Gene Duplication of *endothelin 3* Is Closely Correlated with the Hyperpigmentation of the Internal Organs (*Fibromelanosis*) in Silky Chickens

Ai Shinomiya,* Yasunari Kayashima,*,[1] Keiji Kinoshita,† Makoto Mizutani,† Takao Namikawa,† Yoichi Matsuda,† and Toyoko Akiyama*,[2]

*Department of Biology, Keio University, Yokohama 223-8521, Japan, and †Avian Bioscience Research Center, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan

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 a dorsolateral route of the trunk region and differentiate to melanocytes. However, Silky is an exceptional chicken in which numerous melanoblasts travel via a 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 F_1 individuals between White Silky (WS) and BM-C *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 an ~130-kb interval. It contained five genes including *endothelin 3* (*EDN3*), which encoded a potent mitogen for melanoblasts/melanocytes. *EDN3* with another 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 resulting from the gene duplication in the *Fm* region is the trigger of hypermelanization in internal organs of Silky chickens.

MELANOCYTES are the main cell type, producing pigment that displays 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 the 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, which migrate through the ventral pathway, 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 earlobes, 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 the 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 black, which has led to the belief that it contains unknown physiologically useful substance(s) for Chinese cuisine. Many researchers have studied the specific Silky substance(s) but little has been

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[1]Present address: School of Food and Nutritional Science, Graduate School of Nutritional and Environmental Sciences, University of Shizuoka, Shizuoka 422-8526, Japan.
[2]Corresponding author: Department of Biology, Keio University, 4-1-1 Hiyoshi, Kohoku-ku, Yokohama 223-8521, Japan. E-mail: akiyama@hc.cc.keio.ac.jp

uncovered so far. The origin, history of *Fm*, other chicken breeds with *Fm*, and the molecular mechanism that induces the hyperpigmentation in Silky have not yet been completely clarified.

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, 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). However, 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 (Ortolani-Machado *et al.* 2009). Thus, there are no distinctive differences in the pigment particles and morphologies of melanosomes or 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 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 *Fibromelanosis* (*Fm*). Identification of the gene(s) that controls 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 the neural crest, those in lower vertebrates are often observed to travel through the ventral route (Collazo *et al.* 1993; Raible and Eisen 1994, 1996; Kelsh 2004; Akiyama *et al.* 2006a; Tomlinson *et al.* 2009; Reyes *et al.* 2010). Investigation of Silky chickens could also provide significant clues to recognize the evolutionary 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 that associated with *Id* and *Fm* at 72.3 Mb on chromosome Z and at 10.3–13.1 Mb on chromosome 20, respectively. However, the genomic regions of *Id* and *Fm* extend several megabases 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 2 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 as a *Y* value with a chronometer (CR-221, Minolta, Tokyo, Japan). 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, and RJF) were clearly different from the *Fm* lines (WS and BS) (Supporting Information, 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:

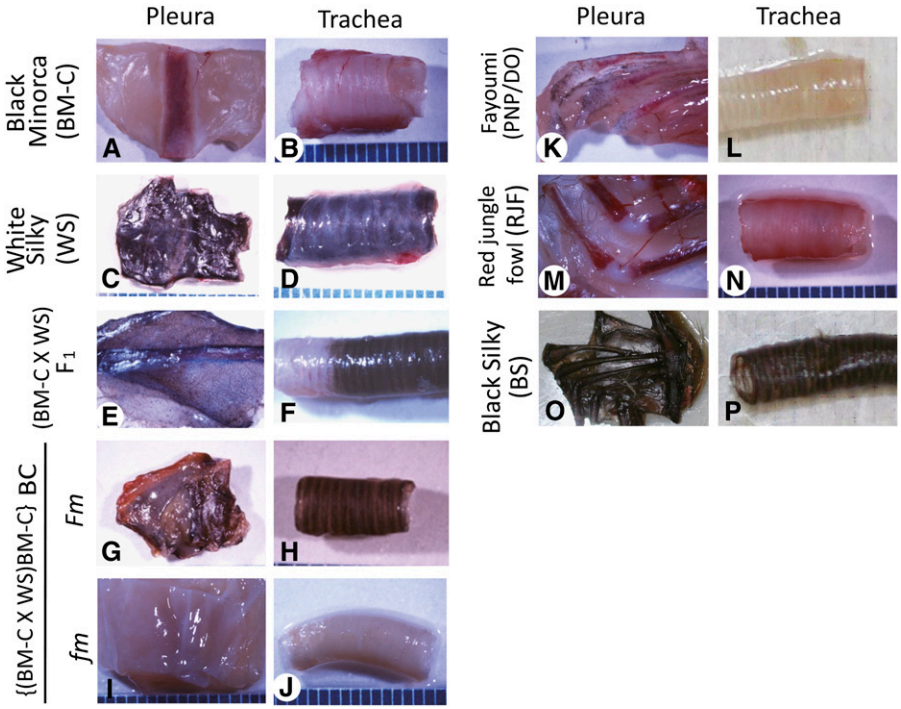

Figure 1 Pigmentation of pleura and trachea in several chicken lines. WS and BS have heavily pigmented internal organs whose colors are clearly different from those in the *fm* lines (BM-C, PNP/DO, and RJF). F_1 between BM-C and WS shows *Fm*, and BC progeny between the F_1 and BM-C are classified into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

