Genetic analysis of the hooded phenotype in the rat

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Abbreviations

BN	Brown-Norway
c DNA	complementary DNA
CI	confidence interval
Chr	chromosome
cM	centi morgan
F (primer)	forward (primer)
h	hooded
h^e	hooded extreme
h^i	hooded irish
h^n	hooded notched
H^{re}	hooded restricted
HE	hematoxylin and eosin
Hm	hooded modifier
Kbp	kilo base pairs
Kit	c-kit tyrosine kinase receptor
LRS	likelihood ratio statistics
Mbp	mega base pairs

NCBI	National Center for Biotechnology Information
PCR	polymerase chain reaction
QTL	quantitative trait loci
R (primer)	reverse (primer)
RT	reverse transcription
TRP2	tyrosinase-related protein 2

Contents

Preface

The genetic studies of the coat color phenotype in rodents have been performed for more than 100 years because of its visible and attractive phenotype. At present, more than 150 loci with nearly 1,000 different alleles are known to affect coat color in the mouse (Steingrimsson et al., 2006; Yamaguchi et al., 2009).

The hooded phenotype is one of the coat color phenotypes, classified as white spotting phenotype, seen peculiarly in the laboratory rat. The typical hooded phenotype shows pigmented hairs covering only head and mid-dorsal regions and non-pigmentation in the rest of body surface. However, it has been reported that the hooded phenotype shows diversity and is categorized from grade 1 to 8 by the extent of non-pigmented coat area (Robinson, 1965). According to this category, most of the body is covered with pigmented hairs in grade1. In grade from 2 to 4, nom-pigmented area is increasing from the abdominal to side with an increase in the grade. Grade 5 is typical hooded phenotype with colored head and shoulders with a thin unbroken stripe of pigmented hairs extending from the head to tail along by mid-dorsal line. In higher grades than 5, the stripe is broken up and the pigmented area becomes to be confined to the head region with an increase in the grade. Furthermore, the

hooded locus showing autosomal recessive inheritance has been mapped on chromosome (Chr) 14 in previous investigations. However, until now, a gene responsible for the hooded locus is not identified.

Generally, the extent of the coat color pigmentation depends on the amount of melanin produced by melanocytes derived from neural crest cells, which arise during gastrulation of embryogenesis at the dorsal neural tube (Donoghue et al., 2008; Thomas et al., 2008). After delamination from neural tube, these neural crest cells migrate to the specific sites where they differentiate into a wide range of lineages including peripheral neurons, endocrine cells, bones, cartilages, connective tissues, and melanocytes (Anderson, 2000). Although the mechanisms by which multi-potent neural crest cells are specified into melanocyte lineage remain obscure, it has been suggested that immature precursors for melanocytes, referred to as melanoblasts, are originated from bi-potent glial-melanoblast progenitors (Dupin et al., 2000; Mollaaghababa and Pavan, 2003). Recent studies suggest that the fate decision of these bi-potent progenitors into melanoblasts is controlled by, at least in part, Wnt signaling pathway (Dunn et al., 2000). Indeed, it has been reported that several neural crest derivatives, including melanoblasts, are markedly reduced in *Wnt1*$^{-/-}$*;Wnt3a*$^{-/-}$ double knockout mice (Ikeya et al., 1997).

Dysregulation in melanoblast development typically exhibits hypopigmentation phenotypes, which is readily discriminated in animals as piebaldism, namely congenital unpigmented spotting in the skin and hairs (Bennett and Lamoreux, 2003). These spotting mutants tend to exhibit characteristic spotting patterns such as belly spots, head spots and piebald spotting (Baxter et al., 2004). Although the mechanisms by which these characteristic patterns occur during development are unclear, recent studies have provided some mechanistic insights into the formation of these spotting phenotypes. It has been demonstrated that melanoblasts exist at higher density in head, cervical, and tail regions, whereas much lower in the trunk region (Wilkie, et al., 2002). This distribution pattern is well correlated with pigmented and unpigmented patterns in the mutant animals (Yoshida et al., 1996). Therefore, based on these observations, it is proposed that the piebald phenotypes are caused by remarkable reduction of the total number of melanoblasts, rather than selectively affecting melanoblast precursor cells responsible for populating a given area (Baxter et al., 2004). On the other hand, the belly and head spotting phenotypes would be explained by failure of melanoblast migration, because melanoblasts have to migrate relatively longer distance to reach belly or head areas from the areas where melanoblast density is high (Baxter et al., 2004).

