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Gene duplication of *endothelin 3* is closely correlated with the hyperpigmentation of the internal organs (*Fibromelanosis*) in Silky chickens

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ABSTRACT

During early development in vertebrates, pluripotent cells are generated from the neural crest and migrate according to their presumptive fate. In birds and mammals, one of the progeny cells, melanoblasts, generally migrate through dorsolateral route of trunk region and differentiate to melanocytes. However, Silky is an exceptional chicken in which numerous melanoblasts travel via ventral pathway and disperse into internal organs. Finally, these ectopic melanocytes induce heavy dermal and visceral melanization known as *Fibromelanosis* (*Fm*). To identify the genetic basis of this phenotype we confirmed the mode of inheritance of *Fm* as autosomal dominant, and then performed linkage analysis with microsatellite markers and sequence-tagged site markers. Using 85 backcross progeny from crossing Black Minorca chickens (BM-C) with (White Silky X BM-C) F_1 individuals, *Fm* was located on 10.2–11.7 Mb of chicken chromosome 20. In addition, we noticed a DNA marker that all Silky chickens and the F_1 individuals showed heterozygous genotyping patterns, suggesting gene duplication in the *Fm*-region. By quantitative real-time PCR assay, Silky-line specific gene duplication was detected as approximately 130 kb interval. It contained five genes including *endothelin 3* (*EDN3*) which encoded a potent mitogen for melanoblasts/melanocytes. *EDN3* with other three of these duplicated genes in Silky chickens expressed almost twofold of those in BM-C. Present results strongly suggest that the increase of the expression levels

resulted from the gene duplication in the *Fm*-region is the trigger of hypermelanization in internal organs of Silky chickens.

INTRODUCTION

Melanocytes are the main cell type producing pigment, which display body color in birds and mammals. The precursor cells of melanocytes, melanoblasts, derived from the neural crest with other progenitor cells during early embryogenesis (Nordlund *et al.* 2006). The neural crest cells in trunk region migrate through either the ventral or dorsolateral route and differentiate according to their presumptive fate (Le Douarin and Kalcheim 2009). The former cells become sensory and autonomic neurons, adrenomedullary cells, and Schwann cells (Weston 1963; Le Douarin and Teillet 1974). The latter cells, melanoblasts, migrate at a slightly later stage than the former cells, differentiate into melanocytes and settle in the integumental basal layer (Erickson and Goins 1995; Le Douarin and Kalcheim 2009).

The Silky chicken (*Gallus gallus*) is a unique chicken breed with numerous characteristics such as brilliant silky feathers, feathered legs, polydactyly, blue ear-lobes, etc. In particular, the hyperpigmentation in tissues and organs such as the dermal layer of skin, bone, muscle, pleura, trachea, blood vessels, abdominal lining, and connective tissue (Kuklenski 1915; Hutt 1949) has been noteworthy for breeders and scientists since 13th century in China (Haw 2006). The huge number of cells containing black melanin particles (melanosomes) in

the connective tissue and sheaths of the internal organs is observed in Silky chickens. Therefore Silky chicken meat looks so black, which is led to the belief that it contains unknown physiologically useful substance(s) for Chinese cuisine. Many researchers have been studied to search the Silky specific substance(s) but little has been still uncovered. None of the origin, history of *Fm*, other chicken breeds with *Fm* nor the molecular mechanism to induce the hyperpigmentation in Silky has been completely clarified yet.

On the pigmentation in dermal tissues of Silky chickens, common melanin was identified as the main pigment substance, and its chemical, physical, and morphological properties were similar to those in other breeds (Muroya *et al.* 2000; Chen *et al.* 2008). Cells having melanosomes in internal organs in Silky contain various stages of immature melanosomes in their cell bodies (Reedy *et al.* 1998; Faraco *et al.* 2001; Ortolani-Machado *et al.* 2007; Ortolani-Machado *et al.* 2009), indicating that melanization occurs inside these cells. Therefore, these cells could be judged as melanocytes. Basically, epidermal melanocytes in humans transfer mature melanosomes to adjacent keratinocytes via melanocyte dendrites (Boissy 2003). On the other hand, melanosomes in the dermal skin, eye, inner ear, and leptomeninx in humans and mice are not transferred to the surrounding cells (Okawa *et al.* 1979, Hori *et al.* 1982, Boissy and Hornyak 2006). Melanocytes in internal organs in Silky produce stage III melanosomes (intermediate phase of melanosomes production with deposit of melanin in

matrix protein) and maintain them inside the cells, as occurs in dermal melanocytes in mammals and other fowl (Ortoloni-Machado *et al.* 2009). Thus, there are no distinctive differences in the pigment particles and morphologies of melanosomes, and in melanogenesis between dermal melanocytes in Silky chicken and melanocytes in other fowl and mammals.

Silky melanoblasts migrate through an unusual ventral pathway with other neural crest derivatives in addition to the common dorsolateral route, and proliferate actively during migration. (Hallet and Ferrand 1984; Erickson 1993; Lecoin *et al.* 1995; Reedy *et al.* 1998; Muroya *et al.* 2000; Faraco *et al.* 2001; Jacobs-Cohen *et al.* 2002; Le Douarin and Kalcheim 2009). These melanoblasts invade into the internal organs and settle among the connective tissues in a similar fashion to fibroblasts. Given the similarity in behavior and localization of these dermal melanoblasts and fibroblasts, Hutt (1949) named this phenotype as *Fibromelanosis* (*Fm*). Identification of the gene(s) that control dermal hyperpigmentation in Silky chickens is particularly important to understand the general mechanism on the cell fate determination and migration of pluripotent cells from the neural crest.

Although melanoblasts in mammals and birds are known to migrate mainly via the dorsolateral route from neural crest, those in lower vertebrates are often observed to travel through ventral route (Collazo *et al.* 1993; Raible and Eisen 1994, 1996; Kelsh 2004; Akiyama *et al.* 2006; Tomlinson *et al.* 2009;

Reyes *et al*. 2010). Investigation of Silky chickens could also provide significant clues to recognize the evolutionally divergence of melanoblast migration.

In addition to the *Fm* locus, *Id* (the sex-linked inhibitor of dermal melanin) affects hyperpigmentation (Bateson and Punnett 1911; Dunn and Jull 1927). *Id* is an incomplete dominant sex-linked gene with a role as a modifier for *Fm*, and combinations of mutants (*Id* and *Fm*) and/or wild-type (id^+ and fm^+) alleles on each locus determine the degree of pigmentation of internal organs in each individual (Stolle 1968). By linkage analyses, *Id* has been localized on the long arm of chicken chromosome Z (Bitgood 1988; Levin *et al.* 1993; Dorshorst and Ashwell 2009). Recently, Dorshorst *et al*. (2010) identified the physical location of *Id* and also *Fm* by a genome-wide single nucleotide polymorphism (SNP)-trait association analysis; they demonstrated SNP markers which associated with *Id* and *Fm* at 72.3 Mb on chromosome Z and at 10.3 to 13.1 Mb on chromosome 20, respectively. However, the genomic regions of *Id* and *Fm* extend several Mb and still contain a large number of candidate genes.

Since 2006, we have been generating families for chromosome mapping to identify the gene responsible for *Fm*. Here, we performed linkage mapping analysis by using White Silky and Black Minorca chickens, after evaluating the effects of other loci on *Fm*. We succeeded in pinpointing the genomic region of *Fm*. And further, we found noteworthy gene duplication completely linked to *Fm*. We discuss the correlation between gene duplication and hyperpigmentation in

internal organs.

MATERIALS AND METHODS

Chicken lines: All the chicken lines have been maintained over seven generations as a closed colony and were supplied by Avian Bioscience Research Center, Nagoya University, Nagoya, Japan. White Silky (WS) and Black Minorca (BM-C) were used for *Fm* mapping. Black Silky (BS), Fayoumi (PNP/DO) and Red jungle fowl (RJF) were used as references for the *Fm* (BS) or *fm* (wild-type) (PNP/DO, and RJF) lines (Figure 1). All experiments were performed in accordance with the Nagoya University and Keio University institutional guidelines for animal experiments.

Identification of the *Fm* phenotype: We observed pigmentation of pleura, trachea, muscle, bone, and skin dissected out from each chicken at least two weeks after hatching (Figure 1); individuals with heavy pigmentation were defined as *Fm* by visual judgment and stereomicroscopy. Although there was a clear difference in color between the *Fm* and *fm* individuals (Figure 1), we confirmed the phenotype quantitatively by measuring the brightness of some internal organs with a chronometer (CR-221, Minolta, Tokyo, Japan) as a Y value. The average Y value of each group was calculated using mean values of pleura in individuals. The Y value of the standard white board was 88.7. Y values in the *fm* lines (BM-C, PNP/DO, RJF) were clearly different from the *Fm* lines (WS and BS) (Figure S1). The Y value in F_1 between WS and BM-C (5.8) was close to but significantly

higher than those in WS (2.6) and BS (3.3) ($P < 0.01$; by Student's t-test) (Figure S1). Y values of *Fm* and *fm* individuals in BC from BM-C and the F_1 showed major differences: BC with *Fm* showed 7.5, whereas BC with *fm* displayed 21.6, which are close to F_1 (5.8) and BM-C (22.2), respectively (Figure S1). Finally, *Fm* or *fm* was determined by visual judgment of the color of the pleura and trachea. Although the colors of the internal organs in *fm* lines were apparently bright (Figure 1), a few numbers of pigment cells were often observed by using stereomicroscopy. Because of the fewest visceral pigment cells among investigated lines, BM-C was used for genetic mapping in this study.

