1, the less probability of alternative splicing occurs. The closer PSI value is to 0 conversely, the more likely alternative splicing occurs [20].

Weighted Gene Co-expression Network Analysis and Network Visualization

To reveal the developmental transcriptional expression network of differentiated gene sets in tailed and rumpless chicken, weighted gene co-expression network analysis (WGCNA) [21] was used for network analysis in this study. The soft thresholding power of PB group was selected 18, R^2 was first greater than 0.64 of topology. RB group was selected 30, R^2 was first greater than 0.45 of topology. The minimum module size was set to 20. Cytoscape3.6.1 software [22] for analyzing network visualization with unsigned network. Finally, hub genes in modules were screened through the degree of gene connectivity.

Analysis of KEGG Pathway

KEGG pathway was analyzed by KOBAS online tool *(http://kobas.cbi.pku.edu.cn/genelist/). Gallus gallus* species, gene symbol, and KEGG pathway were selected. According to

the P value, the top 20 were screened for the display of the bar chart.

Results

Comparative Genomic Analysis of Rumpless Chicken and Multiple Chicken Breeds

Phylogenetic tree analysis was performed on chip data from 988 chickens. According to *Fig. 1,* the tree scale is 0.1. The phylogenetic tree showed that the four types of chicken were divided into four branches, and the commercial breed showed a long distance from the game variety, the wild type, and the local variety, with obvious population differences.

The first 5% of fixation index (F_{ST}) sites were screened for genomic regions that had high population differences between them, as shown in the *Fig. 2,* the degree of differentiation among breeds were C *vs.* G, C *vs.* W, L *vs.* C, L *vs.* W, G *vs.* W, G *vs.* L in sequence, which hit 783, 114, 42, 792, 866 and 744 genes too. The number of highly differentiated loci overlapped genes in CG, CW, LC, LW, GW, LG, and 258 rumpless signal genes was 50, 46, 23, 17, 5 and 4 respectively, which were stripped of duplicates, 76 genes *(Fig. 3)* (Among them, the 258 rumpless signal genes of tailed chicken and rumpless chicken came from our team previous research [13]).

Dynamic Histomorphology and Transparent Staining of Bone in Tailed and Rumpless Chicken

In terms of appearance and histomorphology, there was no significant difference in tail development between tailed chicken and rumpless chicken from 1 d to 4 d, and the tail buds became more prominent from 5 d. The tail buds of tailed chicken were sharp, but those of rumpless chicken were round and blunt. With the increase of age, the tail bud of tailed chicken continued to grow and became, but the tail of embryo of rumpless chicken did not grow significantly on 9 d, and after 10 d, the tail almost did not grow and only had a small bulge *(Fig. 4)*.

The transparent staining method can observe the natural distribution and position of cartilage and hard bone in the body as a stereoscopic visual effect of bone development. Both soft tissue and cartilage were blue after staining the cartilage. Soft tissue decolorized by gradient ethanol is

