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RESEARCH ARTICLE

Inverted duplication including *Endothelin 3* closely related to dermal hyperpigmentation in Silkie chickens

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Abstract The dermal hyperpigmentation phenotype in chickens is controlled by the dominant fibromelanosis allele. One of the ten unique characteristics of Silkie chickens is the fibromelanosis phenotype, which is pigmentation in the dermal layer of the skin and connective tissue. In this study, we found a mutation of fibromelanosis, a genomic rearrangement that included an inverted duplication of *endothelin3* (*EDN3*), is responsible. We show that, as a stimulator of melanoblast proliferation, *EDN3* expression was increased in silkie embryos and in both skin and muscle throughout adulthood. *EDN3* expression led to an increase in expression of the downstream genes *EDNRB2* and *TYRP2,* and was closely relate with the hyperpigmentation phenotype. We examined eight different Chinese chicken breeds showing hyperpigmentation and conclude that this structural genetic variant exists in all fibromelanosis chicken breeds.

Keywords dermal hyperpigmentation, duplication, *endothelin 3*, Silkie chicken

1 Introduction

The Silkie chicken (*Gallus gallus*) is one of the most well-known chicken breeds in China and is a unique breed with many distinctive characteristics, including its crest, walnut comb, blue earlobes, beard, silkie feathering trait, feathered legs and polydactyly [1,2]. In particular, the pigmentation in the dermal layer of the skin, muscles, nerves, tendons, blood vessels, bone and connective tissue [3,4] is one of the most notable traits, and this breed has been used by scientists to identify genes that regulate the mechanisms of melanocyte migration.

The melanocyte is the main dermal cell type that contains melanin, a pigment in birds and other animals. Overproduction of melanin causes hyperpigmentation. Melanoblasts are the precursor cells of melanocytes, and they originate from the neural crest during early embryogenesis [5]. In avian embryos, the neural crest cells at the trunk level migrate in two waves [6]. The first neural crest cells migrate through the ventral pathway in the trunk and become the glia and neurons. The later neural crest cells (melanoblasts) move in the dorsolateral pathway and differentiate into melanocytes 24 h after completion of the ventral migration [7]. One aspect that differs from other vertebrates is that the melanoblasts of Silkie chickens can also reach the ventral regions of the embryo [8] owing to the absence of environmental barrier molecules in tissues that bind peanut agglutinin [9]. In addition, Silkie embryo cultures have been shown to have increased crest cell proliferation *in vitro* [10]. This generalized mesodermal pigmentation is due to environmental factors that promote both the abnormal migration of melanoblasts and their proliferation and differentiation [11], which may result from the classically described inhibitor of dermal melanin (*ID*) and fibromelanosis (*FM*) loci, respectively.

About a century ago, the hyperpigmentation phenotype of Silkie chickens was shown to be closely related to the sex-linked incompletely dominant inhibitor of dermal melanin (Id/id^+) and the autosomal dominant fibromelanosis (Fm/fm^+) loci [12,13]. *Id* has an epistatic effect on *Fm*; pigmentation of the shank in the dermis occurs with id^+; *Fm* with id^+ causes hyperpigmentation in Silkie chickens [2,3]. Linkage analyses showed that *Id* is located near the end of the long arm of chromosome Z [14–18]. Dorshorst et al. [1] performed genome-wide single nucleotide polymorphism (SNP)-trait association analysis to identify the genomic regions in which *ID* and *FM* are located. They identified SNPs that are associated with *ID* at 72.3 Mb on chromosome Z and *FM* at 10.3–13.1 Mb on chromosome 20. However, these genomic regions contain many other genes.

Since 2010, we have worked to establish crossed breeds of chickens for identifying genes that are genetically

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related to *FM*. Here we report that *FM* is associated with an inverted duplication of two genomic regions on chromosome 20, together with increased expression of *endothelin 3* (*EDN3*).

2 Materials and methods

2.1 Ethics statement

All animal work was conducted according to the guidelines for the care and use of experimental animals established by the Ministry of Science and Technology of the People's Republic of China (Approval number: 2006-398). The blood samples were collected from the brachial vein of chickens using the standard venipuncture procedure approved by the Animal Welfare Committee of China Agricultural University (Permit Number: XK622).

2.2 Animal materials

A mapping population of chickens was developed to allow individual segregation of *FM* and clear classification of the dermal hyperpigmentation phenotypes, because of the epistatic interaction between *ID* and *FM*. The cross was generated by mating a male Silkie chicken (*Fm*/*Fm*, id^+/id^+) with a female Gushi chicken (fm^+/fm^+, id^+/*w*). The resulting three males (*Fm*/fm^+, id^+/id^+) were chosen for their conspicuous pigmentation. Each was mated with nine female Youxima chickens (fm^+/fm^+, id^+/*w*). Then, the segregation of *Fm* on the id^+ background was present in 236 individuals. The trait phenotypes of comb color and skin color under the wings were recorded at 4 and 12 weeks. The founding breeds and representative photos of 4-week-old chicks segregating for *FM* are shown in Fig. 1.