Genotyping of DNA markers: DNA was extracted from the blood by using a DNeasy Blood and Tissue Kit (QIAGEN K. K., Tokyo, Japan) according to the manufacturer's instructions. Microsatellite markers were amplified by use of a PCR method as follows: 2 min at 95˚, followed by 10 cycles of 15 sec at 94˚, 30 sec at 55˚, 30 sec at 72˚ and 40 cycles of 15 sec at 94˚, 30 sec at 50˚, 30 sec at 72˚ with a final elongation step of 5 min at 72˚. Length polymorphisms of the PCR products were identified by using 12.5% polyacrylamide gel electrophoresis. PCR for the markers we designed (Table S1) was performed for 2 min at 95˚, followed by 10 cycles of 15 sec at 94˚, 30 sec at 60˚, 45 sec at 72˚ and 30 cycles of 15 sec at 94˚, 30 sec at 55˚, 45 sec at 72˚ with a final elongation step of 5 min at 72˚. Differences in the PCR products were identified through restriction fragment length polymorphisms by using 7.5% polyacrylamide gel electrophoresis.

Quantification of gene copy numbers and expression levels: Gene copy numbers were quantified using genomic DNA extracted from blood. The DNA concentrations of all samples were adjusted to a total of 20 ng for each real-time PCR assay. Primers were designed to amplify 89–136 bp fragments on each locus (Table S2). SYBR *Premix Ex Taq* II (Takara Bio, Otsu, Japan) was used according to the manufacturer's protocol in an Applied Biosystems StepOne Real-Time PCR System (Life Technologies Japan, Tokyo, Japan). The PCR conditions were 1 min at 95˚, followed by 40 cycles of 5 sec at 95˚ and 30 sec at 60˚. To confirm that each amplified PCR product was specific to each locus, we determined each melting temperature from dissociation curves. The melting temperature was the defined unique value for each primer set, indicating that the same product was amplified by the same primer set from any DNA sample. The copy number of each locus was determined by use of the comparative Ct method (Livak and Schmittgen 2001; Pfaffl 2001). Ct values for the BM-C line were set to reference point 1, and sample copy numbers were calibrated (as $2^{-\Delta Ct}$) against the Ct value for the *AS046* locus and calculated as $2^{-\Delta\Delta Ct}$.

Expression levels were quantified by using total RNA extracted from whole embryos at stage 18 (Hamburger and Hamilton 1951) with the RNeasy Plus Mini Kit (QIAGEN K. K.). RNA concentrations were determined by using a NanoDrop ND-2000 spectrophotometer (Thermo Scientific) and were adjusted to 10 ng for each real-time assay. Primers were designed to amplify 83–120 bp

fragments in the exons of each gene (Table S3). A One Step SYBR PrimeScript Plus RT-PCR kit (Takara Bio) was used in accordance with the manufacturer's protocol in the ABI StepOne Real-Time PCR System. The PCR conditions were 5 min at 42°, 10 sec at 95°, then 40 cycles of 5 sec at 95° and 30 sec at 60°. The melting temperature confirmed that the same product had been amplified from all samples. Ct values from the BM-C line were set to 1 and the relative expression levels (as $2^{-\Delta\Delta Ct}$) were calculated after calibration (as $2^{-\Delta Ct}$) against the Ct value for the *GAPDH* (*glyceraldehyde-3-phosphate dehydrogenase*) gene. All real-time quantitative PCR experiments were performed four times.

RESULTS

Chromosome mapping of *Fibromelanosis*: Although the history of chicken breeds are uncertain (Crawfold, 1990), WS has been thought to originate in China. To identify DNA markers for linkage analysis, it is advantageous to cross between breeds with broadly separated genetic origins. Then, we chose BM-C and PNP/DO, which were established in Europe and Egypt, respectively, for mating. First, to understand the modes of inheritance of *Fm* on their genetic backgrounds four female BM-C or one female PNP/DO was crossed with a WS male, respectively, then seven to eight F_1 females and two to three F_1 males from each mating were crossed to obtain F_2 offspring. We gained 34 and 11 F_1 individuals from BM-C X WS and PNP/DO X WS, respectively. All of these F_1 progeny displayed the *Fm* phenotype. Among the F_2 offspring, 71% (N = 17) and

74% (N = 98) showed the *Fm* phenotype, from incrosses of (BM-C X WS) F_1 and (PNP/DO X WS) F_1, respectively. Because all F_1 individuals exhibited *Fm* and the ratio of *Fm* to *fm* in F_2 was almost three to one, we concluded that the expression of the *Fm* phenotype was controlled by a single dominant gene that was not affected by other loci among these lines. Concerning the *Id* locus, these results clearly suggest that both the BM-C and PNP/DO lines had the id^+ (wild-type) allele. Since the BM-C line showed the least pigmentation in internal organs as described in the Materials and Methods, we adopted the BM-C for *Fm* mapping.

For the mapping, we obtained backcross offspring (BC) from three families (Table 1). Among the BC offspring, 44 (51.8%) showed heavy (*Fm*) and 41 (48.2%) showed wild-type (*fm*) pigmentation (Table 1), which corresponded to the expected 1:1 ratio of *Fm*:*fm*. We then searched for DNA markers exhibiting length polymorphisms between the WS and BM-C parents of families 1 and 2. We identified 17 polymorphic markers from 46 known microsatellite markers (Takahashi *et al*. 2005) and genotyped the 29 BC individuals in families 1 and 2. The microsatellite marker *ABR0001*, which is located on chicken chromosome 20, showed a phenotype-specific polymorphism in BC offspring. Concerning this marker, the WS-specific band was slightly longer than the BM-C-specific band, and two F_1-specific bands, which could be the result of DNA heteroduplex formation (Ganduly *et al*. 1993; Hauser *et al*. 1998), were observed in the F_1

individuals (Figure 2). Among the BC progeny, the banding patterns of BM-C and F_1 types were distinguishable. The WS- and F_1-specific bands were linked to the *Fm* phenotype in 93% (27/29) individuals (Figure 2), indicating that the position of *ABR0001* was close to the *Fm* locus. To pinpoint the *Fm* region, we designed new PCR primers by using the draft sequence database for the chicken genome (http://www.ncbi.nlm.nih.gov/sites/entrez?db=genomeprj&cmd=Retrieve&dopt=Overview&list_uids=10804). Restriction fragment length polymorphisms (RFLPs) for the PCR products of the parents and the F_1 of families 1, 2, and 3 were examined and 10 PCR-RFLP markers were adapted. By using these polymorphic markers, linkage analysis was carried out for the 85 BC progeny.

As a result, *Fm* was mapped to the same position with four DNA markers, *AS057, AS044, AS049, and AS055* on chicken chromosome 20 (Figures 3 and S2). *Fm* and adjacent markers located in the following order: *AS056*, *Fm*, and *AS050/AS053/AS051*, each separated by a distance of 1.2 cM. On the basis of the chicken sequence database, the position of *Fm* was 10.2–11.7 Mb on chromosome 20 (Figure 3 and Table S1). Dorshorst *et al.* (2010) reported the position of *Fm* as 10.3–13.1 Mb on chromosome 20 by using SNP-trait association analysis with more than 350 individual backcrossed progeny from F_1 females (from a Polish female and a Silky male), and the same Polish male. The *Fm* region identified in our study is consistent with the *Fm* position mapped by

Dorshorst *et al* (2010). Our results thus confirm the position of *Fm* and narrow down the physical distance of the *Fm* region to 1.46 Mb from 2.8 Mb.

Variations in gene copy number within the *Fm* region: While searching for DNA polymorphisms between BM-C and WS parents, we found a DNA marker linked to *Fm*, *AS044*, showing unusual pattern. Its primer set amplified 1119-bp DNA fragments. All of the PCR-RFLP products in BM-C (n = 9) had a single *Mbo*I recognition site, then we detected two DNA bands on polyacrylamide gel electrophoresis after digestion with it (this pattern is indicated by "*aa*" in Figure 4). On the other hand, all of the products in WS (n = 14) exhibited a non-digested band together with the same two bands of BM-C, which was considered to be a heterozygous banding pattern (this pattern is indicated by "*ab*" in Figure 4). In addition, all F_1 individuals (n = 14) from families 1 and 2 showed the same heterozygous pattern as that of WS. When we examined other DNA markers, the allelic inheritance modes of the PCR-RFLPs followed Mendel's law (Figure 4). These results suggest that the *AS044* locus is duplicated in the WS line and that a sequence polymorphism exists between the original and duplicated DNA sequences.