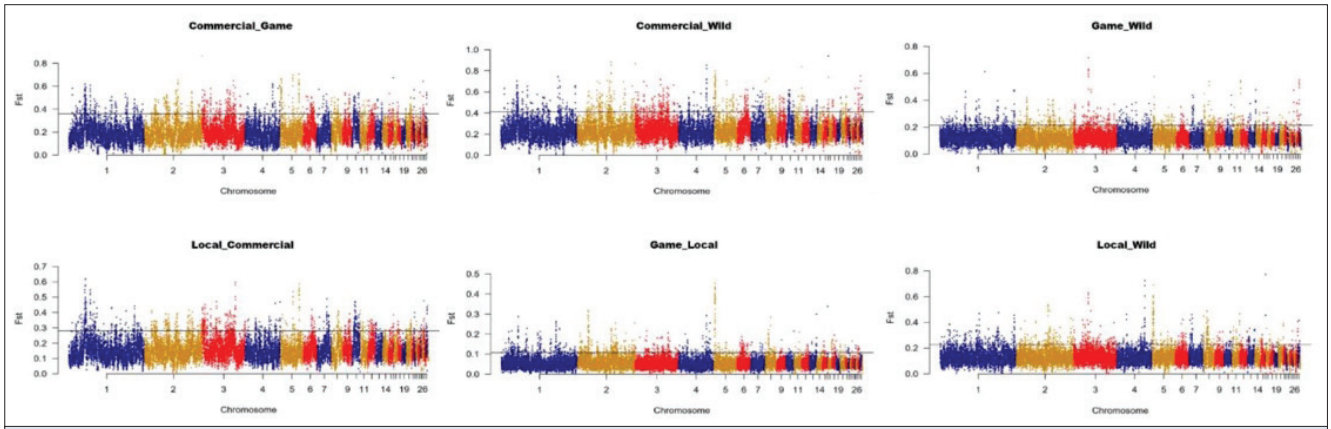

Fig 2. Distribution of F_{ST} among different C, G, W and L breeds. The dividing line represents the first 5% of differentiation sites

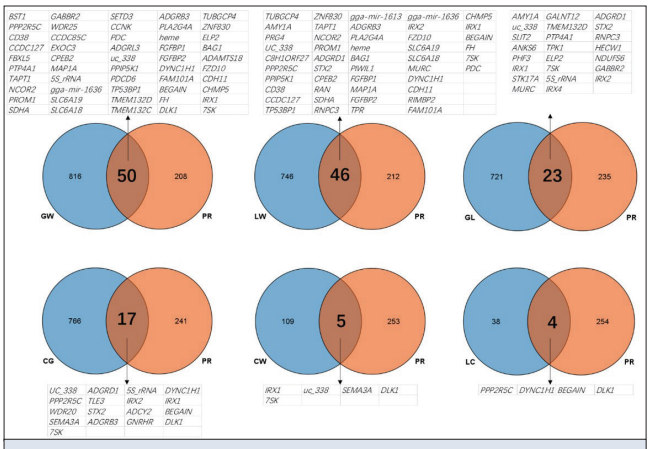

Fig 3. The overlapping genes between rempless chicken differentiation genes and CG, CW, LC, LW, GW, LG breeds. PR represents the gene of significant differentiation of rumpless chicken

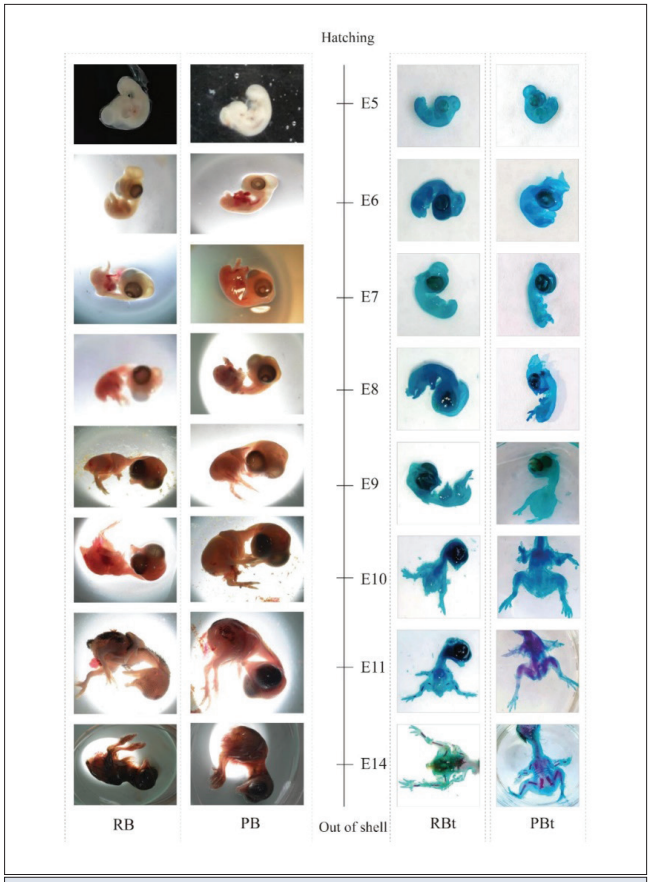

Fig 4. Histomorphology and bone hyalinization of embryo samples from rumpless Piao chicken (PB) and tailed chicken (RB). PB: rumpless Piao chicken, RB: tailed chicken. PBt: transparence dyeing results of rumpless Piao chicken; RBt: Transparence dyeing results of tailed chicken. E: Embryonic day age