DNA samples from adults of other breeds were obtained from the Poultry Institute of Jiangsu Province, Chinese Academy of Agricultural Sciences and Yunnan University and included other *FM* chicken breeds, namely Chuxiong Silkie, Jinhu Silkie, Kuaida Silkie, Tengchong snow, Wuding Silkie and Yanjin Silkie as well as some normally pigmented chicken breeds, namely Anak, Cobb, Gushi, Qingyuan ma, Wahui and White Recessive. DNA samples of Silkie and White Leghorn chicken embryos were obtained from the chicken farm at China Agricultural University.

2.3 Genotyping

Forty-three SNPs (Appendix A) (WUGSC2.1/galGAL3) between 6.2 and 13.7 Mb on chr20 [1] were used for genotyping of the F_2 generation of the crossed population. Each genomic DNA sample was diluted to 30 $ng \cdot \mu L^{-1}$ in double-distilled water and detected with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The haplotype inference software program PHASE (2.1) was used to analyze the haplotype of the crossed population and the haplotype of each individual (http://www.stat.washington.edu/stephens).

2.4 Genomic copy number analysis

High-density array comparative genomic hybridization (aCGH) was designed and produced by Agilent Technologies for comparative genome analysis (Agilent Technologies, Santa Clara, CA, USA). We used an Agilent 2–400K custom-designed high-density microarray and 420288 probes. Twenty-four chickens from 12 breeds (one male and one female of each breed) were used for the assay and included hyperpigmented chicken breeds such as the Silkie and Jinhu. Copy number variants (CNVs) in the genomes of locally raised Chinese chickens were determined using aCGH.

Primers for real-time quantitative PCR (qPCR), which were designed using Primer Express 2.0 software (Applied Biosystems, Carlsbad, CA, USA), were used to confirm the existence of CNVs (Appendix B, Table S1). The primers for the control gene, *PCCA* (encoding mitochondrial propionyl-CoA carboxylase), were as described [19]. The BLAT web tool, accessed at the University of California, Santa Cruz, website (http://genome.ucsc.edu/cgi-bin/hgBlat?command=start) showed that the sequences were specific for each region of interest. Melting curve and amplification analyses were used to validate the primers. qPCR was carried out as follows: 95°C for 5 min, 40 cycles of amplification (95°C for 10 s, 60°C for 10 s and 72°C for 10 s) and a final dissociation step (95°C for 5 s, 60°C for 1 min and 97°C for 5 s). Each genomic DNA sample was diluted to 10 $ng \cdot \mu L^{-1}$ in double-distilled water, and the concentrations verified using a NanoDrop instrument. A standard curve was prepared by taking the average of triplicate measurements for the reference genomic DNA of a Youxima chicken at five concentrations (2.5, 5, 10, 20 and 40 $ng \cdot \mu L^{-1}$) in the same plate as the test samples. SYBR Green-based real-time qPCR assays were performed using a LightCycler480 instrument with a 96-well block (Roche Applied Science, Indianapolis, IN, USA). All qPCR samples were assayed in quadruplicate. Each reaction contained 10 ng template and all results were analyzed using LightCycler480 software 1.5 with a *C*t threshold of 0.2. Relative copy numbers were assigned by comparing the *C*t values with the standard curve and the number of copies in 1 ng reference DNA (arbitrarily defined as one unit).

Pyrosequencing was performed to test the existence of the uniqueSNPs Gga_rs16172722 and Gga_rs16172768 in Silkie and other hyperpigmented breeds using the PyroMark™ ID system (Biotage, Sweden). Assay Design Software was used to design the primers (Appendix B, Table S2). The pyrosequencing PCR system contained 10 pmol upstream primer, 10 pmol downstream primer, 2.5 μL 10 × buffer II, 2 μL $MgCl_2$ (25 $mmol \cdot L^{-1}$), 0.5 μL

Fig. 1 Founding breeds of the mapping populations and chick pigmentation phenotypes. Individuals of the chicken breeds used to develop the population for pigmentation: (a) Silkie, (b) Gushi and (c) Youxima; after hatching, four-week-old chickens from the mapping populations displaying different pigmentation phenotypes in the comb, which represents the color of the skin: (d) a putative Fm/fm^+ (black comb) individual and (e) a putative fm^+/fm^+ (white comb) individual.

dNTP (10 mmol·L^{-1}) and 0.2 μL TaqGold polymerase (Applied Biosystems, Foster City, CA, USA). The thermal cycling protocol was 95°C for 5 min, followed by 45 cycles of 95°C for 15 s, 57°C for 30 s and 72°C for 30 s. Then the PCR product was detected with the sequencing primer on the pyrosequencing platform according to the manufacturer's protocol.