To reveal the gene duplication, we analyzed the genomic copy number of several loci by using quantitative real-time PCR. Initially, we analyzed the genomic copy numbers of four loci, *AS048*, *AS044*, *AS049*, and *AS050* in two BM-C and three WS individuals (Figures 3 and 5, details of primers were

indicated in Table S2). Gene copy numbers of three of these loci (*AS048*, *AS049,* and *AS050*) were the same for BM-C and WS chickens (Figure 5 **A**, **C**, **D**). However, the relative copy number of the *AS044* locus*,* in WS was twice that in BM-C (Figure 5 **B**). Because there were no differences in copy number among individuals in the same line, this copy number duplication was considered to be WS-specific. To further clarify this gene duplication, we analyzed eight loci including the *AS044* locus in the 140-kb area of the *Fm* region and increased sampling to five and six individuals of BM-C and WS, respectively (Figures 3 and 5). For five loci from *AS072* to *AS845*, the genomic copy number in WS was almost twofold of that in BM-C (Figure 5 **F-J**), whereas those for the other three loci, *AS717*, *AS8465* and *AS058*, in WS were similar to BM-C individuals (Figure 5 **E**, **K**, **L**). For all loci except *AS8465*, there were no considerable differences among individuals in the same line. Although a small variation was observed on the *AS8465* locus, it was not strain-specific. These results clearly demonstrate that the genome in the WS line has a duplicated part in the *Fm* region and that this duplicated area was within 130 kb from the border between *AS717* and *AS072* to that between *AS845* and *AS8465* (Figure 3).

Correlation between gene duplication and *Fm*: To determine whether both copies of the duplicated area were located in the *Fm*-linked region, we investigated the linkage of the copy number of *AS044* to the *Fm* phenotype by using families 1 and 2. Two or three F_1 individuals, as well as three and two with *Fm* and *fm*

phenotypes in BC, respectively, were randomly selected from each family and analyzed together with the parent chickens. The copy number in the F_1 was 1.5 times that of BM-C, which was the middle value between the parents (Figure 6 **A**). The copy number in BC with *Fm* was also 1.5 times, whereas that for BC with *fm* was almost equal to that in BM-C (Figure 6 **A**). There were no differences among individuals in the same group and between families. These results indicate that the gene duplication is linked to the *Fm* phenotype and that WS chickens have two, namely an original and a duplicated, segments in the *Fm* region on each chromosome 20, whereas BM-C chickens have only the original segment on this chromosome.

To elucidate the correlation between the *Fm* phenotype and copy number variation, we examined the copy numbers of the *AS044* locus in other chicken lines that display the *Fm* or *fm* phenotype. In *fm* lines (RJF and PNP/DO), the copy numbers were equal to that in BM-C, whereas the value in BS (*Fm*) was almost twice that in BM-C (Figure 6 **B**). These data show that: (1) part of the *Fm*-linked region in chromosome 20 is duplicated in Silky lines, and (2) this gene duplication is specific to *Fm* chickens; *fm* chickens have only one copy on chromosome 20. These results strongly suggest that gene duplication is responsible for the *Fm* phenotype.

We maintain another chicken line that has heavy melanization in its internal organs, similar to that in Silky chickens. This line originated from two

Indonesian chickens, Ayam cemani and Ayam Arab. The internal organs of Ayam cemani were black, whereas those of Ayam Arab showed the wild-type phenotype. We crossed an Ayam cemani female with an Ayam Arab male and all of the F_1 progeny displayed black internal organs, indicating that the hyperpigmentation in Ayam cemani was a dominant trait like *Fm* in Silky. This line has been maintained by incrosses among these siblings, and the progeny included two types of individuals of which internal organs showed dark- and wild-type-color. By using one generation (N = 11) in this line, we examined copy number of *AS044*. The copy number of individuals with dark color internal organs (N = 6) was almost 1.5 times that in the siblings with wild-type color (N = 5), and there was a statistically significant difference between them ($P < 0.01$; by Student's t-test). Namely, the copy number of siblings in the Ayam cemani line is also correlated with the color of internal organs, suggesting that the original Ayam cemani chicken, which displays hyberpigmentation in internal organs, also had a duplicated segment containing *AS044*.

Expression levels of genes located on the duplicated segment: In the 130-kb area duplicated in the WS genome, five genes, which are homologous to *endothelin 3* (*EDN3*), *HIVEP1*, *slowmo homolog2* (*SLMO2*), *H+ transporting F1 ATP synthase epsilon subunit* (*F1ATPase-e*), and *tubulin beta 3* (*TUBB3*), are annotated based on information in the ENTREZ Genome Project database (*Gallus gallus* Build 2.1;

http://www.ncbi.nlm.nih.gov/sites/entrez?db=genomeprj&cmd=Retrieve&dopt=Overview&list_uids=10804) (Figure 3). In chicken embryos, melanoblasts first begin to migrate from the neural crest at stage 18 and enter the dorsolateral path at around stage 20. Only a few of them generally enter the ventral area after that. In Silky embryos, a greater number of the melanoblasts are observed in the ventral area at stage 22 than those in other fowls (Reedy *et al.* 1998; Faraco *et al.* 2001). To clarify whether the five genes are expressed in chicken embryos during melanoblast migration, we analyzed the mRNA expression of these genes by using RNA extracted from whole embryos at stage 18. Four of the five genes (not *HIVEP1*) were detected by using RT-PCR (data not shown). We then quantified the expression levels of these four genes and compared them between BM-C and WS embryos. For all four genes, the mRNA levels in the WS embryos were significantly higher than those in the BM-C embryos; that of *EDN3, SLMO2*, *F1ATPase-e,* and *TUBB3* was 1.9, 2.4, 1.9 and 1.9 times higher, respectively (Figure 7). These results correspond with the copy number duplication in the WS line, and suggest that the gene duplication caused the hyper-expression of these genes.

DISCUSSION

Our findings in the present study confirm that the *Fm* phenotype in Silky chickens is mainly controlled by a single locus. Other unique characteristics, silky feathers, feathered legs, polydactyly, a mulberry crest and

hair bulb in head, were not linked to the *Fm*, demonstrating that these are controlled by other loci. The *Fm* region was within a 1.46-Mb on chicken chromosome 20 and further narrowed relative to a previous report by Dorshorst *et al.* (2010). Furthermore, we found gene duplication in the *Fm* region of the genome in White and Black Silky chickens, and this duplication was not observed in other chicken breeds with wild-type pigmentation in their dermal tissues. We also detected a linkage between hypermelanization and gene duplication in independent chicken lines, that is, White Silky and Ayam cemani. These results strongly suggest that the gene duplication affects the hypermelanization in dermal tissues.

Chromosomal segmental copy number variation (CNV) has been recently recognized as a very important source of genetic variability (Jiang *et al.* 2004; Redon *et al.* 2006; MacCarrol *et al.* 2008; Conrad *et al.* 2010). Some CNV loci contain genes or conserved regulatory elements that affect mRNA expression levels (Stranger *et al.* 2007). Furthermore, recent studies have found CNVs related to various human diseases (Wain *et al.* 2009; Zhang *et al.* 2009; Stankiewicz *et al.* 2010). CNVs are also observed in Aves both in inter-, and intra-species (Griffin et al. 2008; Skinner et al. 2009; Völker et al. 2010; Wang *et al.* 2010). In addition, an association between CNVs and a specific phenotype has been documented (Wright *et al.* 2009). Intraspecific CNVs for the chicken genome were analyzed by using NimbleGen whole genome tiling arrays with

385,000 probes; the mean probe spacing was approximately 2.6 kb (Wang *et al.* 2010). However, the 130-kb CNV in this study was not found by them; we are, therefore, the first to report this CNV between Silky fowl and other chicken breeds. Since Wang *et al.* (2010) used three *fm* lines (broilers, Leghorns, and Rhode Island Reds), we believe that none of these lines has a duplicated copy of the region. This may explain why the CNV was not detected by their whole chicken genome assay. Chickens represent a very important farm animal species that has also long-served as a model for biological and biomedical research. Further studies will disclose the correlation between CNVs and various distinctive traits that are segregated and established as chicken breeds.

In the 130-kb duplicated area, five genes were annotated based on information in the ENTREZ Genome Project database. mRNAs were expressed from four of these five genes in whole embryos at stage 18 just before initiation of melanoblast migration (Reedy *et al.*, 1998; Faraco *et al.* 2001). The expression levels of all four of these genes in Silky chickens were 1.9 to 2.4 times those in BM-C, which concurred with the gene copy numbers. On the other hand, distribution of the *EDN3* mRNA detected by *in situ* hybridization studies was not significantly different between Silky and other *fm* fowl embryos (data not shown). These results suggest that the mRNAs are transcribed from both the original gene and the duplicated copy and that there are no obvious differences in cis-regulatory elements between the two sequences of these genes. Although

all F_1 progeny between BM-C and WS displayed the *Fm* phenotype, the degree of pigmentation in their internal tissues was significantly lighter than that in WS (Figure S1). This observation indicates that the hyperpigmentation in the *Fm*/*Fm* homozygote is more severe than that in the *Fm*/*fm*$^+$ heterozygote. Therefore, the *Fm* phenotype can be considered as a semi-dominant rather than a dominant trait. It corresponds well to the copy numbers of the duplicated area (Figure 6 **A**). From these results, we propose the following hypothesis: gene duplication leads to high levels of mRNA expression, which, in turn, triggers hypermelanization in internal organs. The degree of pigmentation could correlate with the mRNA expression level.