transparent and light blue. Hyalinization results in blue cartilage, deep red hard bone, and light blue jelly outside the bone. The results of cartilage staining showed that the bone development of tailed chicken and rumpless chicken embryos showed blue staining on the spindle bone from incubation 6 d, and then blue staining on the long bones of limbs, ribs, phalanges, wing roots, and tailbone. The caudal bone of rumpless chick embryo was very short and did not develop in late incubation. Bone staining showed that spotty-like red staining appeared in the main vertebra and long bones of limbs from the 7 d of incubation, and then the red staining gradually increased. Red staining appeared in the bones of phalanx, ischium, ribs and wing root, but no red staining appeared in the coccyx, indicating that the coccyx was not ossification. These results indicated that during embryo development of rumpless chicken, the development of coccyx was normal in the early stage of embryo (before the 4 d to 6 d stage of incubation), and degenerated or stopped in the late stage. This rumplessness may be related to the inhibition of genes related to bone development. The following analysis will focus on genome and transcriptome data after 8 d (Scoop sample on 6 d could not extract complete RNA, so it was abandoned).

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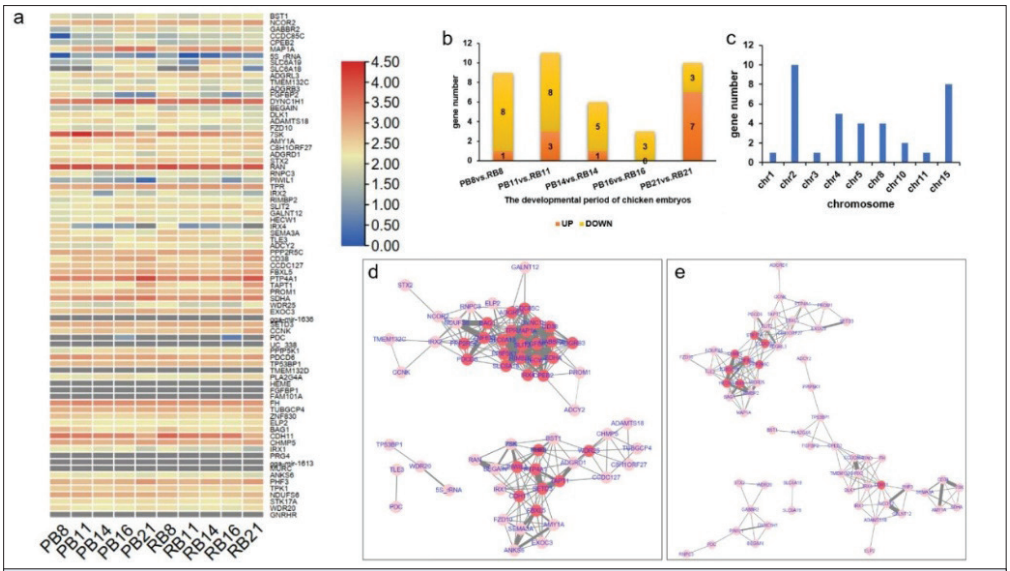

Fig 5. a) Expression distribution of differentiation signal gene sets (76 genes) of rumplessnesss in different breeds at different developmental stages of tail chicken and rumpless chicken (P<0.05, adjP<0.05, |logFC|>1. All genes from *BST1* to *ADCY2* were differentially expressed); **b)** Differential expression and distribution of differentiation signal gene sets (76 genes) of rumplessnesss in different developmental stages of tailed and rumpless chicken (P<0.05, adjP<0.05, |logFC|>1. Up represents up-regulated genes and down represents down-regulated genes); **c)** Distribution of 36 DEGs in chromosomes; **d)** Differentiation signal gene sets (76 genes) in the co-expression network of rumpless Piao chicken; **e)** Differentiation signal gene sets (76 genes) in the co-expression network of tailed chicken

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Developmental Transcriptome Analysis Reveals Rumpless Dynamic Expression of Rumpless Signaling Gene Set

According to bone transparent staining, blue staining was found from the 6 d, but the RNA acquisition of the 6 d of the failed, which may be due to sample degradation and other reasons, so the data of the 8 d was obtained. The differential expression of 76 strongly differentiated genes between tailed chicken and rumpless chicken was analyzed by transcriptome sequencing at embryonic stage 8, 11, 14, 16, 21 d respectively. These 76 genes indicated the characteristics of tail in the selection process of different chicken breeds.