2.5 Boundary finding PCR

The Genome Walker Universal kit (Clontech, Mountain View, CA, USA) was used to identify the boundary of the duplication variations. At each of four duplication boundary regions, four outward facing primers near the boundary were used to determine the location of the endpoints of the duplicated region via PCR (Table 1). According to the manufacturer's protocol, the size of the genome and purity of the genomic DNA of a Silkie chicken were checked on a 0.6% agarose gel before beginning the library construction. The DNA was digested with four restriction enzymes: *Dra* II, *Eco*R V, *Pur* II and *Stu* I. After purification, the DNA was ligated to Genome Walker adaptors. A two-step PCR system was used. The first step of the PCR was performed with 10 pmol the outer adaptor primer (AP1) provided in the kit, 10 pmol gene-specific primer (GSP1) provided by ourselves, 2.5 μL 10 × LA PCR buffer, 1 μL dNTP (10 mmol·L^{-1}), 0.5 μL LA Taq polymerase (Tiangen, Beijing, China) and 1 μL of each DNA library. The thermal cycling protocol was seven cycles of 94°C for 25 s and 72°C for 4 min, followed by 32 cycles of 94°C for 25 s and 67°C for 4 min, and finally 67°C for 7 min. The product was diluted 50-fold for the second step of the PCR, which was performed with 10 pmol AP2 primer, 10 pmol GSP2 primer, 5 μL 10 × LA PCR buffer, 1 μL dNTP (10 mmol·L^{-1}), 0.5 μL LA Taq polymerase (Tiangen) and 1 μL diluted PCR product. The thermal cycling protocol was five cycles of 94°C for 25 s and 72°C for 4 min, followed by 20 cycles of 94°C for 25 s and 67°C for 4 min, and finally 67°C for 7 min. After gel extraction, the PCR product was sequenced using the Sanger method.

2.6 Boundary testing PCR

Based on the sequencing results obtained with Genome Walker PCR, two pairs of primers that were suitable for the two special boundaries, Dup-1-5′and Dup-2-5′, and Dup-1-3′and Dup-2-3′ were designed to test the presence of the newly identified structural variation in hyperpigmented breeds. The primer sequences are shown in Table S3

Table 1 Primer sequences for locating the boundary with PCR

Primer name	Orientation	Primer sequence (5′ to 3′)
Dup-CNV-1-5′-GSP1	R	CACAATAACATAGGTAAGGGCACACACTG
Dup-CNV-1-5′-GSP2	R	CTGTGAGGCATTAGTAATCCCACCAAA
Dup-CNV-1-3′-GSP1	F	CCCTCCTTCAAAATCCCCATCTGTTA
Dup-CNV-1-3′-GSP2	F	GACTTGGTAGCAACGATAGCACTTATTCCT
Dup-CNV-2-5′-GSP1	R	CAATGGCTCTCCAAAGGAATGGCTCT
Dup-CNV-2-5′-GSP2	R	ACAACTGCCTAAAACTTTACTCGACTTCTC
Dup-CNV-2-3′-GSP1	F	GTCCATTATCCCAGAGACAGCCTTGC
Dup-CNV-2-3′-GSP2	F	GCTCAGTGAAACACCCAACATAAAATTAC
AP1	F	GTAATACGACTCACTATAGGGC
AP2	F	ACTATAGGGCACGCGTGGT

(Appendix B). Standard PCR was performed as follows: 5 pmol of each of the two primers, 10 × PCR buffer, 5 mmol·L^{-1} dNTPs, Taq DNA Polymerase (CWBIO, Beijing, China) and 30 ng DNA in a total volume of 20 μL. The thermal cycling protocol used for the test was 95°C for 5 min, followed by 35 cycles of 95, 59 and 72°C for 30 s each.

2.7 Target DNA capture and sequencing data analysis

DNA from 18 Silkie chickens, 21 Jinhu Silkie chickens, 21 White Leghorn chickens and 20 Gushi chickens was pooled into one pool for each breed. The Agilent SureSelect DNA Capture array was designed according to the manufacturer's protocol to capture the target genomic regions on chr20 from 9390989 to 11768733 bp (WUGSC 2.1/galGal3) and the libraries for the target genome were collected using the Agilent SureSelect^{TM} Target Enrichment system. Illumina HiSeq 2000 (Illumina, San Diego, CA, USA) mate-pair sequencing was performed with these libraries by BerryGenomics Company (Beijing, China). The reads were mapped to the chicken genome (WUGSC 2.1/galGal3) reference assembly and used for *de novo* assembly mapping using VELVET (1.2.01 version) and SOAPdenovo (1.05 version) software (http://metavelvet.dna.bio.keio.ac.jp/, http://soap.genomics.org.cn/soapdenovo.html).