Through the extensive studies of the white spotting mutant animals, a number of key genes for melanoblast development have already been identified and characterized. Instance of these genes include *Pax3* (*Paired box 3*), *Sox10* (*Sex-determining region Y-box 10*), *Mitf* (*Microphthalmia-associated transcription factor*), *Edn3* (*endothelin 3*), *Ednrb* (*endothelin receptor B*), *Kit* (*c-Kit tyrosine kinase receptor*), *Kitl* (*Kit ligand, also called SCF or steel factor*), and *Snail2* (*also called Slug*) (Bennett and Lamoreux, 2003).

In part 1, the attempt to identify a gene responsible for the hooded locus was conducted by performing the high-resolution linkage mapping. In part 2, studies were conducted to unveil genetic mechanisms for modifying the extent of hooded phenotype by quantitative trait loci (QTL) analysis.

Part1

High-resolution linkage mapping of the rat hooded locus

Introduction

The hooded phenotype is one of the coat color phenotypes, which are peculiarly seen in laboratory rats. Typically, the hooded phenotype shows non-pigmented coat except for the head and shoulders and a mid-dorsal stripe. The hooded locus has been reported to show linkage to plasma protein markers, Gl-1, Gc protein, and albumin loci, all of which are now mapped to Chr 14 (Moutier et al., 1973; Syumiya and Nagase, 1982; Syumiya and Nagase, 1988). Therefore, the hooded locus is believed to locate on Chr 14. However, a gene responsible for the hooded locus is not yet identified.

Pigmentation of the hair is achieved with melanin granules secreted by melanocytes in the skin. Melanocytes are derived from neural crest cells and migrate through the skin in dorsal and caudal directions during embryogenesis. There are many rodent mutants deficient in neural crest cell migration (Potterf et al., 2000; Stanchina et al., 2006; Tachibana et al., 2003), all of which show non-pigmentation of the hair with loss of enteric ganglions and

deafness, known as Waardenburg syndrome. However, hooded phenotype is unique from the point showing non-pigmented hairs with normal development of enteric ganglions and auditory functions. Therefore, studying the mechanism of generation of the hooded phenotype may provide new insight into the understanding of the mechanism of migration of neural crest cells and development of melanocytes.

Materials and Methods

Animals

IS and LEA rats were provided from the National Bio Resource Project for Rat. F_1 progenies were obtained from female LEA rats mated with male IS rats. Backcrossed progenies were produced by mating female LEA rats with male F_1 rats. Animals were maintained in specific pathogen-free conditions with feeding and drinking *ad libitum*. In the experimental animal care and handling, the investigators adhered to the Regulation for the Care and Use of Laboratory Animals, Hokkaido University. Animal experimentation protocol was approved by President of Hokkaido University through the judgment by Institutional Animal Care and Use Committee of Hokkaido University.

Histological and immunohistological analyses

Dorsal and abdominal skins were sampled from the neck and the vicinity of navel, respectively, from both adult and neonatal rats. Formalin-fixed, paraffin-embedded skin samples were used for hematoxylin and eosin (HE) staining and Schmorl's staining according to the standard method. Paraformaldehyde-fixed, paraffin-embedded skin samples were used for immunohistochemical staining with anti-c-Kit antibody (sc-168, 1:100 dilution; Santa Cruz Biotechnology, CA).

Linkage analysis

Extraction of genomic DNA from tail clips was performed according to the standard method. Linkage analysis was performed using microsatellite markers *D14Rat84* [National Center for Biotechnology Information (NCBI), UniSTS:226657], *D14Got40* (UniSTS:115468) and original microsatellite marker designated as *D14Hok1* [forward (F) primer: ggtaatgtctcctccaagcagg and reverse (R) primer: gactcaaatgagtttcccaagg].