As shown in *Fig. 5-a*, log10 (FPKM+1) transformation of differentially expressed genes (DEGs) was carried out and heat map was drawn. Among them, 36 genes located between heat map *BST1* and *ADCY2* were all DEGs, and 2, 4, and 15 were abundant. The up-regulation distribution of these DEGs at different developmental stages showed that 12 of them were higher and 1 of them were lower in Piao chicken than in tailed chicken at 8 d of embryo. At 11 d of embryo, there were 13 higher and 3 lower in Piao chicken than in tailed chicken. At 14 d of embryo, there were 14 higher and 1 lower expression in Piao chicken than in tailed chicken. At 16 d of embryo, there were 6 higher and 0 lower expression in Piao chicken than in tailed chicken. At 21 d of embryo, there were 6 higher and 10 lower expression in Piao chicken than in tailed chicken *(Fig. 5-b)*.

Table 1. *Differentially expressed genes with high network connectivity in embryos of tailed chicken and rumpless chicken*

Genes	Degree	Modules	Average Shortest PathLength	Betweenness Centrality	Neighborhood Connectivity
SLC6A19	22	PB-turquoise	1.406	0.093	15.591
FGFBP2	20	PB-turquoise	1.469	0.050	16.500
TPR	20	PB-turquoise	1.469	0.046	16.400
MAP1A	19	PB-turquoise	1.500	0.017	17.211
DYNC1H1	19	PB-turquoise	1.500	0.017	17.211
RIMBP2	19	PB-turquoise	1.500	0.017	17.211
SLIT2	19	PB-turquoise	1.500	0.017	17.211
GABBR2	18	PB-turquoise	1.688	0.022	16.444
SLC6A18	18	PB-turquoise	1.563	0.037	16.778
CCDC85C	17	PB-turquoise	1.594	0.053	16.765
HECW1	11	RB-turquoise	2.192	0.028	9.182
RAN	10	RB-turquoise	2.231	0.019	9.600
IRX2	9	RB-grey	2.040	0.139	7.000
IRX4	9	RB-grey	2.240	0.064	6.889
RIMBP2	9	RB-turquoise	2.308	0.012	10.000
ADGRL3	9	RB-turquoise	1.808	0.080	8.667
C8H1ORF27	9	RB-turquoise	2.115	0.104	7.222
CCDC85C	8	RB-grey	2.080	0.096	7.375
CPEB2	7	RB-grey	2.400	0.383	5.714
TMEM132C	7	RB-grey	2.120	0.073	8.143

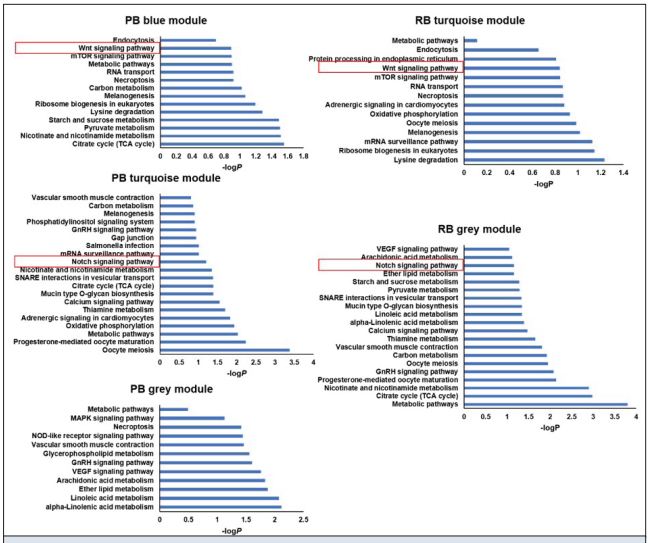

Fig 6. Enrichment of signal pathways of different gene co-expression modules in tailed and rumpless chicken