2.8 RNA isolation and mRNA qPCR

Tissue from the Silkie and White Leghorn chickens was obtained at incubation times of 1.5, 3, 5, 8, 13 and 4 days before hatching, which represents the gradual change in pigmentation in the embryo (not shown). Beginning at embryonic day 5, tissue was collected from the head, heart and hind legs. At least three biological replicates of each breed were collected per time point. Tissue was homogenized with TissueLyser LT (QIAGEN, Dusseldorf, Germany) in 1 mL TriZol reagent (Tiangen, Beijing, China) and RNA was isolated according to the manufacturer's protocol. The associated primer sequences for all genes are shown in Table S4 (Appendix B). Total RNA (1 μg) from each sample was used to synthesize first-strand cDNA using M-MLV (Promega, Beijing, China). Reactions were performed using the Roche SYBR Green-based real-time qPCR kit on a Roche LightCycler480 instrument and contained 30 ng cDNA and 3 pmol each primer in a total volume of 15 μL. Thermal cycling parameters were the default settings and the PCR products were subjected to melting curve analysis. The Basic Relative Quantification module of the LightCycler480 software was used to analyze the data with a ΔΔCT algorithm. Expression in each sample was normalized to *GAPDH* expression. Then, the samples from hyperpigmented and normally pigmented chickens were tested for significance using an unpaired *t*-test. Error bars in all gene expression figures represent 95% confidence intervals.

3 Results

3.1 The genomic region associated with *FM* and discovery of two CNV regions

The 2.8 Mb region of chr20 was identified as being completely associated with the dermal hyperpigmentation phenotype corresponding to the *FM* locus that had been identified by Dorshorst [1]. A crossed population of Silkie, Gushi and Youxima chickens was created to segregate *Fm* alleles on an *id*$^{+}$ background in 187 individuals. We refined the 7.5 Mb region with 43 markers (6.2–13.7 Mb) in the F_2 generation of the crossed population (markers are according to the May 2006 assembly; WUGSC 2.1/galGAL3) and obtained 91 kb (10724771–10816347 bp) of chr20 that was completely associated with *FM* according to genotyping analysis (Appendix A). We identified two SNPs, Gga_rs16172722 and Gga_rs16172768, that were heterozygous in all *Fm/fm*$^{+}$

chickens. These two SNPs were genotyped in Silkie, Jinhu Silkie, Kuaida Silkie, Youxima, Anak and White Recessive chickens, as well as *Fm* and fm^+ individuals of the crossed population (Appendix C, Fig. S1). All individuals with Gga_rs16172722 (G/A) were hyperpigmented, whereas all individuals with Gga_rs16172722 (G/G) were pigmented. We concluded that a CNV existed in this region (data for Gga_rs16172768 are not shown). We next performed an aCGH assay with 14 Chinese local breeds including Silkie and Jinhu Silkie chickens. Two duplicated CNV regions were detected on the region of chr20 that is uniquely found in the Silkie and Jinhu Silkie breeds (Appendix C, Fig. S2). Dup-CNV-1 was on chr20: 10718139–10844289 bp (126.15 kb); Dup-CNV-2 was on chr20: 11263937–11435137 bp (171.2 kb). Both were located in the region that is associated with *FM*.

3.2 Identification of a structural rearrangement of CNVs that is associated with *FM*

qPCR analysis of genes and fragments was performed to confirm the duplication of the first genomic region in *FM* chickens (Table 2) (crossed individuals with gray skin color; crossed individuals with black skin color; Silkie, Jinhu Silkie, Tengchong, Yanjin, Wuding and Chuxiong chickens) with a relative copy number of approximately 1.5–2 × that of wild-type individuals (Youxima and Qingyuan ma chickens; crossed individuals with white skin color). Here, 1.5 × indicates that some *FM* individuals were heterozygous for a duplicated allele (Fig. 2) (the data for the second genomic region are shown in Appendix C (Fig. S3).

Because the boundaries of the duplicated regions correspond to the probes of aCGH, the accuracy of the boundaries of both duplicated CNV regions needed to be confirmed. After unsuccessful determination of conventional duplication arrangements, PCR (Genome Walker) was performed between the outward facing primers (Table 1) from the edges of each end of both duplicated CNV regions (Fig. 3). After performing BLASTN analysis with the sequence of the PCR products and the May 2006 (WUGSC 2.1/ galGal3) assembly, a complex structural rearrangement was identified: each duplicated region was joined to the other in an inverted orientation. The exact boundary of the first duplicated region was verified at 10717294 to 10846232 bp and the exact boundary of the second duplicated region was verified at 11262904 to 11435256 bp. We also confirmed the accuracy of the coordinates of the duplications using a *de novo* assembly map, which contained two duplicated regions (9390989 to 11768733 bp) (Appendix D). Contigs C130791 and C134745 showed an unexpected distance from the two pair ends, and they exactly matched the locations of the duplications.