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, 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°, and 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°, and 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 (RFLPs) 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- to 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). 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- to 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 is uncertain (Crawford 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. Therefore, 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 × WS and PNP/DO × 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 × WS) F_1 and (PNP/DO × WS) F_1, respectively. Because all F_1 individuals exhibited *Fm* and the ratio of *Fm* to *fm* in F_2 was almost 3:1, 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 were the id^+/id^+ (wild type) homozygotes. Since the BM-C line showed the least pigmentation in internal organs as described in *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). 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 (Figure 3 and Figure S2). *Fm* and adjacent markers were 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 >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 an 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, and 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 nondigested 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

Table 1 The *Fm* phenotype in backcross progeny used for *Fm* mapping

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

BC, backcross.

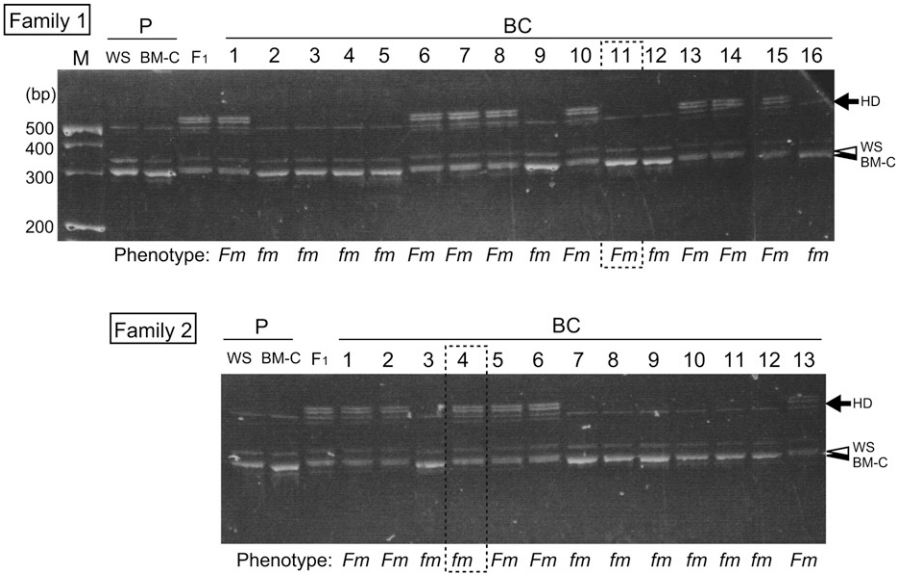

Figure 2 An example of linkage analysis using microsatellite markers on the BC progeny. Electrophoretic patterns of *ABR0001* PCR products in families 1 and 2 are shown. PCR products in WS (open arrowhead) are slightly longer than those in BM-C (solid arrowhead). Heterozygote-specific heteroduplex bands (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).

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 are 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, and D). However, the relative copy number in WS of the *AS044* locus was twice that in BM-C (Figure 5B). 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, and 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 6A). 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 6A). 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 6B). 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