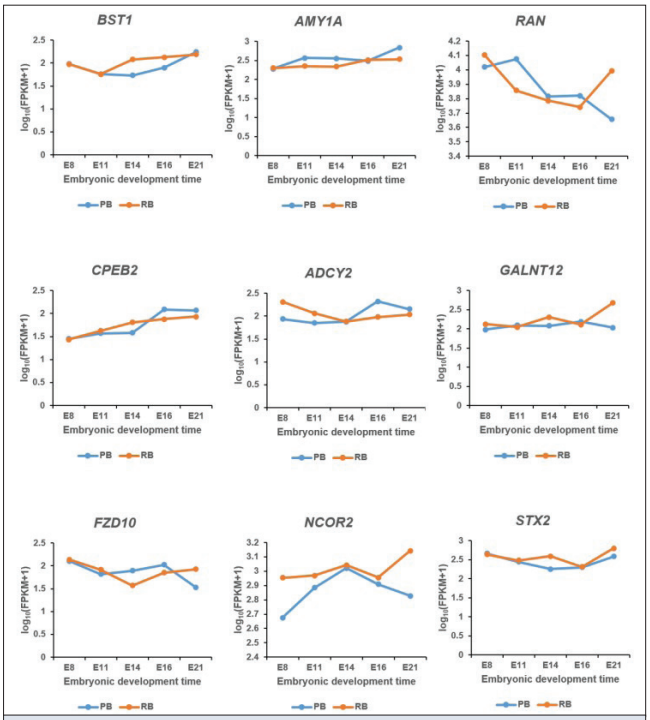

Fig 7. Dynamic FPKM temporal expression of differentially expressed genes in common signaling pathways in tailed and rumpless chicken

WGCNA Reveals Co-expression Network of Rumpless Signal Gene

The global network construction results of WGCNA show that the Piao chicken β value is 18 and the network topology fit index is 0.64. It is divided into three co-expression modules, namely turquoise, blue and grey. There are 12 and 8 hub genes with degree greater than 10 in turquoise and blue modules respectively *(Fig. 5-c)*. The β value of tailed chicken was 30, and the network topology fit index is 0.45, which was divided into two modules turquoise and grey. Genes with degree greater than 10 were mainly distributed in turquoise (8 genes) *(Fig. 5-d)*. The expression of these differentiation signals of rumplessness was more closely interacted with each other and the weight between genes was greater than that in tailed chicken.

As shown in *Table 1*, we sorted out the network attribute parameters of the top 10 genes with differentially expressed genes and large degree among the network genes of tailed chicken and rumpless chicken. The network connectivity of the rumpless chicken is larger than that of the tailed chicken. The first three hub genes were *SLC6A19*, *FGFBP2* and *TPR* of rumpless chicken network, which were 22, 20 and 20 genes that had very similar expression patterns. The first three hub genes were *IRX2*, *IRX4* and *RIMBP2* of tailed chicken network, which were 9, 9 and 9 genes that had very similar expression patterns. The *IRX2* and *IRX4* genes related to rumplessnesss reported in previous literatures were included *(Table 1)*.

KEGG (Kyoto Encyclopedia of Genes and Genomes) Analysis Delineate the Signaling Pathway Relevant to Posterior Patterning

KOBAS (KEGG Orthology Based Annotation System) signal pathway enrichment analysis was performed for different modules of genes in tailed chicken and rumpless chicken respectively *(http://kobas.cbi.pku.edu.cn/)*. The result is shown in *Fig. 6*, except for PB enrichment in 6 pathways (glycerophospholipid metabolism, NOD-like receptor signaling pathway, MAPK signaling pathway, salmonella infection, gap junction, phosphatidylinositol signaling system). RB is specifically enriched in protein processing in endoplasmic reticulum. The other 31 pathways were common enrichment pathways, all of these include: nicotinate and nicotinamide metabolism, metabolic pathways *(BST1)*; starch and sucrose metabolism, metabolic pathways *(AMY1A)*; ribosome biogenesis in eukaryotes, RNA transport *(RAN)*; melanogenesis, mTOR signaling pathway, wnt signaling pathway *(FZD10)*; notch signaling pathway *(NCOR2)*; oocyte meiosis, progesterone-mediated oocyte maturation *(CPEB2)*; progesterone-mediated oocyte maturation, adrenergic signaling in cardiomyocytes, calcium signaling pathway *(ADCY2)*; mucin type O-glycan biosynthesis *(GALNT12)*; SNARE interactions in vesicular transport *(STX2)*. Red boxes indicate some typical signaling pathways related to traits reported by previous studies.