Of the four genes that displayed high mRNA expression, we propose that *EDN3*, in particular, is as a candidate gene for *Fm*. Vasoactive endothelin (EDN) was first described by Yanagisawa *et al.* (1988). The endothelin family comprises three 21-amino acid peptides, EDN 1, 2, and 3, that are highly conserved. In mammals, two endothelin receptors (EDNRs), EDNRA and EDNRB, which belong to a G-protein-coupled heptahelical superfamily (Arai *et al.* 1990; Sakurai *et al.* 1990, 1992; Kusserow and Unger 2004), have been identified. EDNRA has high affinities for EDN1 and EDN2 and a significantly lower affinity for EDN3 (Arai *et al.* 1990), whereas EDNRB exhibits similar affinities for all three EDNs (Sakurai *et al.* 1990, 1992). *EDN3* and *EDNRB* are both allelic to the spontaneous mouse mutations that occur at the *piebald lethal* (*s*) and *lethal*

spotting (*ls*) loci, respectively. Recessive mutants of these loci yield similar phenotypes that consist of differing degrees of hypopigmentation and aganglionic megacolon (Baynash *et al*. 1994; Hosoda *et al*. 1994). The hypopigmented phenotype has been attributed to a decrease in the melanoblast population and to abnormal cell migration (Pavan and Tilghman 1994; Lee *et al*. 2003). A paralogue of EDNRB (designated EDNRB2), found to be specific to the melanocytic lineage, was cloned in quail by Lecoin *et al*. (1998). A mutant in quail, which had an amino acid substitution and reduced gene expression in the *ENDRB2* gene, displayed white plumage with wild-type-colored spots (Miwa *et al*. 2007). In the mutant embryos, pigment production in the integument and feather bud was strongly suppressed from the early developmental stage, and few melanoblasts survived (Akiyama *et al*. unpublished data). These analyses clearly demonstrate that the signal transduction system of EDN3–EDNRB in mammals and EDN3–EDNRB2 in birds has a crucial role in melanoblast/melanocyte development from neural crest cells. Of the other three genes that were highly expressed in Silky embryos (Figure 7), *H^+ transporting F1 ATP synthase epsilon subunit* may relate with the phenotype because *F1F0 mitochondrial ATP synthase* has been reported as a target for modulating pigmentation of melanocytes (Jung *et al*. 2005). More studies are necessarily to determine whether these three genes are involved in the melanoblasts/melanocytes development.

In Silky embryos, two distinctive events occur during melanoblast development: accelerated proliferation and unusual ventral migration. Numerous *in vitro* studies using quail and mouse embryos have reported that EDN3 affects the melanocyte lineage population by increasing their number in a dose-dependent manner (Lahav *et al*. 1996, 1998; Reid *et al*. 1996; Opdecamp *et al*. 1998; Dupin *et al*. 2000). In addition, exogenous overexpression of *EDN3* driven by keratin 5 in transgenic mouse embryos induced proliferation of melanocyte precursors, and led to hyperpigmentation on most areas of their skin (Garcia *et al*. 2008). Here, the high-level expression of *EDN3* mRNA in Silky chickens was detected at stage 18, and we obtained the same result at stages 15 and 24 (data not shown). These data suggest that Silky embryos are exposed to high doses of EDN3 before and during differentiation of melanoblasts from neural crest cells, and that abundant EDN3 *in vivo* could induce the accelerated proliferation of melanocyte precursors. Experiments with grafts between embryos of Silky and other fowl (Hallet and Ferrand 1984; Ferrand and L'Hermite 1985) and the culture of neural crest cells isolated from quail embryos in medium containing embryonic extract from Silky or other fowl (Lecoin *et al*. 1994), suggest that Silky embryos contain a growth factor(s) for melanocyte proliferation and that the *Fm* phenotype is not attributable to the melanocyte lineage but rather to other cell-types in the melanoblast environment. *EDN3*, which encodes a ligand of *EDNRB2*, is expressed in the ectoderm and in gut

mesenchyme (Nataf *et al.* 1998), whereas *EDNRB2* is expressed throughout the melanocyte lineage (Lecoin *et al.* 1998). From these data and our present results, we suggest that a mitogen contained in Silky embryos for the melanocyte lineage is abundant EDN3 due to the gene duplication.

It remains unclear whether the unusual ventral migration of melanoblasts in Silky embryos is a result of excess EDN3. In *Xenopus* embryos, many melanoblasts have been observed migrating through the ventral pathway (Collazo *et al.* 1993). Kawasaki-Nishihara *et al.* (2011), suggest that EDN3–EDNRB2 signaling in *Xenopus* embryos is essential for normal migration of melanoblasts by *in vivo* experiments of *EDNRB2* overexpression and inhibition of *EDN3* expression. In the case of Aves, EDNRB2 is thought to be important for melanoblast migration toward the usual "dorsolateral" pathway (Pla *et al.* 2005; Harris *et al.* 2008). On the other hand, Aoki *et al.* (2009) demonstrated that non-cutaneous and dermal melanocytes are more sensitive to EDN3 for growth and differentiation compared with epidermal melanocytes. In Silky fowl, excess EDN3 may affect only the proliferation of the dermal melanocyte cell lineage during the early differentiating stage. Thereafter, these proliferated cells would disperse to other accessible sites. It may result in the abnormal migration of melanoblasts and the distribution of melanocytes in internal organs. Excess EDN3 may actually induce the sequential expression of other genes involved in signal transduction or of extracellular matrix proteins,

which could lead to melanoblast proliferation and ventral migration. Even so, the excess production of EDN3 in Silky fowl as a result of gene duplication could be the first trigger for hypermelanization.

Our results and hypothesis seem appropriate to explain the hyperpigmentation in Silky chickens. Silky hyperpigmentation is a valuable model to study one of the basic important biological themes, that is, cell migration and fate determination of pluripotent neural crest cells. Additional studies are necessary to fully understand this phenomenon, some of which are now in progress.

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FIGURE LEGENDS

FIGURE 1: Pigmentation of pleura and trachea in several chicken lines. WS and BS have heavily pigmented internal organs whose colors are clearly different from those in the *fm* lines (BM-C, PNP/DO, RJF). F_1 between BM-C and WS shows *Fm*, and BC progeny between the F_1 and BM-C are classified into *Fm* or *fm* groups. BM-C, A, B; WS, C, D; F_1, E, F; BC judged as *Fm*, G, H; BC judged as *fm* I, J; PNP/DO, K, L; RJF, M, N; BS, O, P; pleura, A, C, E, G, I, K, M, O; trachea; B, D, F, H, J, L, N, P. (Size) one pitch of the scales: 1 mm.

FIGURE 2: An example of linkage analysis using microsatellite markers on the BC progeny. Electrophoretic patterns of *ABR0001* PCR products in families 1 and 2 are shown. PCR products in WS (white arrowhead) are slightly longer than those in BM-C (black arrowhead). Heterozygote-specific heteroduplex bands (HD: arrow) appear in F_1 individuals. The WS-specific band and HD cosegregated with *Fm* in BC individuals except for two individuals (indicated by dotted rectangles).

FIGURE 3: Genetic (left) and physical (right) maps of *Fm* on chicken chromosome 20. These maps were derived by our study and drawn with a reference of *Gallus gallus* draft genome sequence data (http://www.ncbi.nlm.nih.gov/sites/entrez?db=genomeprj&cmd=Retrieve&dopt=Overview&list_uids=10804). The 1.46 Mb *Fm* region and the 130 kb duplicated area are shown by double headed arrows. Positions of DNA markers used in this

study are indicated in the physical map. Details of these markers are shown in Tables S1 and S2. Loci analyzed by qPCR were indicated by rectangles. A primer set for *AS046* (arrowhead) was served as a reference for qPCR analyses. Abbreviations of gene names are indicated in the Results. Arrows indicate the direction of the genes.

FIGURE 4: Genotyping data for five *Fm*-linked DNA markers in BM-C (n = 9), WS (n = 14), and F_1 (n = 14) between BM-C and WS in families 1 and 2. A haplotype pattern of PCR-RFLP is indicated as "*a*" or "*b*". Individuals that showed the "*a*" or "*b*" band pattern are described as "*aa*" or "*bb*", respectively, and individuals that displayed the "*a*" plus "*b*" band pattern are shown as "*ab*". On the *AS044* locus (arrows), all WS and F_1 individuals displayed the "*ab*" band pattern. ID, identification number. Details of these loci are shown in Table S1.

FIGURE 5: Quantitative PCR analysis of genomic copy numbers in the BM-C and WS lines. Copy numbers in BM-C (light gray columns) and WS (dark gray columns) individuals, relative to that for the average of BM-C chickens, were calculated by use of the comparative Ct method. Data were normalized to the reference locus (*AS046*). Two asterisks indicate a statistically significant difference between BM-C and WS lines ($P < 0.01$; by Student's t-test). Locations of analyzed loci are represented in Figure 3.

FIGURE 6: Quantitative PCR analyses of genomic copy numbers of the *AS044* locus in the families used for the linkage mapping and other breeds. Copy

numbers relative to that for BM-C were calculated by using the comparative Ct method. Data were normalized to the reference locus (*AS046*). Light gray and dark gray columns indicate the *fm* and *Fm* phenotypes, respectively. Sample numbers used for the analysis are indicated in parentheses. The gene duplication was linked to the *Fm* phenotype in BC progeny in families 1 and 2 (A). Chicken breeds displaying *fm* (RJF, PNP/DO) and *Fm* (BS) demonstrated equal and almost twofold of the copy number in BM-C, respectively (B). Two asterisks and taggers indicate a statistically significant difference for BM-C and WS lines, respectively ($P < 0.01$; by Student's t-test). Bar, ± standard error.