To test our conclusion of a complex structural rearrangement on chr20 in *FM* individuals, a PCR assay was developed with primers designed according to the inverted rearranged sequence. All *FM* breeds (Silkie and Jinhu Silkie) produced amplicons, whereas fm^+ breeds (White Leghorn, Cobb, White Recessive, Gushi and Wahui) did not (Appendix E, Fig. S4).

3.3 Increased expression of genes in CNVs is related to *FM*

Four known genes are present in the first duplicated region: *EDN3* (*endothelin 3*), *SLMO2* (*slowmo homolog 2*), *ATP5e* (*ATP synthase epsilon subunit*) and *TUBB1* (*tubulin, beta 1*). The second duplicated region contains no known coding or regulatory elements according to the UCSC Genome Browser (http:// genome.ucsc.edu). *EDN3* plays a role in melanocyte regulation [20,21]. In the Silkie (*Fm*) embryo, *EDN3* showed significantly ($p < 0.01$) increased expression from 1.5- to 3-day when melanoblasts are migrating (Fig. 4). The expression of *EDN3* was maintained at a higher level in Silkie embryos than in White Leghorns (fm^+) during development. On the fourth day after hatching, *EDN3* expression in Silkie chickens reached a remarkably high level ($p < 0.001$) in skin and

Table 2 Chinese chicken breeds used for confirmation of copy number variation regions

Chicken breed	Abbreviation	Skin color	Shank color
Youxima	YX	White	Black
Qingyuan	QY	White	Black
Crossed *FM* family: white skin	*FM* W	White	Black
Crossed *FM* family: gray skin	*FM* G	Gray	Black
Crossed *FM* family: black skin	*FM* B	Black	Black
Silkie	WJ	Black	Black
Jinhu	JH	Black	Black
Tengchong snow	TC	Black	Black
Yanjin Silkie	YJ	Black	Black
Wuding Silkie	WD	Black	Black
Chuxiong Silkie	CX	Black	Black