The genes that were not differentially expressed at 8 to 11 d and were present after 14 d included the *BST1*, *CPEB2*, *FZD10*, *STX2*, *AMY1A*, *GALNT12*, *RAN*. *FZD10* and *RAN* were differentially expressed only on 21 d. The difference between 8 d and 11 d was *ADCY2*, and only the difference between 8 d was *NCOR2* *(Fig. 7)*.

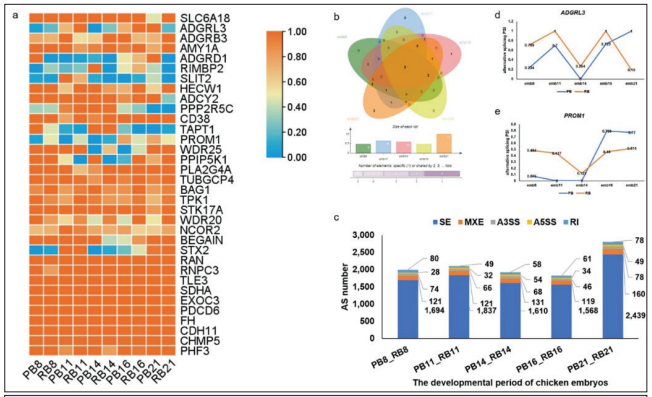

Fig 8. a) Differential variable splice number expression heat map (PSI) of transcriptome at different developmental stages; **b)** Venn analysis of SE at different developmental stages; **c)** Differentially expressed alternative splicing and differentiation factor genes at each stage; **d)** The dynamic distribution of PSI of *ADGRL3* at each embryonic development stage; **e)** The dynamic distribution of PSI of *PROM1* at each embryonic development stage

Alternative Splicing of Differentiation Signal Gene Sets for Rumplessnesss in Embryo Development Differences Between Tailed Chicken and Rumpless Chicken

Transcriptome sequencing was used to analyze the global distribution of differential alternative splicing in tailed chicken and rumpless chicken, and it was found that there more alternative splicing in late embryo, especially on 21 d. The exon skip (SE) splicing form was the most prominent among all the splicing forms. With the development process, 1 694, 1 837, 1 610, 1 568 and 2 439 SE were identified respectively *(Fig. 8-c)*. Next, the expression of splicing PSI on SE of 34 genes *(Fig. 8-a)* among 76 differentiated genes that had differential alternative splicing between tailed chicken and rumpless chicken breeds was compared, which can reveal the pre-transcriptional splicing characteristics of differentiation signal gene sets during embryonic development (due to multiple splicing in a gene, only the most significant PSI was screened in this study). We obtained 9 genes that were differentially expressed at a certain stage of embryo and underwent differential alternative splicing: *SLC6A18*, *ADGRL3*, *ADGRB3*, *AMY1A*, *ADGRD1*, *RIMBP2*, *SLIT2*, *HECW1* and *ADCY2 (Fig. 8-a)*. In addition, *ADGRL3* and *PROM1* were differentially expressed in all 5 periods *(Fig. 8-b,d,e)*.

Discussion

In this study, we investigated the differences of the whole genome among different breeds, especially the overlapping genes with rumplessness candidate genes involved in selection reported by focused on the dynamic transcriptome expression of these overlapping genes during embryonic development of tailed chicken and rumpless chickens. As reported that caudal vertebral bodies are formed from different forms of cartilage that remodel to bone at distinct stages [24]. First, the eggs of tailed chicken (Camellia chicken) and rumpless chicken (Piao chicken) were incubated in this study. After death, the chicken embryos were stained with alcian blue and alizarin red [23,24] for cartilage and hard bone, and transparent specimens were made. This study observed the changes of caudal embryo development, it was found that the caudal bud appeared sharp protrusion from the 4 d of tailed chicken hatching. After that, the tail buds grew relatively with the increase of body size. However, round and blunt tail buds appeared from the 4 d of rumpless chicken, and after the 9 d, tail buds stopped growing and became round and blunt more obvious. Zwilling as early as 1942 proposed that dominant rumpless embryos have a reduced tail at the end of the 4 day [25]. Some studies suggest that the tail bud of chicks begins to produce mitotic inhibitors on the 3 d of hatching [26], and lasts until the 4 d to 5 d of hatching, when the tail bud reaches the maximum length, and then a 3-step remodeling process is carried out: 1.Differential growth between the tip of the tail and the more anterior regions; 2. The anterior regions become incorporated into the caudal portions of the trunk. 3. Cell at the tip of the tail die, And then you end up retaining the proximal part to form the final tail [27,28].The results of this study showed that during embryo development, tailbone development was no significantly difference in the early stage of embryo between tailed chicken and Piao chicken (hatching 4-6 d), and degenerated or stopped in the late stage of Piao chicken, which is a visualization of the developmental characteristics of the tail bone of a rumpless chicken.