FIGURE 7: Quantitative PCR analysis of expression levels of genes located in the duplicated 130-kb area in whole embryos at stage 18 in BM-C (n = 5, light gray column) and WS (n = 7, dark gray column). Gene expression levels in WS relative to those in BM-C were obtained by using the comparative Ct method. Data were normalized to a reference gene (*GAPDH*; *glyceraldehyde-3-phosphate dehydrogenase*). The gene positions are indicated in Figure 3. Asterisks indicate a statistically significant difference (*$P < 0.05$, ** $P < 0.01$; by Student's t-test). Bar, ± standard error.

TABLE 1

The *Fm* phenotype in backcross progeny (BC) used for *Fm* mapping

Family	Crossing		Phenotype in BC		
	♀	♂	*Fm*	*fm*	Total
1	BM-C	(BM-C X WS) F_1	9	7	16
2	(WS X BM-C) F_1	BM-C	5	8	13
3	(WS X BM-C) F_1	BM-C	30	26	56
		Total	44 (51.8%)	41 (48.2%)	85

REFERENCES

AKIYAMA, T., J. MATSUMOTO and K. KITAMURA, 2006 Immunocytochemical studies on the differentiation of melanophores and its relation to migratory behaviors in goldfish *Carassius auratus.* Hiyoshi Review of Nat. Sci. Keio Univ. 39: 1-20.

AOKI, H., Y. YAMADA and T. KUNISADA, 2009 Two distinct types of mouse melanocyte: differential signaling requirement for the maintenance of noncutaneous and dermal versus epidermal melanocytes. Development 136: 2511-2521.

ARAI, H., S. HORI, I. ARAMORI, H. OHKUBO and S. NAKANISHI, 1990 Cloning and expression of cDNA encoding an endothelin receptor. Nature 348: 730-732.

BATESON, W. and R. PUNNETT, 1911 The inheritance of the peculiar pigmentation of the silky fowl. J. Genet. 1: 185-203.

BAYNASH, A. G. , K. HOSODA, A. GIAID, J. A. RICHARDSON, N. EMOTO *et al.*, 1994 Interaction of Endothelin-3 with Endothelin-B Receptor Is Essential for Development of Epidermal Melanocytes and Enteric Neurons. Cell 79: 1277-1285.

BITGOOD, J. J., 1988 Linear relationship of the loci for barring, dermal melanin inhibitor, and recessive white skin on the chicken Z chromosome. Poult. Sci. 67: 530-533.

BOISSY, R. E., 2003 Melanosome transfer to and translocation in the keratinocyte. Exp. Dermatol. 12: 5-12.

BOISSY, R. E. and T.J. HORNYAK 2006 Extracutaneous melanocytes. In: J.J. Nordlund, R.E. Boissy, V.J. Hearing, R.A. King, W.S. Oetting and J.P. Ortonne, Editors, The Pigmentary system: Physiology and pathophysiology, Second edition, Oxford University Press, New York, pp. 91-107.

CHEN, S., B. JIANG, J. ZHENG, G. XU, J. LI *et al.*, 2008 Isolation and characterization of natural melanin derived from silky fowl(*Gallus gallus domesticus Brisson*). Food Chem. 111: 745-749.

COLLAZO A, M. BRONNER-FRASER and S.E. FRASER, 1993 Vital dye labelling of *Xenopus laevis* trunk neural crest reveals multipotency and novel pathways of migration. Development 118: 363-376.

CONRAD D. F., D. PINTO, R. REDON, L. FEUK, O. GOKCUMEN *et al.*, 2010 Origins and functional impact of copy number variation in the human genome. Nature 464: 704-712.

CRAWFORD, R.D. 1990 Poultry breeding and genetics. Elsevier Science Publishers. Amsterdam.

DORSHORST, B. J. and C. M. ASHWELl, 2009 Genetic mapping of the sex-linked barring gene in the chicken. Poult. Sci. 88: 1811-1817.

DORSHORST, B., R. OKIMOTO and C. ASHWELL, 2010 Genomic regions associated with dermal hyperpigmentation, polydactyly and other

morphological traits in the Silkie chicken. J. Hered. 101: 339-350.

DUNN, L. and M, JULL, 1927 On the inheritance of some characters on the silky fowl. J. Genet. 19: 27-63.

DUPIN, E., C. GLAVIEUX, P. VAIGOT and N. M. LE DOUARIN, 2000 Endothelin 3 induces the reversion of melanocytes to glia through a neural crest-derived glial-melanocytic progenitor. Proc Natl. Acad. Sci. USA 97: 7882-7887.

ERICKSON, C. A., 1993 From the crest to the periphery: control of pigment cell migration and lineage segregation. Pigment Cell Res. 6: 336-347.

ERICKSON, C. A. and T. L. GOINS, 1995 Avian neural crest cells can migrate in the dorsolateral path only if they are specified as melanocytes. Development 121: 915-924.

FARACO, C. D., S. A. VAZ, M. V. PASTOR and C. A. ERICKSON, 2001 Hyperpigmentation in the Silkie fowl correlates with abnormal migration of fate-restricted melanoblasts and loss of environmental barrier molecules. Dev. Dyn. 220: 212-225.

FERRAND, R. and A. L`HERMITE, 1985 Experimental analysis of the extensive pigmentation in the Silkie fowl embryo: evidence for an environmental regulatory process. Experientia 41: 512-514.

GANDULY, A, M. J. ROCK and D. J. PROCKOP, 1993 Conformation-sensitive gel electrophoresis for rapid detection of single-base differences in

double-stranded PCR products and DNA fragments: evidence for solvent-induced bends in DNA heteroduplexes. Proc. Natl. Acad. Sci. USA 90: 10325-10329.

GARCIA, R. J., A. ITTAH, S. MIRABAL, J. FIGUEROA, L. LOPEZ *et al.*, 2008 Endothelin 3 induces skin pigmentation in a keratin-driven inducible mouse model. J. Invest. Dermatol. 128: 131-142.

GRIFFIN, D. K., L. B. ROBERTSON, H. G. TEMPEST, A. VIGNAL, V. FILLON *et al.*, 2008 Whole genome comparative studies between chicken and turkey and their implications for avian genome evolution. BMC Genomics 9: 168.

HALLET, M. M. and R. FERRAND, 1984 Quail melanoblast migration in two breeds of fowl and in their hybrids: evidence for a dominant genic control of the mesodermal pigment cell pattern through the tissue environment. J. Exp. Zool. 230: 229-238.

HAMBURGER, V. and H. L. HAMILTON, 1951 A series of normal stages in the development of the chick embryo. J. Morphol. 88: 49-92.

HARRIS, M. L., R. HALL and C. A. ERICKSON, 2008 Directing pathfinding along the dorsolateral path – the role of EDNRB2 and EphB2 in overcoming inhibition. Development 135: 4113-4122.

HAUSER M.T., F. ADHAMI, M. DORNER, E. FUCHS and J. GLOSSL, 1998 Generation of co-dominant PCR-based markers by duplex analysis on high resolution gels. Plant J. 16: 117-125.

HAW, S. G., 2006 Marco Polo's China: a Venetian in the realm of Khubilai Khan. Routledge studies in the early history of Asia 3 London (NY): Routledge.

HORI, Y., Y. OHARA, M. NIIMURA and A. KUKITA, Electron microscopy, ultrastructural observations of the extracellular sheath of dermal melanocytes in the nevus of Ota, 1982 Am. J. Dermatophathol. **4**: 245–251.

HOSODA, K., R. E. HAMMER, J. A. RICHARDSON, A. G. BAYNASH, J. C. CHEUNG *et al.*, 1994 Targeted and natural (Piebald-Lethal) mutations of endothelin-B receptor gene produce megacolon associated with spotted coat color in mice. Cell 79: 1267-1276.

HUTT F. B., 1949 Genetics of the fowl. McGraw-Hill, New York.

JACOBS-COHEN, R. J., P. R. WADE and M. D. GERSHON, 2002 Suppression of the melanogenic potential of migrating neural crest-derived cells by the branchial arches. Anat .Rec. 268: 16-26.

JIANG L. J., J. H. MAO, A. BALMAIN, L. PETERSON, C. HARRIS *et al.*, 2004 Genomic segmental polymorphisms in inbred mouse strains. Nat. Genet. 36: 952-954.

JUNG, D., D. WILLIAMS, S. M. KHERSONSKY, T. KANG, N. HEIDARY, Y. CHANG and S. J. ORLOW. 2005 Mol. BioSyst., Identification of the F1F0 mitochondrial ATPase as a target for modulating skin pigmentation by screening a tagged triazine library in zebrafish. 1: 85-92.

KAWASAKI-NISHIHARA, A., D. NISHIHARA, H. NAKAMURA and H. YAMAMOTO, 2011 ET3/Ednrb2 signaling is critically involved in

regulating melanophore migration in *Xenopus*. Dev. Dyn. 240: 1454-1466.

KELSH, R. N., 2004 Genetics and evolution of pigment patterns in fish. Pigment Cell Res. 17: 326-336.

KUKLENSKI, J., 1915 Über das vorkommen und die verteilung des pigmentes in den organen und geweben bei japanischen seidenhühnern. Arch. Micro. Anat. Entwickl. 87: 1-37.