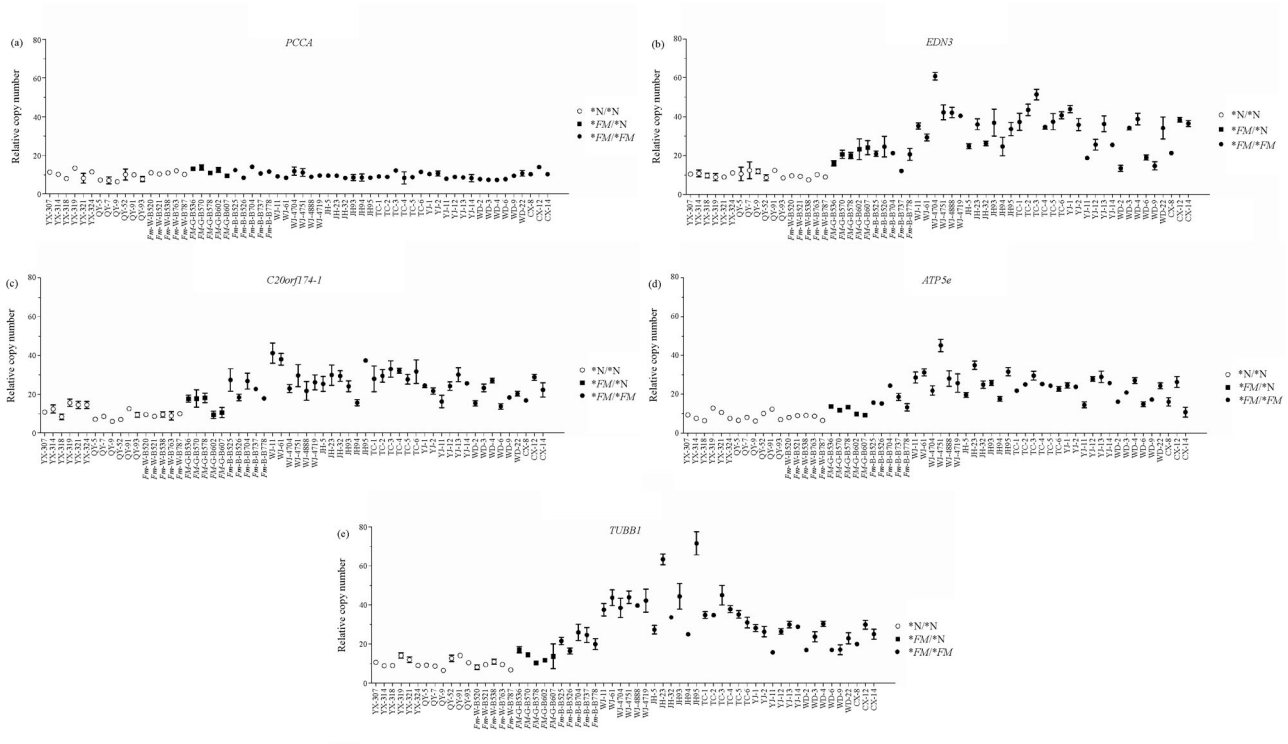

Fig. 2 Results of qPCR analysis of genes and fragments for Dup-CNV-1 detection. Dup-CNV-1: (a) *PCCA* (control); (b) *EDN3*; (c) *C20orf174-1*; (d) *ATP5e*; (e) *TUBB1*. Genomic copy number was detected with qPCR in 11 breeds of chicken. The heterozygotes (*FM* G) showed an estimated copy number of approximately 1.5 × compared to wild-type individuals (YX, QY, *FM* W). Homozygotes (*FM* B, WJ, JH, TC, YJ, WD, CX) showed an estimated copy number of approximately 2 × compared to wild-type individuals. The numbers after the hyphen mean the label when feeding; "N"means the individual has no *Fm* loci; the data are the mean ± SD.

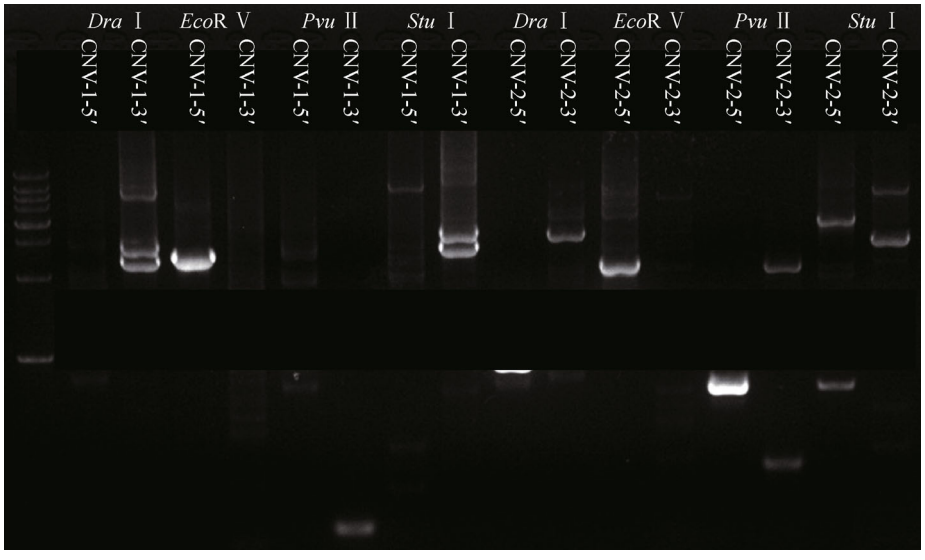

Fig. 3 Genome Walker PCR. PCR products were digested with four restriction enzymes, namely *Dra* II, *Eco*R V, *Pvu* II, *Stu* I, producing fragments of different lengths from the same lines and indicating differential amplification of segments with the same primers (i.e., polymorphic segments).

muscle tissue. In Silkie chickens, the other genes (*ATP5e, SLMO2* and*TUBB1*), which are downstream of *EDN3* and located in the first duplicated region, also showed significantly high expression from the embryo to the adult in skin and muscle tissue (Fig. 4).