Next, we compared the genomes of large chicken species with different uses to try to find out the differences and relationships between them and the tailless chicken. While the results of this study do not prove mutated genes in each genome versus the tailless genome, this interesting comparison of overlapping genes could reflect genome-level differences between results showed that the differentiation degree of several chicken breeds was obvious, and there were some overlapping genes with these cultivars [29], especially commercial *vs.* game chicken and commercial *vs.* wild, which had more overlapping genes with tailless selection signal, with 50 and 46 genes, respectively. This suggests that some of the rumplessness signal genes reported by Hu [13] are involved in the difference between other varieties. These results demonstrated there is rich genetic diversity and extensive genetic basis of the provided excellent molecular materials for further heterosis and high-yield breeding. This helps to explain the history and divergence of these breeds and to facilitate genetic breeding.

Gene expression variation is a key underlying factor influencing phenotypic can occur via pre- transcriptional

and transcriptional expression [30]. In this study, we also focused on the dynamic transcriptional patterns of these genes during embryonic development of tailed chicken and Piao chicken. Among the 76 rumpless signaling gene set, 36 genes were differentially expressed, and most of them were concentrated on chromosome 2, 4 and 15. They were distributed at different embryonic stages, and most of them were differentially expressed at 11 and 21 days. There are research reports that through genome-wide association and linkage analyses, the candidate region was fine-mapped to 798.5 kb (chromosome 2: 86.9 to 87.7 Mb). Whole-genome sequencing analyses identified a single variant, a 4.2 kb deletion, which was completely associated with the rumpless phenotype [31].

Co-expression gene clusters of development are paradigms for the study of gene regulation [32]. We used WGCNA to identify regulatory networks of these signaling genes during embryonic development in tailed and rumpless chicken. A key characteristic of scale-free networks is a small number of highly interconnected hub genes (hubs). Because hubs are more likely than nohubs to be necessary for the integrity of the network and the survival of an organism, the identification of so-called "hubs genes" is of great significance. Therefore, we identified highly co-expression hub genes in each co-expression network. The centrality of nodes is measured by three parameters: shortest path length, betweenness centrality and degree. The shortest path length is the probability of functional correlation between gene/protein and gene/protein, the larger it is, the stronger the gene/protein regulation function to other genes/proteins [33]. Degree is used to measure the centrality of a node. A node with a higher degree reflects the higher connectivity between two sub-networks, but the connectivity within the sub-network is not high. Often acts as a communication gene/protein between two modules. Degree attribute indicates the degree of connection between a gene/protein and other genes/proteins. The higher the connection degree, the more critical the hub gene/protein is in the regulation of biological organism. The major evolutionary maintains the association of the hub and expression genes in clusters. These genes were differentially expressed at different stages of embryo development between tailed and rumpless chicken, and their distribution was not balanced. These genes were co-expressed and interacted more strongly in Piao chicken than in tailed chicken. The first three hub genes were *SLC6A19*, *FGFBP2* and *TPR*. The first three hub genes were *IRX*2, *IRX*4 and *RIMBP2*. The Iroquois gene is expressed in clusters in most vertebrates and misexpression of *IRX1* and *IRX2* within the tailbud precedes all observed genetic and morphological changes. The *IRXA* cluster contains *IRX1*, *IRX2* and *IRX4,* which have similar expression patterns [32-34]. In this study, *IRX2* appeared in the comparison results of genome differences of multiple breeds, and *IRX2* appeared in the core of gene co-expression network of tailed chicken, and the connectivity of gene co-expression network of tailless chicken was results indicate one of the characteristics of *IRX2* gene expression during embryonic development between tailed chicken and Piao chicken.