KUSSEROW, H. and T. UNGER, 2004. Vasoactive peptides, their receptors and drug development. Basic Clin. Pharmacol. Toxicol. 94: 5-12.

LAHAV, R., C. ZILLER, E. DUPIN and N. M. LE DOUARIN, 1996 Endothelin 3 promotes neural crest cell proliferation and mediates a vast increase in melanocyte number in culture. Proc. Natl. Acad. Sci. USA 93: 3892-3897.

LAHAV, R., E. DUPIN, L. LECOIN, C. GLAVIEUX, D. CHAMPEVAL *et al.*, 1998 Endothelin 3 selectively promotes survival and proliferation of neural crest-derived glial and melanocytic precursors *in vitro*. Proc. Natl. Acad. Sci. USA 95: 14214-14219.

LECOIN, L., R. LAHAV, F. H. MARTIN, M. A. TEILLET and N. M. LE DOUARIN, 1995 *Steel* and *c-kit* in the development of avian melanocytes: a study of normally pigmented birds and of the hyperpigmented mutant silky fowl. Dev. Dyn. 203: 106-118.

LECOIN, L., P. MERCIER and N. M. LE DOUARIN, 1994 Growth of neural crest cells in vitro is enhanced by extracts from silky fowl embryonic tissues.

Pigment Cell Res. 7: 210-216.

LECOIN, L., T. SAKURAI, M. NGO, Y. ABE, M. YANAGISAWA *et al.*, 1998 Cloning and characterization of a novel endothelin receptor subtype in the avian class. Proc. Natl. Acad. Sci. USA 95: 3024-3029.

LE DOUARIN, N.M. and C. KALCHEIM, 2009 The neural crest. Cambridge Univ. Press, Cambridge 472 pp.

LE DOUARIN, N. M. and M. A. TEILLET, 1974 Experimental analysis of the migration and differentiation of neuroblasts of the autonomic nervous system and of neurectodermal mesenchymal derivatives, using a biological cell marking technique. Dev Biol. 41: 162-184.

LEE, H., J. M. LEVORSE and M. K. SHIN, 2003 The endothelin receptor-B is required for the migration of neural crest-derived melanocyte and enteric neuron precursors. Dev. Biol. 259: 162-175.

LEVIN, I., L. B. CRITTENDEN and J. B., 1993 Genetic map of the chicken Z chromosome using random amplified polymorphic DNA (RAPD) markers. Genomics 16: 224-230.

LIVAK, K. J. and T. D. SCHMITTGEN, 2001 Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. METHODS 25: 402–408.

MAUL, G. G. and J. A. BRUMBAUGH, 1971 On the possible function of coated vesicles in melanogenesis of the regenerating fowl feather. J. Cell Biol. 48:

41-48.

MCCARROLL, S. A., F.G. KURUVILLA, J. M. KORN, S. CAWLEY, J. NEMESH *et al.*, 2008 Integrated detection and population-genetic analysis of SNPs and copy number variation. Nat. Genet. 40: 1166-1174.

MIWA, M., M. INOUE-MURAYAMA, H. AOKI, T. KUNISADA, T. HIRAGAKI *et al.*, 2007 *Endothelin receptor B2* (*EDNRB2*) is associated with the *panda* plumage colour mutation in Japanese quail. Anim. Genet. 38: 103-108.

MUROYA, S., R. TANABE, I. NAKAJIMA and K. CHIKUNI, 2000 Molecular characteristics and site specific distribution of the pigment of the Silky fowl. J. Vet. Med. Sci. 62: 391-395.

NATAF, V., A. AMEMIYA, M. YANAGISAWA and N. M. LE DOUARIN, 1998 The expression pattern of endothelin 3 in the avian embryo. Mech. Dev. 73: 217-220.

NORDLUND, J.J., R. E. BOISSY, V. J. HEARING, R. A. KING, W. S. OETTING *et al.*, 2006 The pigmentary system. Blackwell Publishing. Oxford.

OKAWA, Y., R. YOKOTA and A. YAMAUCHI 1979 On the extracellular sheath of dermal melanocytes in nevus fuscoceruleus acromiodeltoideus (Ito) and Mongolian spot. An ultrastructural study, J. Invest. Dermatol. **73**: 224–230.

OPDECAMP, K., L. KOS, H. ARNHEITER and W. J PAVAN 1998 Endothelin signalling in the development of neural crest-derived melanocytes.1998 Biochem. Cell Biol. 76: 1093-1099.

ORTOLANI-MACHADO, C., P. DE FREITAS, M. E. BORGES and C. FARACO, 2007 Special features of dermal melanocytes in white silky chicken embryos. Anat. Rec. 291: 55-64.

ORTOLANI-MACHADO, C.F., P. F. FREITAS and C. D. FARACO, 2009 Melanogenesis in dermal melanocytes of Japanese Silky chicken embryos. Tissue Cell 41: 239-248.

PFAFFL, M. W., 2001 A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29: 2002-2007.

PAVAN, W. J. and S. M. TILGHMAN, 1994 Piebald lethal (*s^l*) acts early to disrupt the development of neural crest-derived melanocytes. Proc. Natl. Acad. Sci. USA 91: 7159-7163.

PLA, P., C. ALBERTI, O. SOLOV'EVA, M. PASDAR, T. KUNISADA *et al.*, 2005 Ednrb2 orients cell migration towards the dorsolateral neural crest pathway and promotes melanocyte differentiation. Pigment Cell Res. 18: 181-187.

RAIBLE, D. W. and J. S. EISEN, 1994 Restriction of neural crest cell fate in the trunk of the embryonic zebrafish. Development 120: 495-503.

RAIBLE, D. W. and J. S. EISEN, 1996 Regulative interactions in Zebrafish neural crest. Development 122: 501-507.

REDON, R., S. ISHIKAWA, K. R. FITCH, L. FEUK, G. H. PERRY *et al.*, 2006 Global variation in copy number in the human genome. Nature 444: 444-454.

REEDY, M. V., C. D. FARACO and C. A. ERICKSON, 1998 Specification and migration of melanoblasts at the vagal level and in hyperpigmented Silkie chickens. Dev. Dyn. 213: 476-485.

REID, K., A. M. TURNLEY, G. D. MAXWELL, Y. KURIHARA, H. KURIHARA *et al.*, 1996 Multiple roles for endothelin in melanocyte development: regulation of progenitor number and stimulation of differentiation. Development 122: 3911-3919

REYES M, K. ZANDBERG, I. DESMAWATI and M. E. DE BELLARD, 2010 Emergence and migration of trunk neural crest cells in a snake, the California Kingsnake (*Lampropeltis getula californiae*). BMC Dev. Biol. 10: 52.

SAKURAI, T., M. YANAGISAWA, Y. TAKUWA, H. MIYAZAKI, S. KIMURA *et al.*, 1990 Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. Nature 348: 732-735.

SAKURAI, T., M. YANAGISAWA and T. MASAKI, 1992 Molecular characterization of endothelin receptors. Trends Pharmacol. Sci. 13: 103-108.

SKINNER, B.M., L. B. ROBERTSON, H. G. TEMPEST, E. J. LANGLEY, D. IOANNOU *et al.*, 2009 Comparative genomics in chicken and Pekin duck using FISH mapping and microarray analysis. BMC Genomics 10: 357.

STANKIEWICZ, P. and J. R. LUPSKI, 2010 Structural Variation in the Human

Genome and its Role in Disease. Annu. Rev. Med. 61: 437-55.

STRANGER, B. E., M. S. FORREST, M. DUNNING, C. E. INGLE, C. BEAZLEY *et al.*, 2007 Relative impact of nucleotide and copy number variation on gene expression phenotypes. Science 315: 848-853.

TAKAHASHI, H., M. TSUDZUKI, O. SASAKI, J. NIIKURA, M. INOUE-MURAYAMA *et al.*, 2005 A chicken linkage map based on microsatellite markers genotyped on a Japanese Large Game and White Leghorn cross. Anim. Genet. 36: 463-467.

TOMLINSON, M. L., P GUAN, R. J. MORRIS, M. D. FIDOCK, M. REJZEK *et al.*, 2009 A Chemical genomic approach identifies matrix metalloproteinases as playing an essential and specific role in *Xenopus* melanophore migration. Chem. Biol. 16: 93-104.

VÖLKER, M., N. BACKSTRÖM, B. M. SKINNER, E. J. LANGLEY, S. K. BUNZEY *et al.*, 2010 Copy number variation, chromosome rearrangement, and their association with recombination during avian evolution. Genome Res. 20:503-511.

WAIN L.V., J. A. L. ARMOUR and M. D. TOBIN, 2009 Genomic copy number variation, human health, and disease. Lancet 374: 340-350.

WANG, X., S. NAHASHON, T. K FEASTER, A. BOHANNON-STEWART and N. ADEFOPE, 2010 An initial map of chromosomal segmental copy number variations in the chicken. BMC Genomics 11: 351.

WESTON, J. A. 1963 A radioautographic analysis of the migration and localization of trunk neural crest cells in the chick. Dev. Biol. 6: 279-310.

WRIGHT, D., H. BOIJE, J. R. S. MEADOWS, B. BED'HOM, D. GOURICHON *et al.*, 2009 Copy number variation in intron 1 of *SOX5* causes the pea-comb phenotype in chickens. PLoS Genetics 5: 6.