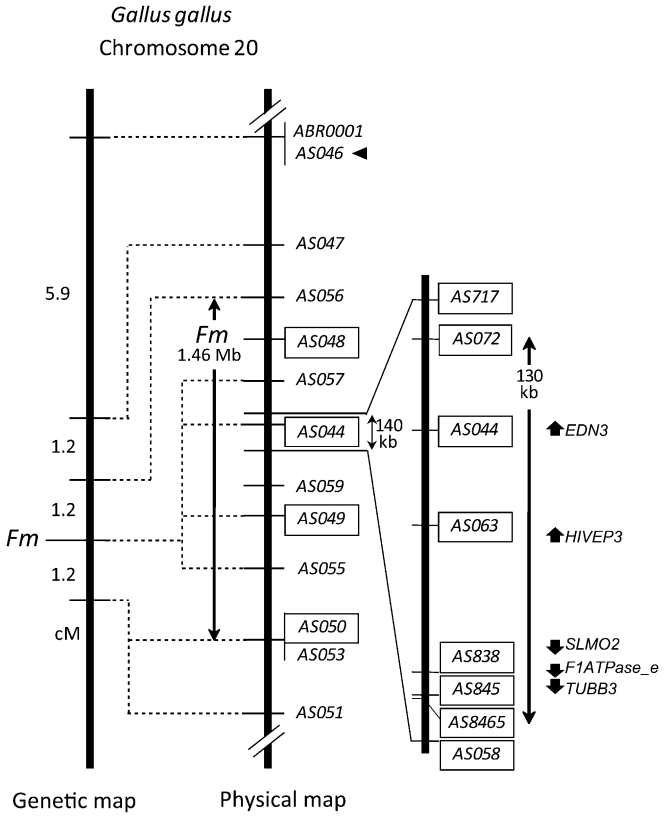

Figure 3 Genetic (left) and physical (right) maps of *Fm* on chicken chromosome 20. These maps were derived by our study and drawn with a reference of *Gallus gallus* draft genome sequence data (http://www.ncbi.nlm.nih.gov/sites/entrez?db=genomeprj&cmd=Retrieve&dopt=Overview&list_uids=10804). The 1.46-Mb *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 Table S1 and Table S2. Loci analyzed by qPCR were indicated by rectangles. A primer set for *AS046* (arrowhead) served as a reference for qPCR analyses. Abbreviations of gene names are indicated in *Results*. Arrows indicate the direction of the genes.

internal organs showed dark and wild-type color. By using one generation ($N = 11$) in this line, we examined the 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 on the basis of 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 hyperexpression 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 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; McCarroll *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 and Lupski 2010). CNVs are also observed in Aves both in inter- and intraspecies (Griffin *et al.* 2008; Skinner *et al.* 2009; Völker *et al.* 2010; Wang *et al.* 2010). In addition, an association between CNVs and

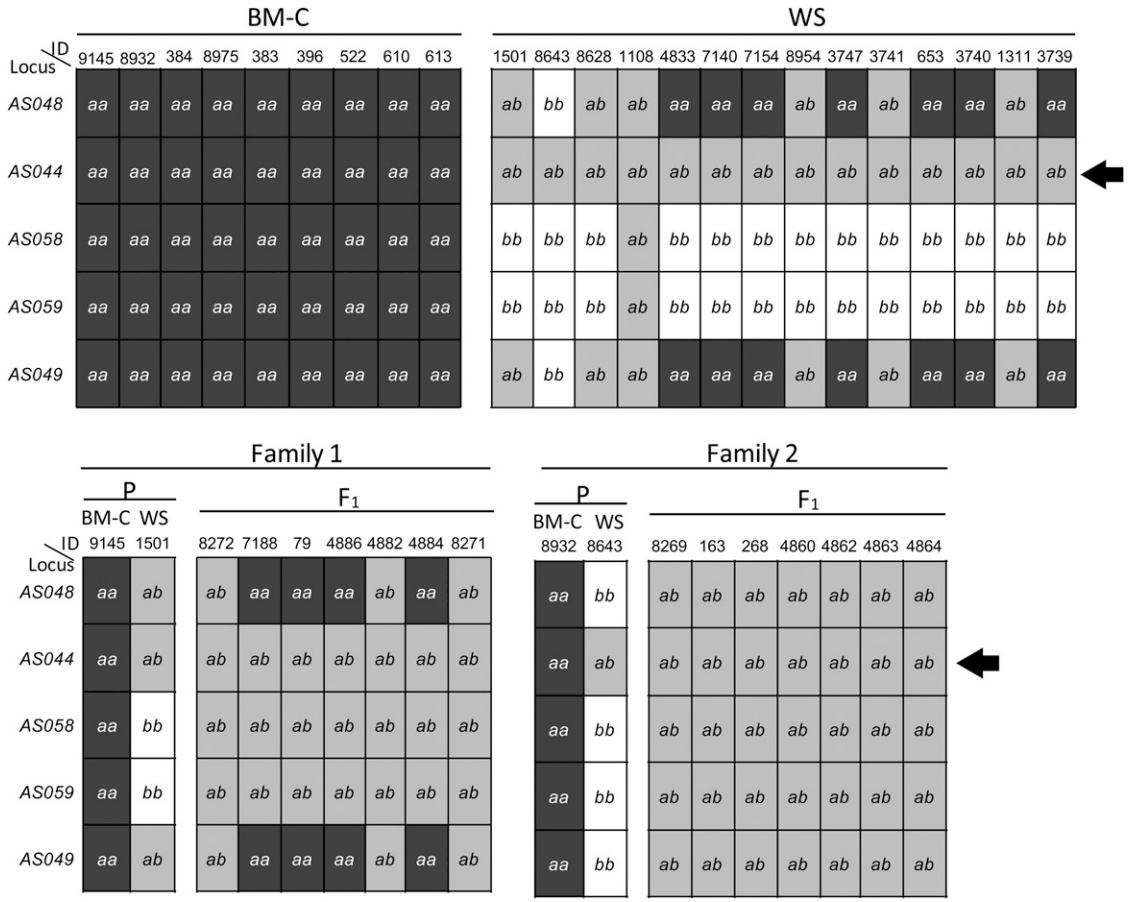

Figure 4 Genotyping data for five *Fm*-linked DNA markers in BM-C (n = 9), WS (n = 14), and F_1 (n = 14) between BM-C and WS in families 1 and 2. A haplotype pattern of PCR-RFLP is indicated as *a* or *b*. Individuals that showed the *a* or *b* band pattern are described as *aa* or *bb*, respectively, 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.