The results of functional enrichment of these differentially expressed genes showed that *ADCY2* and *NCOR2* genes were inhibited on the 8 d of embryo. On 11 d, the repressed gene was *ADCY2*. Notch signaling pathway, oocyte meiosis, progesterone-mediated oocyte maturation, adrenergic signaling in cardiomyocytes, calcium signaling pathway was affected. Most genes began to be differentially expressed after 14 d, which significantly affected the development of coccyx. The genes that were inhibited on 14 d were *BST1*, *CPEB2*, *STX2*, and *GALNT12*, which affected signal pathway include nicotinate and nicotinamide metabolism, metabolic pathways, oocyte meiosis, progesterone-mediated oocyte maturation, SNARE interactions in vesicular transport, mucin type O-glycan biosynthesis. The genes that were inhibited on 21 d were *FZD10* and *RAN*, affecting melanogenesis, mTOR signaling pathway and wnt signaling pathway. It has been reported that notch and wnt signaling pathways play an important role in regulating segmental formation and tail bone termination and extension [35]. According to Rashid's review, a prevalent pleiotropic effect of mutations that cause fused caudal vertebral bodies is tail truncation, and at least half of the mutated genes are located in the notch/wnt pathway, leading to changes in somite number or size of short-tailed birds [36]. Notch signaling pathway can mediate embryonic development and tissue renewal and is highly conserved during evolution. Studies have shown that it regulates chondrocyte, osteoblast and osteoclast differentiation [37]. It is expressed in the early stage of osteoblast differentiation, leading to bone formation inhibition and bone loss [37]. When the wnt signaling pathway extends in the tail, it can coordinate with the concentration of RA and Fgf signaling pathways to form a gradient balance and regulate the formation of body segments [38]. Notch signaling pathway is activated when wnt3a/Fgf8 reaches the gradient equilibrium point. However, when RA concentration is too high, wnt3a/Fgf8 is inhibited, which in turn inhibits notch [39]. In conclusion, notch is expressed in coccyx of both tailed and rumpless chicken in preembryonic stage, and the expression of notch is higher in tailed chicken, and the wnt signaling pathway was inhibited by the low expression of *FZD10*. Since no RNA was detected from 4 to 7 days of embryo, notch expression was inhibited earlier in rumpless chicken, which was also the main reason why PCR verification was not carried out in this study.

In addition, the mechanisms that mediate phenomena such as alternative splicing elusive. Alternative splicing is the core mode of gene regulation in higher eukaryotes, which can affect plant and animal growth and development [40], signal transduction [41] and regulatory responses under biological/abiotic stress [42,43], play a key role in regulating osteoblast function and bone formation [44]. In this study, it was found that the SE splicing was the most prominent alternative splicing occurred more at embryo 21 d than at the early stage. Among the 76 rumpless signaling gene set, only 34 genes underwent differential alternative splicing, among which the PSI of *ADGRL3* and *PROM1* were significantly different at each embryo time, again indicating the existence of transcriptional differential characteristics of some differentiation factors. Therefore, different exon splicing forms represented by *ADGRL3* and *PROM1* may be one of the factors leading to the differences of these genes. In conclusion, this study highlight genetic charateristics of transcriptome and genome in tailed chicken and Piao chicken.

In conclusion, genomic differences in different breeds and genetic characteristics of bone in embryo indicate the molecular mechanism of tailbone development in a Piao chicken by comprehensive analysis of the histomorphology, genome and transcriptome of embryo skeleton, which may enhance useful resources for rumplessness breeding, also in-fluence the selection process of other breeds.

Declarations

Availability of Data and Materials: The datasets used and/or analyzed during the current study are available from the corresponding authors (YYS & YC) on reasonable request.

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Author Contributions: WMQ performed the experiments, analysed the results, and drafted the manuscript. YC and ZXL assisted in the experimental design and summarized the experimental results. YYS and WMQ conceived and designed the study, revised the manuscript and funded the study. All authors have read and agreed to the published version of the manuscript.

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