Endothelin 3 has two receptors: EDNRB (endothelin

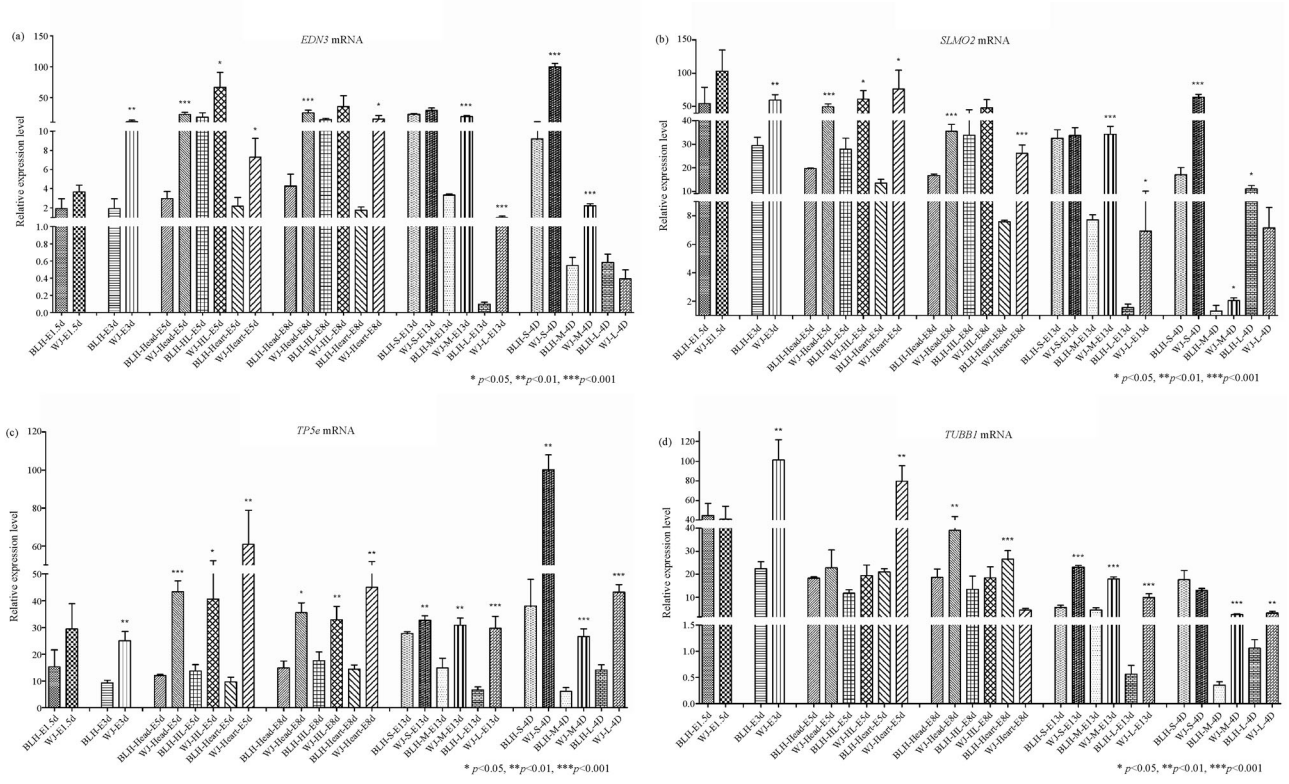

Fig. 4 Results of qPCR analysis of the expression of duplicated genes in Dup-CNV-1. (a): *END3* mRNA; (b) *SLMO2* mRNA; (c) *ATP5e* mRNA; (d) *TUBBa* mRNA. Gene expression analysis in Silkie (FM, WJ) and White Leghorn (fm^+, BLH) chickens with SYBR Green qPCR normalized to expression of *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*). Genes in the first duplicated region (*EDN3*, *SLMO2*, *ATP5e*, *TUBB1*) showed significantly increased expression from embryonic tissue (E3d) through adult skin and muscle tissue (4D) of the Silkie chicken. HL: hind leg; S: skin; M: muscle; L: liver.

receptor B) and EDNRB2 (endothelin receptor B subtype 2). The *EDNRB* product prevents non-melanocyte derivatives of the neural crest from traversing the dorsoventral pathway [22]. *EDNRB2* is expressed by melanocytes and is involved in migration [23]. We assayed *TYRP2* (*tyrosinase-related protein 2*) as an indicator of the biosynthesis of eumelanin, one of the three broad classes of melanin. We examined the expression of these three genes. At embryonic day 13 and day 4 after hatching, *EDNRB* expression was not significantly different between Silkie (*Fm*) and White Leghorn (fm^+) skin and muscle tissue (Fig. 5). The expression of *EDNRB2* and *TYRP2* was significantly higher in Silkie than in White Leghorn chickens ($p < 0.001$).

4 Discussion

Our current findings confirm that the *FM* phenotype is completely due to a complex structural variant that contains two duplicated genomic regions of 129 and 172 kb that are separated by 417 kb on wild-type chromosomes [24]. The discovery of this structural variant was facilitated by our refinement of the region of 7.5 Mb on chr20 to identify the most closely correlated region for *FM*, followed by precise identification with the aCGH assay (Appendix C, Fig. S2). By comparing the 14 Chinese local breeds and four special Yunnan local dermal hyperpigmented chickens, this structural variant was shown to exist only in *FM* breeds. Sequencing the duplication junctions revealed complete sequence conservation at both duplication junctions, suggesting that this structural variant exists exclusively in *FM* breeds. Using Genome Walker PCR, we analyzed the structural variant containing the joined 5′ ends and the joined 3′ ends of the two duplicated regions. We were able to PCR amplify the wild-type duplicated boundary sequences.