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 ~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 on the basis of 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 semidominant rather than a dominant trait. It corresponds well to the copy numbers of the duplicated area (Figure 6A). 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.

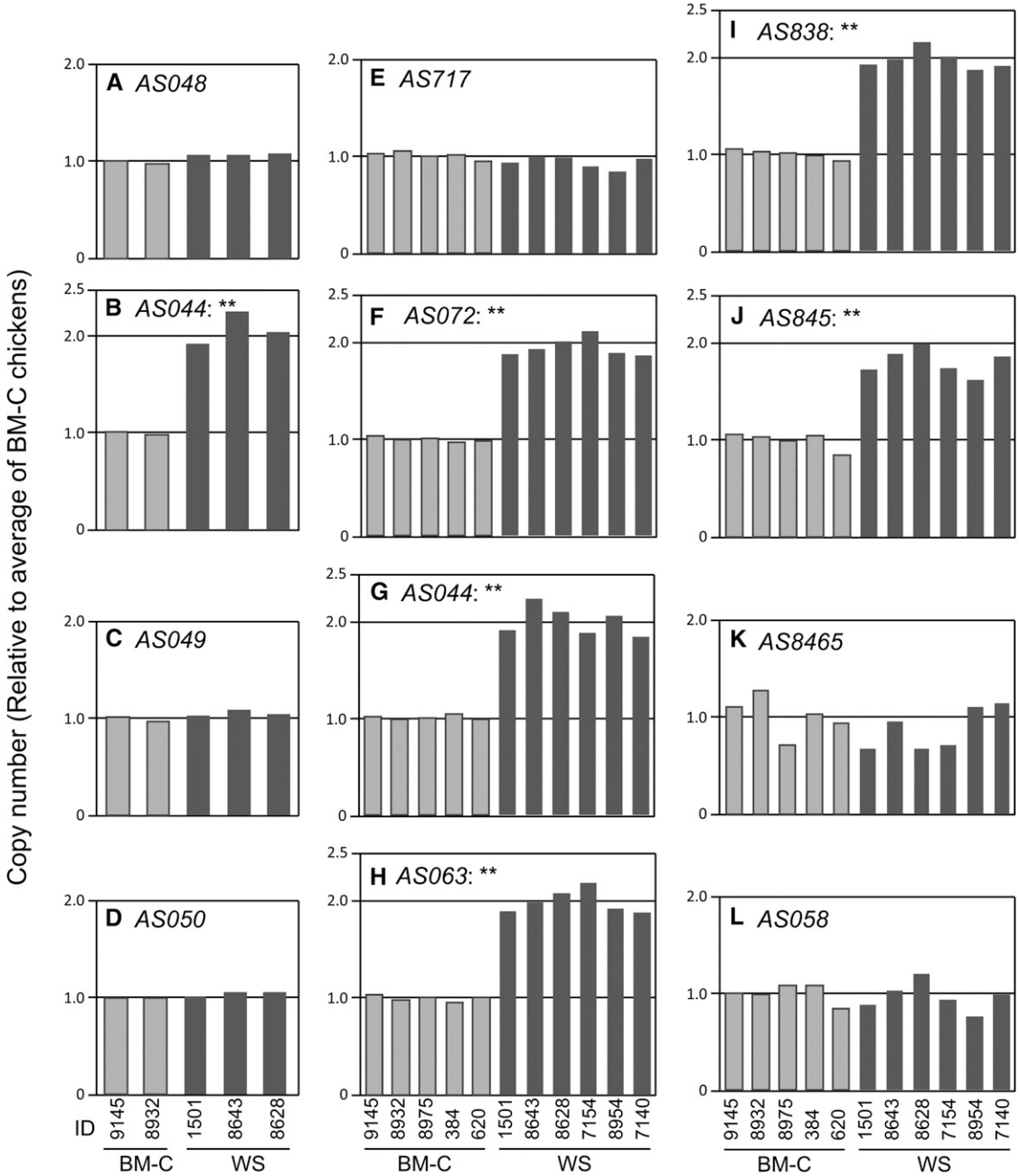

Figure 5 Quantitative PCR analysis of genomic copy numbers in the BM-C and WS lines. Copy numbers in BM-C (light-shaded columns) and WS (dark-shaded columns) individuals, relative to that for the average of BM-C chickens, were calculated by use of the comparative Ct method. Data were normalized to the reference locus (*AS046*). Double 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.