YANAGISAWA, M., H. KURIHARA, S. KIMURA, Y. TOMOBE, M. KOBAYASHI *et al.*, 1988 A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 332: 411-415.

ZHANG F, W. L. GU, M. E. HURLES and J. R. LUPSKI, 2009 Copy number variation in human health, disease, and evolution. Annu. Rev. Genomics. Hum. Genet. 10: 451-481.

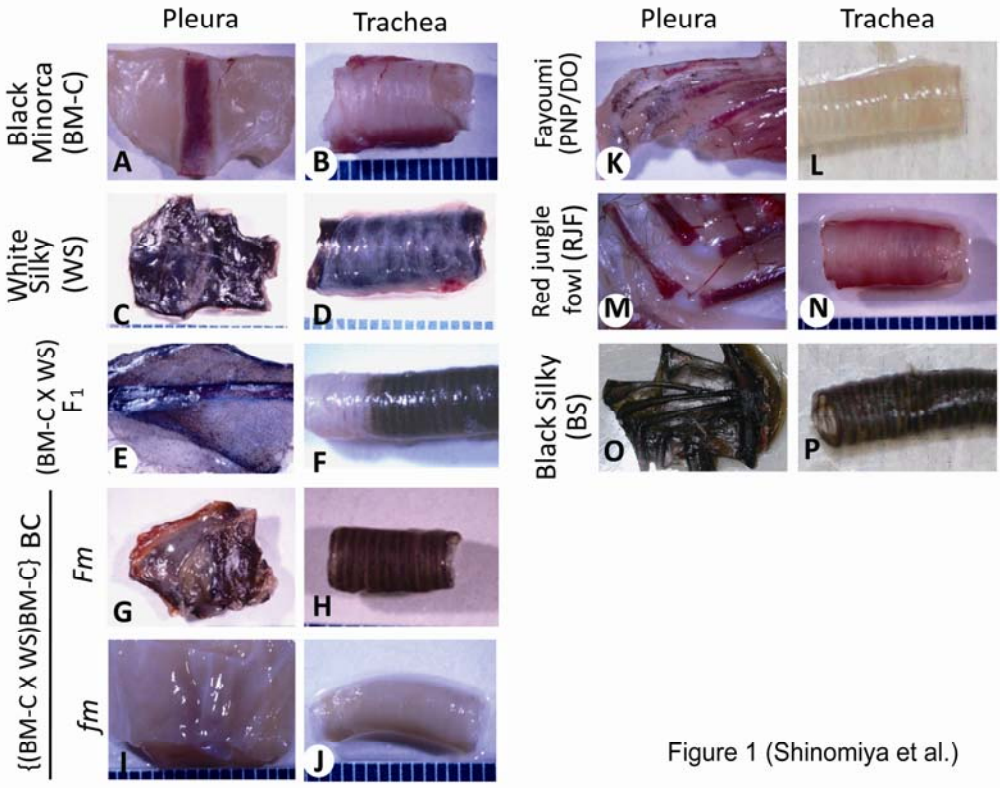

Figure 1 (Shinomiya et al.)

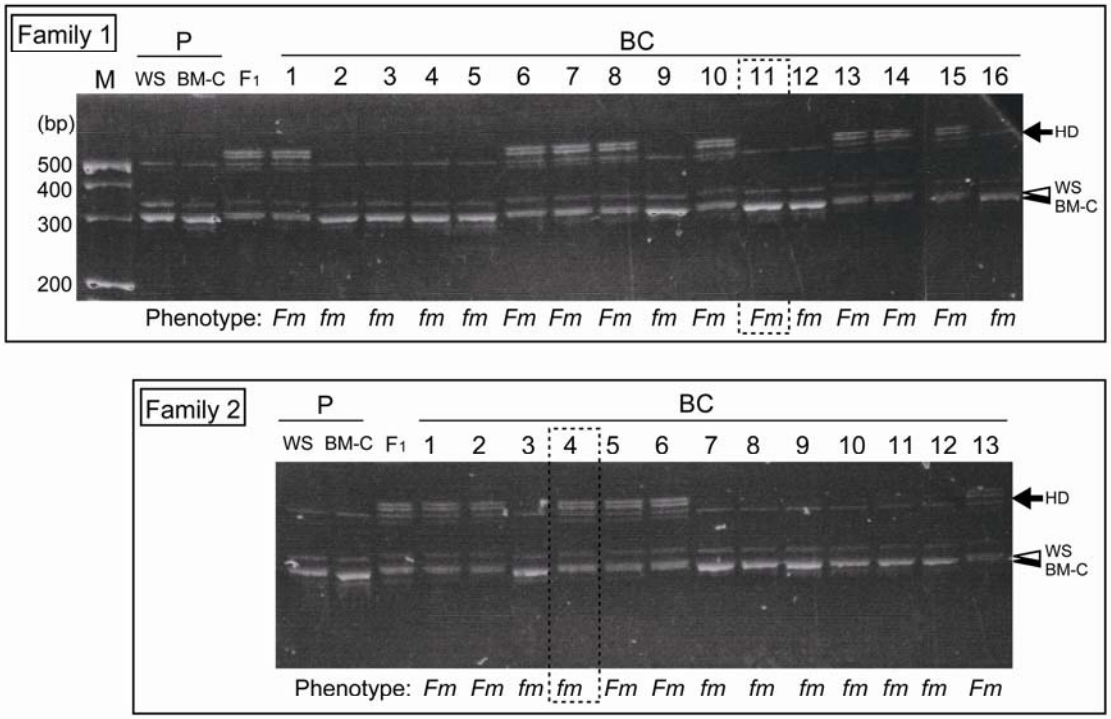

Figure 2 (Shinomiya et al.)

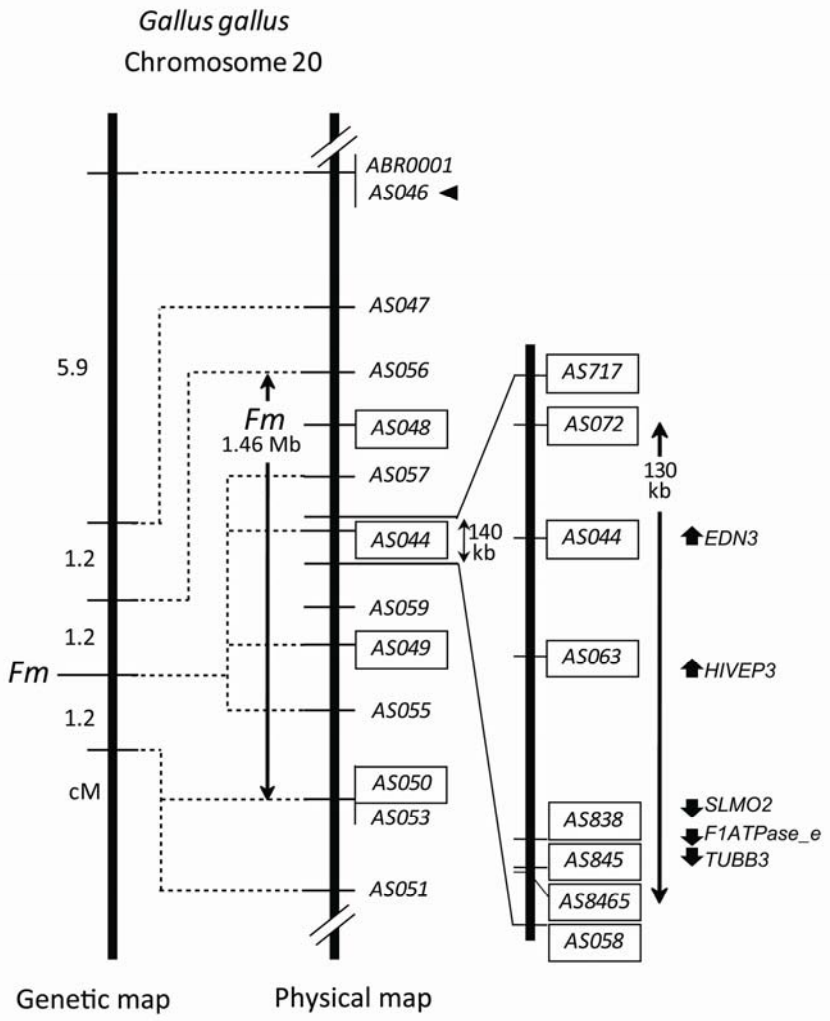

Figure 3 (Shinomiya et al.)