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from tissues using TRIzol reagent (Invitrogen, Carlsbad, CA)

following the manufacture's protocol. Complimentary (c) DNAs were synthesized by the RT reaction using the Rever Tra Ace reverse transcriptase (Toyobo, Osaka, Japan) and random primers (Toyobo). RT-PCR was performed using specific primers as follows: Kit1 F and R, ctgtagcagagagaggagctcag (nt. 2-24) and tgagggttcaactttatccacat (nt. 777-799).

Nucleotide sequence of the Kit cDNA

Total RNA was extracted from adult dorsal skin using TRIzol reagent and cDNAs were synthesized as described above. RT-PCR was performed using 6 specific primer sets designed that both ends of each PCR product were overlapped to amplify entire coding region of the *Kit* gene. The sequences of the primers are as follows: Kit1 F and R as described above; Kit2 F and R, acggacctgaagttcgtcccc (nt. 516-536) and ggaagatgttgatgaatccc (nt. 981-999); Kit3 F and R, aggggacacatttacggtga (nt. 719-738) and tatggaactctgaaccaccagtt (nt. 1462-1484); Kit4 F and R, ggagcccacaatagattggtatt (nt. 1351-1373) and aggaggttgacgatattcatgtg (nt. 1998-2020); Kit5 F and R, gtagttgaggccactgcctatg (nt. 1857-1878) and gtgcatttccttttaccacgta (nt. 2514-2535); Kit6 F and R, agatttgctgagcttttcctacc (nt. 2348-2370) and gtgctcataaaaggcagaatcac (nt. 3118-3140). PCR was performed with BigDye terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA). Samples were sequenced by

an outsourcing supplier (FASMAC, Atsugi, Japan).

Quantitative real-time PCR analysis

Purified RNAs were treated with DNase (Ambion, Foster, CA) for DNA digestion and cDNAs were synthesized by the same method as RT-PCR. Quantitative real-time PCR analysis was performed using Brilliant SYBR Green QPCR Master Mix and real-time thermal cycler, MX 3000 (Stratagene, La Jolla, CA) according to the manufacture's instructions. The mRNA expression levels of target genes were normalized to the expression of beta-actin (*Actb*). The sequences of the primers used for each gene are as follows: *Kit* F and R, cgcagcttccttatgaccac (nt. 1771-1790) and tgagcatcttcacggcaac (nt. 1908-1926); *Trp2* (tyrosinase-related protein 2) F and R, ccaattgcagcgtgtatgac (nt. 645-664) and aatggtacctgtgccatgtg (nt. 766-785); *Actb* F and R, ctaaggccaaccgtgaaaagat (nt. 413-434) and tggtacgaccagaggcatacag (nt. 502-523).

Statistical analysis

Statistical analysis was performed using non-parametric statistical methods. The Mann-Whitney test was used to compare two groups. The Kruskal-Wallis test was used for

multiple comparisons. These values were represented by means ± SE and difference with $p < 0.05$ was considered to be significant.

Results

Hair pigmentation in IS and LEA rats

To perform genetic analysis of hooded phenotype, I used IS, which is an inbred strain derived from Japanese feral rats and shows non-hooded phenotype, and LEA, which is an inbred strain possessing hooded allele with pigmented hairs. As shown in Figure 1A, IS rats are covered with pigmented hairs in whole body. Abdominal hairs of IS rats appear to be non-pigmented. Thus, the tips of abdominal hairs are less pigmented, whereas bases of hairs are substantially pigmented, of which phenotype appears due to the agouti phenotype, different from the hooded phenotype (Figure 1A, right-upper inset). On the other hand, LEA rats were covered with pigmented hairs in the dorsal skin and part of the ventral skin from mouth to forelimb regions (Figure 1A). Although LEA rats also have the agouti gene, the abdominal hairs around navel are completely non-pigmented (Figure 1A, right-lower inset). Next, I confirmed the difference in hair pigmentation histologically. Melanin was detected in

dorsal hairs but not in abdominal hairs of adult LEA rats (Figure 1B), whereas melanin was detected in both dorsal and abdominal hairs of adult IS rats (Figure 1B) and abdominal pigmented hairs around forelimbs of LEA rats (data not shown). To confirm the melanin-less phenotype of hooded rats at the neonatal period, I examined the generation of melanin by HE staining. I could detect melanin from neonatal day 4 in IS whole body surface and LEA dorsal skins; however, could not in LEA abdominal skins in any postnatal day stages (Figure 1 C and D).