Of the four genes that displayed high mRNA expression, we propose that *EDN3*, in particular, is 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 *lethal spotting* (*ls*) and *piebald lethal* (s^l) 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

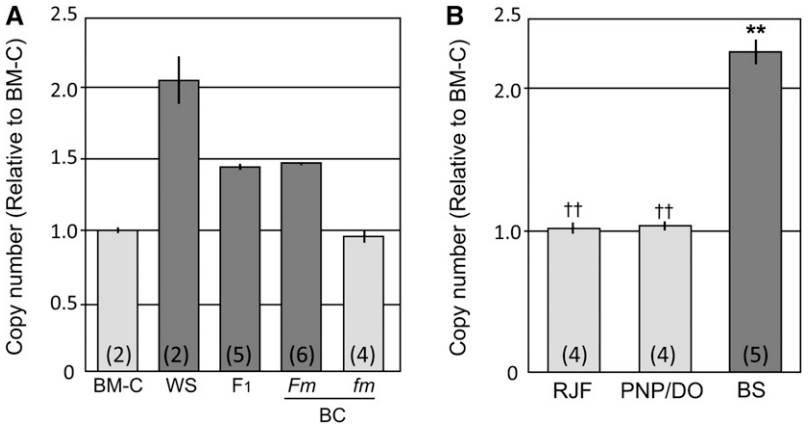

Figure 6 Quantitative PCR analyses of genomic copy numbers of the *AS044* locus in the families used for the linkage mapping and other breeds. Copy numbers relative to that for BM-C were calculated by using the comparative Ct method. Data were normalized to the reference locus (*AS046*). Light-shaded and dark-shaded 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 and PNP/DO) and *Fm* (BS) demonstrated equal and almost twofold of the copy number in BM-C, respectively (B). Double asterisks and daggers indicate a statistically significant difference for BM-C and WS lines, respectively ($P < 0.01$, by Student's *t*-test). Bar, ±SE.

and to abnormal cell migration (Lee *et al.* 2003; Pavan and Tilghman 1994). 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*. 2006b). 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 necessary 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

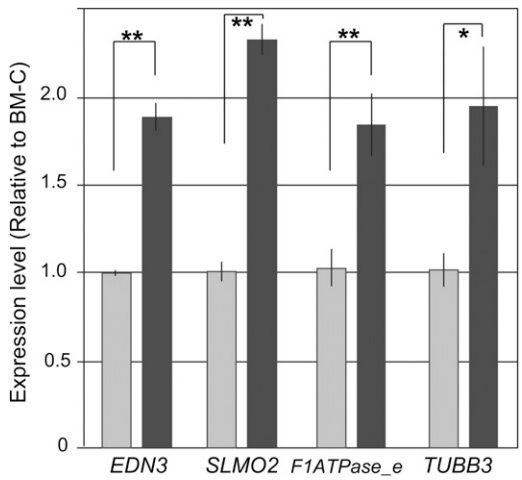

Figure 7 Quantitative PCR analysis of expression levels of genes located in the duplicated 130-kb area in whole embryos at stage 18 in BM-C (n = 5, light-shaded column) and WS (n = 7, dark-shaded column). Gene expression levels in WS relative to those in BM-C were obtained by using the comparative Ct 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, ±SE.

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 noncutaneous 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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Supporting Information
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Gene Duplication of *endothelin 3* Is Closely Correlated with the Hyperpigmentation of the Internal Organs (*Fibromelanosis*) in Silky Chickens

Ai Shinomiya, Yasunari Kayashima, Keiji Kinoshita, Makoto Mizutani, Takao Namikawa, Yoichi Matsuda, and Toyoko Akiyama

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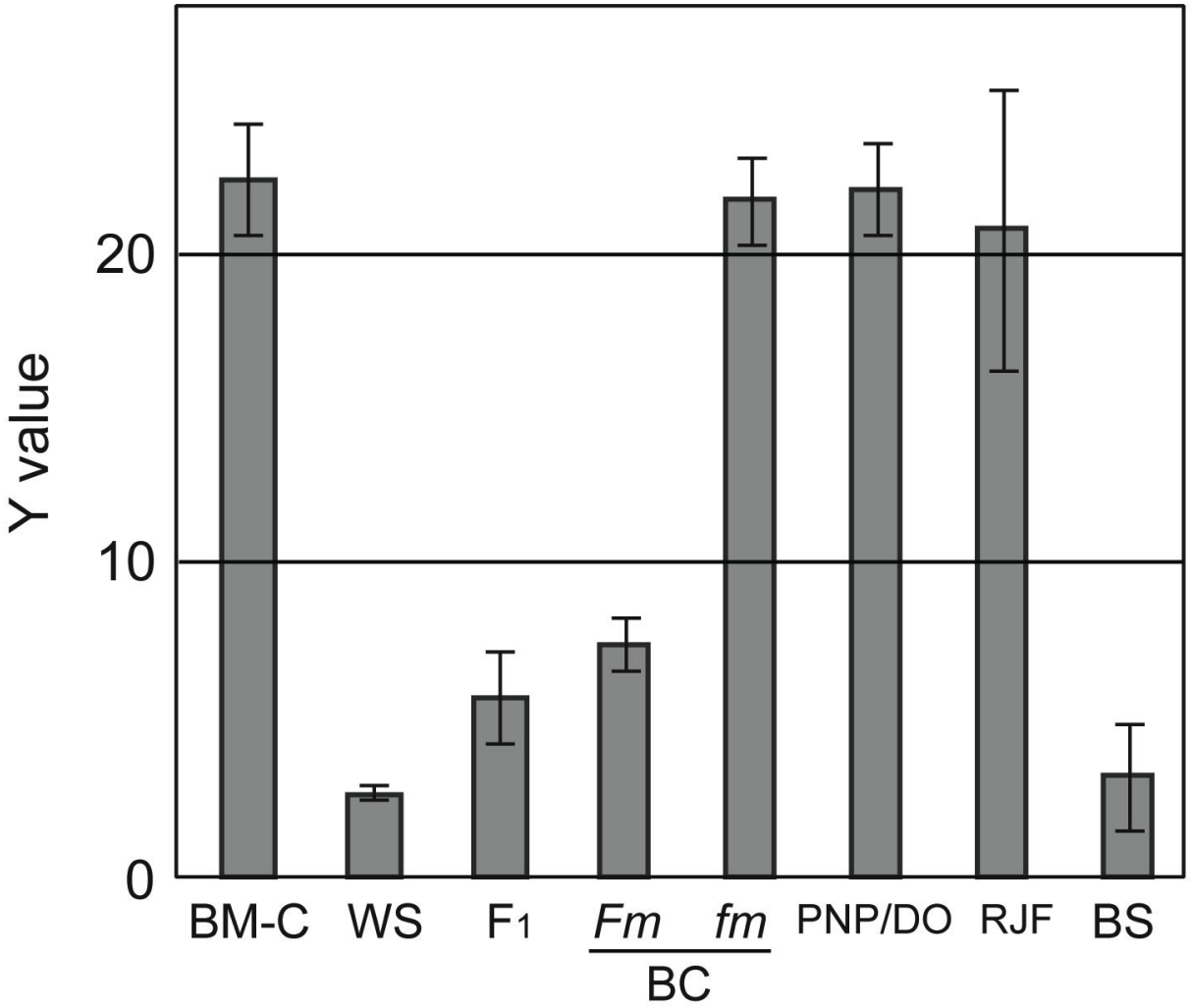