The structural variant we identified produces a segmental CNV. When a CNV contains genes or conserved regulatory elements, mRNA levels can be affected [25]. In the first duplication, four genes were annotated. The mRNAs of *EDN3*, *SLMO2*, *ATP5e* and *TUBB1* were expressed at higher levels in *FM* breeds in the whole embryo. *EDN3* has a mitogenic effect on melanocytes [26]. For example, ectopic *EDN3* expression results in dermal hyperpigmentation in a transgenic mouse model [20]. In Silkie embryos, the spatial characteristics of melanoblast migration and the timing are very different compared to

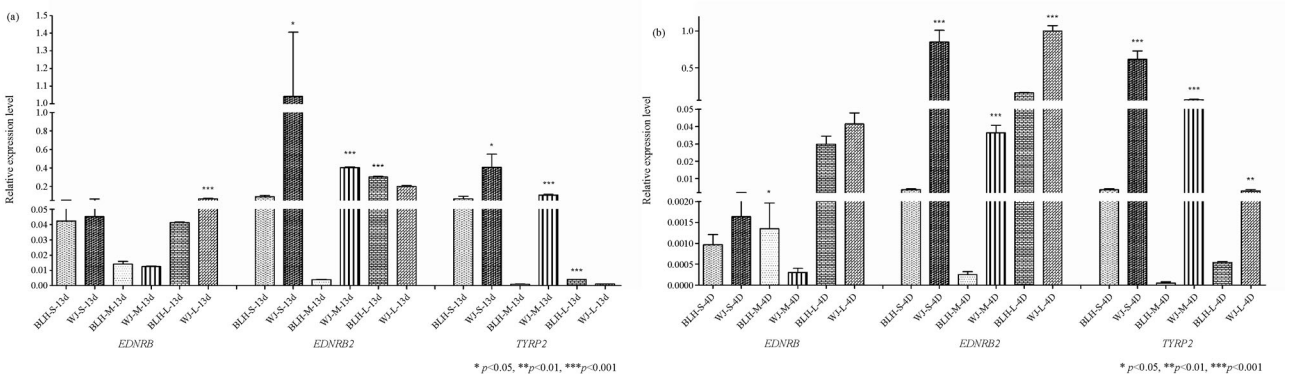

Fig. 5 Expression of genes downstream of *EDN3*. Gene expression analysis of the EDN3 receptor genes *EDNRB* and *EDNRB2* and the melanin biosynthesis pathway gene *TYRP2*. *EDNRB* expression in skin or muscle showed no significant difference between Silkie and White Leghorn chickens at either embryonic day 13 (a) or adult day 4 (b). However, the expression of *EDNRB2* and *TYRP2* was strongly increased in Silkie skin and muscle tissue in both the embryo and adult. In contrast, the expression of *EDNRB2* and *TYRP2* was very low in White Leghorn chickens. HL: hind leg; S: skin; M: muscle; L: liver.

wild-type embryos [9]. We showed there was increased expression of *EDN3* at the time of melanocyte migration. *EDN3* expression remained high in adult Silkie skin, perhaps owing to maintenance of an environment that allows melanoblast development. However, this observation was not a consequence of a simple dosage effect because duplication of *END3* resulted in a 10-fold increase in expression of *EDN3* in Silkie tissues; the reasons for this are unknown. The other genes in the first duplication, namely *SLMO2*, *ATP5e* and *TUBB1*, also showed increased expression. We do not know if these genes are involved in the *FM* phenotype, but *EDN3* duplication may be a primary reason for dermal hyperpigmentation in *FM* chickens because a similar observation has been reported in mouse [20].

Hyperpigmentation in chickens is influenced by both *FM* and *ID* (inhibitor of dermal melanin). Thus, to avoid the influence of *ID*, we developed a population with id in which all individuals have black shanks. We hypothesized that the abnormal migration pattern and the increase in melanoblasts was due to increased *EDN3*-related signaling. In fm^{+}/id individuals, skin pigmentation was only seen in the dermis of the shank. This observation may be interpreted as *ID* controlling the migration of melanoblasts and *FM* controlling proliferation, because only the shanks express *EDN3* in conditions of differential regulation. However, how expression in the shank is accurately controlled is unknown. Identifying the *ID* causal mutation located on the Z chromosome will be required to determine the complete mechanism of hyperpigmentation in chickens.

5 Conclusions

In chickens, the dominant *FM* allele causes dermal hyperpigmentation. Our current research confirmed that the *FM* phenotype is due to a complex structural variant that contains two duplicated genomic regions on wild-type chromosomes. *EDN3* expression was increased in Silkie embryos through adulthood and was maintained in adult skin and muscle. The expression of *EDN3* was closely correlated with the pigmentation phenotype. We examined eight different Chinese chicken breeds displaying hyperpigmentation and conclude that this structural variant exists in all *FM* chicken breeds.

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Compliance with ethics guidelines Ming Tian, Suyun Fang, Yanqiang Wang, Xiaorong Gu, Chungang Feng, Rui Hao, Xiaoxiang Hu and Ning Li declare that they have no conflict of interest or financial conflicts to disclose.

All applicable institutional and national guidelines for the care and use of animals were followed.

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