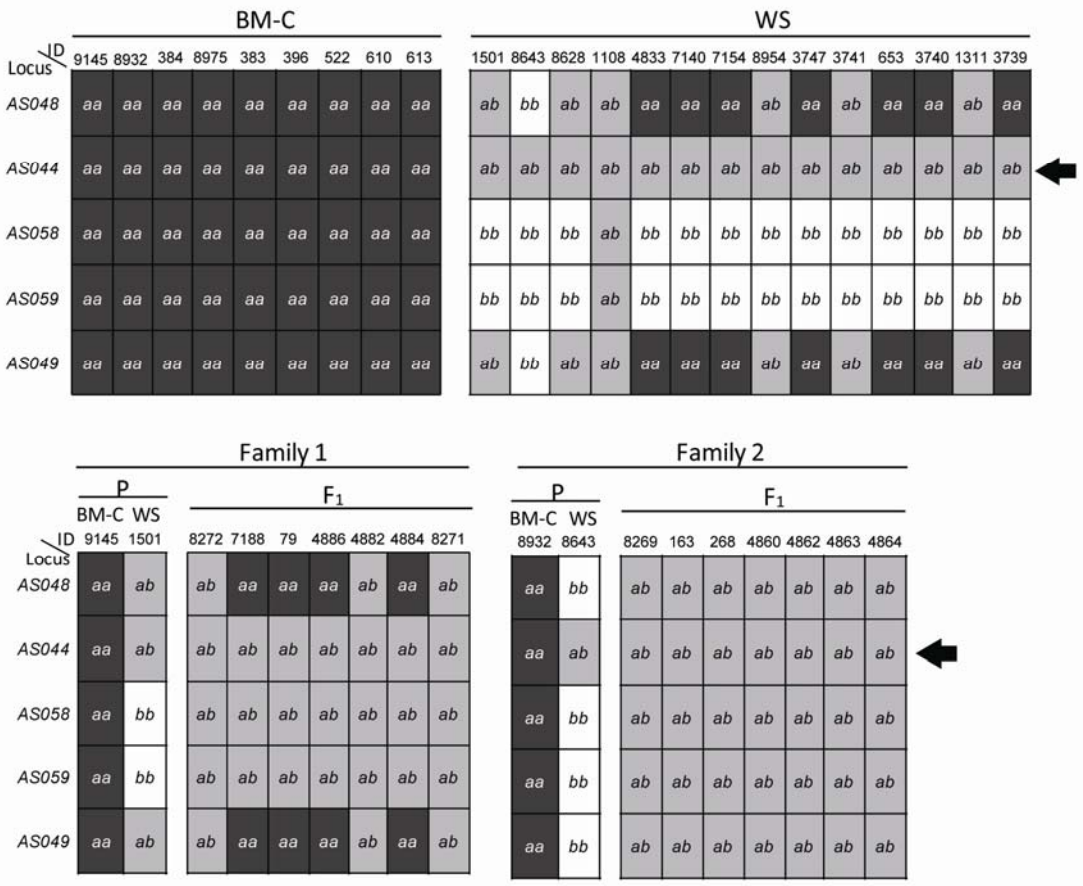

Figure 4 (Shinomiya et al.)

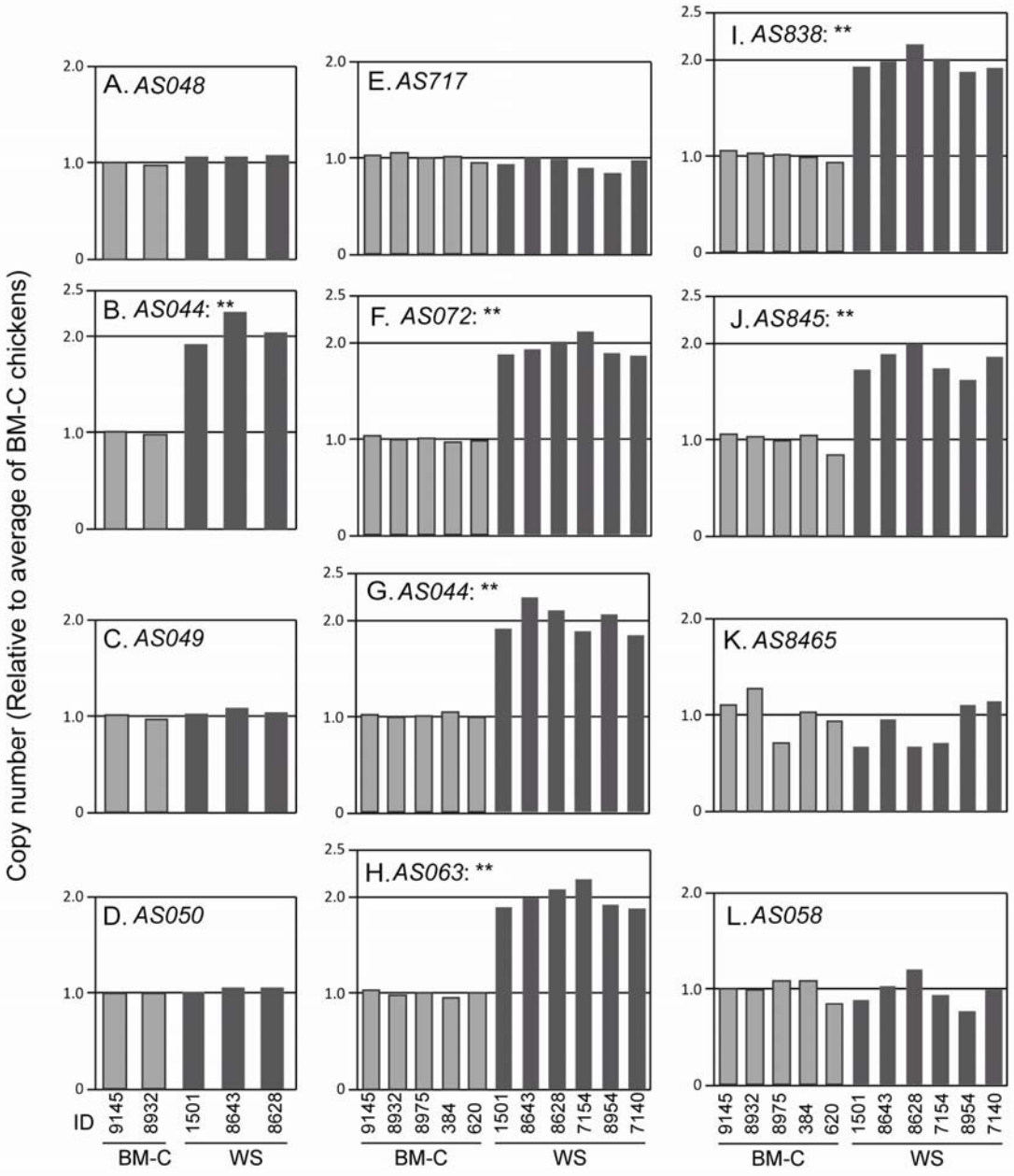

Figure 5 (Shinomiya et al.)

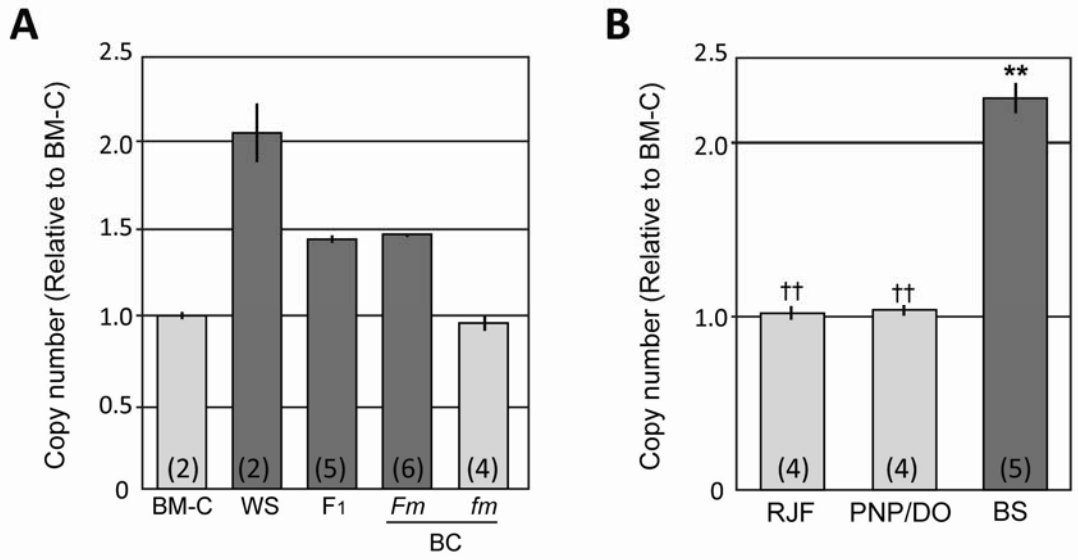

Figure 6 (Shinomiya et al.)

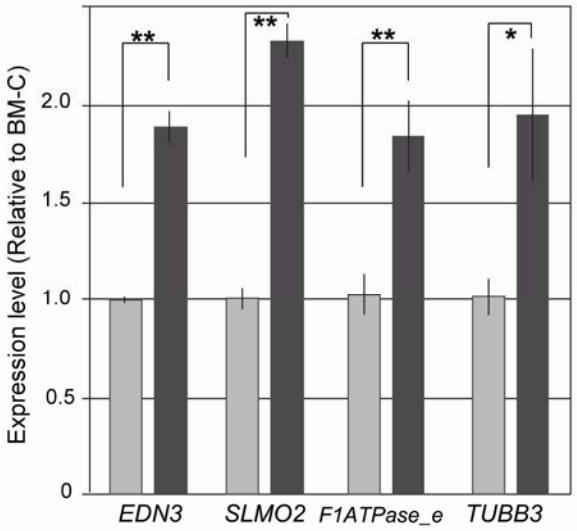

Figure 7 (Shinomiya et al.)