Figure S1 The degree of pigmentation revealed by measuring brightness with a chronometer (CR-221, Minolta, Tokyo, Japan). The Y value of the white board as a reference is 88.7. Chicken breeds with light-colored tissue (*fm*) show high values, whereas the values for Silky chickens with dark-colored tissue (*Fm*) are low. F_1 between BM-C and WS indicates an intermediate value, and a group of BC judged as the *Fm* phenotype exhibits a value close to that of F_1. The other group of BC that judged as the *fm* phenotype indicates a similar value seen in the *fm* lines. Individual numbers: BM-C, n = 2; WS, n = 6; F_1, n = 23; BC with dark color, n = 33; BC with bright color, n = 33; PNP/DO, n = 2; Red jungle fowl, n = 2; BS, n = 5. Bar, $\hat{B}$ (B standard deviation).

(1) Family 1

	BMC	WS		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Generation	F0	F0	F1	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC
Phenotype	*fm*	*Fm*	*Fm*	*Fm*	*fm*	*fm*	*fm*	*fm*	*Fm*	*Fm*	*Fm*	*fm*	*Fm*	*Fm*	*fm*	*Fm*	*Fm*	*Fm*	*fm*
Locus																			
ABR0001	1	2	3	3	1	1	1	1	3	3	3	1	3	1	1	3	3	3	1
AS046	1	2	3	3	1	1	1	1	3	3	3	1	3	1	1	3	3	3	1
AS047	1	2	3	3	1	1	1	1	3	3	3	1	3	3	1	3	3	3	1
AS044	1	3	3	3	1	1	1	1	3	3	3	1	3	3	1	3	3	3	1
AS049	1	3	3	3	1	1	1	1	3	3	3	1	3	3	1	3	3	3	1
AS050	1	2	3	3	1	1	1	1	3	3	3	1	3	3	1	3	3	3	1
AS051	1	2	3	3	1	1	1	1	3	3	3	1	3	3	1	3	3	3	1

(2) Family 2

	BMC	WS	3/W	1	2	3	4	5	6	7	8	9	10	11	12	13
Generation	F0	F0	F1	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC
Phenotype	*fm*	*Fm*	*Fm*	*Fm*	*Fm*	*fm*	*fm*	*Fm*	*Fm*	*fm*	*fm*	*fm*	*fm*	*fm*	*fm*	*Fm*
Locus																
ABR0001	1	2	3	3	3	1	3	3	3	1	1	1	1	1	1	3
AS046	1	2	3	3	3	1	3	3	3	1	1	1	1	1	1	3
AS047	1	3	3	3	3	1	3	3	3	1	1	1	1	1	1	3
AS056	1	2	3	3	3	1	1	3	3	0	0	0	0	0	0	0
AS048	1	2	3	3	3	1	1	3	3	1	1	1	1	1	1	3
AS044	1	3	3	3	3	1	1	3	3	1	1	1	1	1	1	3
AS049	1	2	3	3	3	1	1	3	3	1	1	1	1	1	1	3
AS055	1	3	3	3	3	1	1	3	3	1	1	1	1	1	1	3
AS053	1	2	3	3	1	1	1	3	3	1	1	1	1	1	1	3
AS051	1	2	3	3	1	1	1	3	3	1	1	1	1	1	1	3

(3) Family 3

	BMC	WS	3/W	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Generation	F0	F0	F1	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC
Phenotype	*fm*	*Fm*	*Fm*	*Fm*	*Fm*	*fm*	*Fm*	*fm*	*fm*	*Fm*	*fm*	*Fm*	*Fm*	*fm*	*fm*	*Fm*	*Fm*	*fm*	*Fm*	*fm*	*fm*	*Fm*	*fm*	*Fm*	*fm*	*Fm*	*Fm*	*fm*	*fm*	*fm*	*Fm*
Locus																															
AS046	1	2	3	3	1	1	3	1	1	3	3	3	3	1	1	1	3	1	3	1	1	3	1	3	1	3	3	1	1	3	3
AS047	1	3	3	3	1	1	3	1	1	3	1	3	3	1	0	3	3	1	3	1	1	3	1	3	1	3	3	1	1	1	3
AS056	1	2	3	3	1	1	3	1	1	3	1	3	3	1	1	3	3	1	3	1	1	3	1	3	1	3	0	1	1	1	3
AS057	1	3	3	3	3	1	3	1	1	3	1	3	3	1	1	3	3	1	0	1	1	3	1	0	0	0	3	1	0	1	0
AS044	1	3	3	3	3	1	3	1	1	3	1	3	3	1	1	3	3	1	3	1	1	3	1	3	1	3	3	1	1	1	3
AS049	1	2	3	3	3	1	3	1	1	3	1	3	3	1	1	3	3	1	3	1	1	3	1	3	1	3	3	1	1	1	3
AS051	1	2	3	3	3	1	3	1	1	3	1	3	3	1	1	3	3	1	3	1	1	3	1	3	1	3	3	1	1	1	3

	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56
Generation	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC
Phenotype	*Fm*	*Fm*	*fm*	*fm*	*fm*	*Fm*	*Fm*	*Fm*	*fm*	*Fm*	*fm*	*Fm*	*Fm*	*fm*	*fm*	*fm*	*fm*	*Fm*	*Fm*	*Fm*	*fm*	*fm*	*Fm*	*fm*	*Fm*	*Fm*	*Fm*	*Fm*
Locus																												
AS046	3	3	1	1	1	3	1	3	1	3	1	3	3	1	1	1	1	3	3	3	1	1	3	1	3	3	3	3
AS047	3	3	1	1	1	3	3	3	1	3	1	3	3	1	1	1	1	3	3	3	1	1	3	1	3	3	3	3
AS056	3	3	1	1	1	3	3	3	1	3	1	3	3	0	1	1	1	3	3	3	1	1	0	1	3	3	3	0
AS057	3	3	1	1	0	3	3	3	1	3	1	0	3	1	1	1	1	3	0	0	0	0	0	0	0	0	0	0
AS044	3	3	1	1	1	3	3	3	1	3	1	3	3	1	1	1	1	3	3	3	1	1	3	1	3	3	3	3
AS049	3	3	1	1	1	3	3	3	1	3	1	3	3	1	1	1	1	3	3	3	1	1	3	1	3	3	3	3
AS051	3	3	1	1	1	3	3	3	1	3	1	3	3	1	1	1	1	3	3	3	1	1	3	1	3	3	3	3

Figure S2 Genotyping data in 85 backcross progeny. 1, BM-C homozygous type; 2, WS homozygous type, 3; heterozygous type, 0; missing data.

Table S1 List of *Fm*-linked markers, primer sequences and positions, and types of polymorphism between Black Minorca and White Silky chickens

Locus	Primer	Forward primer (5'-3')	Reverse primer (5'-3')	Position[a]	Fragment length	Polymorphism
ABR0001[b]	ABR0001	ACGTCCATCCAGCTCTGCTC	ACTCCATCCAAGTGCCTATTTCT	9439859 - 9440160	302	MLP[c]
AS044	AS044_1	CCCAGCCTTCATTTCGGTGC	CCCTCCAAGCTCTGCTACTG	10753918 - 10752800	1119	MspI
AS046	AS046_1	CGGGAAGAAGTACAAATCCAAGGC	GCTTCACCTCTGGCTCCTCTGG	9457096 - 9457883	788	HinfI
AS047	AS047_1	CATTGACTTTGGGCTGGCTCG	CGTATTTCTTGAGCATCACCTGG	10009721 - 10010768	1048	MboI or HinfI
AS048	AS048_1	CTGAGAGGGCAGCAGAGGACG	CTCTCCATCAATCTTCTTATCAGC	10396755 - 10395542	1214	HhaI
AS049	AS049_1	CCGACGGAAAATGATAAGCC	CCTCTGAACTTCTACTTGAAGCC	11140132 - 11138848	1285	RsaI
AS050	AS050_1	GATCATCGCTCCAGAGGGC	GTTTCGGCACAGTCTCTGGG	11682221 - 11683585	1365	DdeI
AS051	AS051_1	CGCCATCTGAACAACTGCTG	GCACTGTCATCATTCTCCATCC	11980807 - 11979485	1323	DdeI or RsaI
AS053	AS053_1	CAGGAGGGAAGCAGAGGAGCC	CGTGCTTCTTGCAGGCTTGGC	11679789 - 11681581	1793	HaeIII
AS055	AS055_1	GCACTGTGTTCCATGTAAGGG	GGGATGTGGGCATGGTCATCTG	11410080 - 11411181	1102	MspI
AS056	AS056_1	CATGGACATGCTCTTCCCAGG	GGGTTTGTTCACCACCGGAGC	10217316 - 10218429	1114	HinfI or MspI
AS057	AS057_1	GCAGGGCTTTGGCTTTGTGGC	CCCTTTCACCTTCACCACGAGC	10569168 - 10570175	1008	MspI or HaeIII
AS058	AS058_1	GGAGGTGTTTTCCCGAGTTCTGCG	CGTGTGCTCCCCATGGCATACC	10859829 - 10858472	1358	DdeI
AS059	AS059_1	CCCGCTCTACCTGAACTACGCC	GGAGCGATGCGTGAACAACGGG	11025852 - 11024528	1325	MspI

aThe Position of Microsatellite or STS marker is based on 2006 whole chicken genome assembly (http://www.ncbi.nlm.nih.gov/genomeprj/13342).

bACCESSION number: AB186519

cMicrosatellite length
polymorphism

Table S2 List of sequences and positions of primers used for quantification of gene copy numbers among chicken strains

Locus	Primer	Forward primer (5'-3')	Reverse primer (5'-3')	Position[a]	Fragment length
AS044	AS044_2	TTCCATCCCAGTCTCTTGCC	TATTGCTGACTGGTGCTTGC	10753352 - 10753251	102
AS046	AS046_2	GTTCTGTTGATCCCTGCTGG	CTCCATACTTTCACCCTCCTC	9457542 - 9457638	97
AS048	AS048_2	CTTGTTCATGTTGCTGCCTGC	TCAGCAGAGAGACACAGCAC	10396540 - 10396405	136
AS049	AS049_2	GTCTTTGTTGCAGTTGTGGATACG	CCTCCTGAGTATCAGACGTGGTG	11138951 - 11139060	110
AS050	AS050_2	AGGAAGGTCTGCATTAGCTCAC	GCAAAGACAGGGTAATTCCTCG	11682418 - 11682521	104
AS058	AS058_2	TTACTTTCGGATCTGCCCTC	GCCAAGGAACTGATTAGACTG	10858974 - 10858886	89
AS063	AS063_2	CTTCAGCTCGATCTTCTGCTC	GCAAGCCAGGAAAAAGTACC	10793958 - 10794060	103
AS072	AS072_2	GAACAGTTACACAAGACTATTGCC	CAAATACCATCCTTCACCCTCTAC	10729667 - 10729759	93
AS717	AS717_2	GAGGGAACTGATGTGTTATGTGG	CTTCCTTCCTCTACATATTGCAGCCC	10716886 - 10717000	114
AS838	AS838_2	GCTACATCCGGTACTCCCAG	CCCGCTACTCCTTCTTGGTC	10838285 - 10838413	129
AS845	AS845_2	CCAGTGACTAACTTGCACAGAGGC	CCTGGGAAATTCTGATGCTACACC	10845060 - 10845179	120
AS8465	AS8465_2	GAAACCTCAAGCCTGTTAGCTGC	CAGAGCCAACCCTTCCACCACATC	10846361 - 10846486	126

aThe location of the fragment is based on 2006 whole chicken genome assembly (http://www.ncbi.nlm.nih.gov/genomeprj/13342).

Table S3 List of primer sequences for quantification of expression levels of duplicated genes

Gene symbol	Forward primer (5'-3')	Reverse primer (5'-3')	Fragment length
GAPDH[a]	CAGAACATCATCCCAGCGTCCAC	CAACAGAGACATTGGGGGTTGGC	120
endothelin 3	CATGACAAACAGTGTACGCAG	CTGCATCGGTCCTTCTCTGTTG	92
HIVEP1	CTCAGAAATCCCGCAGGTGAAGC	CAGACAAACACACGGGTGGCAAC	83
slowmo homolog2	GCAAACACGATTTCCTCCAACGC	CCTCTTGCTGAAGCTGTGAACTCC	105
H+ transporting F1 ATP synthase epsilon subunit	GAGCAGCTCAGGTCTCTTCTGGGC	GACAGGTTGTGGGCCCATCAGTG	106
tubulin beta 3	CCCTTAGCCCAGTTGTTTCCAGC	GCCTGGTACAATGGACAGTGTACG	109

[a] *glyceraldehyde-3-phosphate dehydrogenase (GAPDH*) was used as a reference gene.