Identification of a gene responsible for the hooded locus by fine mapping

To identify a gene responsible for the hooded locus, I created a high-resolution genetic map surrounding the hooded locus using 795 backcrossed rats determined their phenotypes of homozygotes (*h*/*h*) or heterozygotes (*H*/*h*) by their pigmented hair patterns. None of the rat among 795 backcrossed progenies showed recombination between hooded/non-hooded phenotype and genotype of a marker, *D14Hok1*, indicating that the hooded locus locates very close to the *D14Hok1* locus (Figure 2A). As shown in Figure 2B, some genes were found to locate in the vicinity of the *D14Hok1*. Among them, only two genes, *Kit* and *LOC679908,* were disclosed to locate between *D14Rat84* and *D14Got40* in NCBI Map Viewer database,

RGSC v3.4. Because *LOC679908* is a pseudogene, it is likely that the *Kit* is a strong candidate gene responsible for the hooded locus. When compared chromosomal regions showing homologous synteny among rat, mouse, and human, 4 predicted genes are shown to locate in this homologous region of the mouse genome based on the NCBI Map Viewer database, Build 37.1 (data not shown). However, little is known about the function of these genes. There is no gene found in this homologous region in human genome based on the NCBI Map Viewer database, Build 37.2 (data not shown). Thus, I examined the expression of *Kit* gene in the adult dorsal skin of both IS and LEA rats with RT-PCR. However, no quantitative and qualitative differences were observed (data not shown). I next compared nucleotide sequences of the *Kit* gene between IS and LEA rats and identified a G to C transversion in exon 2, which was, however, synonymous substitution (Figure 2B).

Quantitative analysis of the expression of Kit mRNA

I next examined the expression level of *Kit* mRNA, because it was considered that disorder of the *Kit* gene expression in developing stages might cause hooded phenotype. I first examined the expression of *Kit* mRNA in tissues containing neural tubes at embryonic day 10.5, when melanoblasts start delaminating from neural tube and migrating into dorsolateral

pathway in the rat. Although the expression of *Kit* mRNA in LEA rats tended to be lower than that of IS rats, there was no significant difference between them (Figure 3A). I then examined expression of *Kit* mRNA in the skin of postnatal rats. As shown in Figure 3B upper panels, there was no difference in the expression of *Kit* mRNA at both dorsal and abdominal skins. This result raises a possibility that melanocytes are present in the abdominal skin, of which hairs are even non-pigmented. To confirm this possibility, the expression of *Trp2* mRNA, another marker for melanocyes, was examined. As shown in Figure 3B lower panes, *Trp2* mRNA was expressed in the LEA rat abdominal skin as compared to that of IS rats, confirming that melanocyts reside in the LEA abdominal non-pigmented skin.

Melanin staining and immunohistochemical analysis at infant period

To examine the location site where melanocytes reside in the LEA rat abdominal non-pigmented skin, I performed immunohistochemical examination for Kit and Schmorl's staining, specific staining for melanin, using adjacent tissue sections. Melanin was observed in both dorsal and abdominal skins of postnatal day 14 in IS rats as well as in dorsal skin of postnatal day 14 LEA rats, whereas melanin was not observed in abdominal skin of LEA rats as expected (Figure 4 lower panels). Kit-positive cells, possibly melanocytes, were detected in

dorsal and abdominal skin of IS rats in agreement with the result of Schmorl's melanin staining (Figure 4). However, Kit-positive cells were observed in both dorsal and abdominal skins of LEA rats, in disagreement with the result of Schmorl's staining (Figure 4). These results indicate that melanocytes are present even in non-pigmented hair follicles of LEA rats, suggesting that melanocytes residing non-pigmented hair follicles are impaired to synthesize melanin by certain mechanism in LEA rats.

Discussion

Analyses of coat color mutations in rodents have been providing mechanistic insights into melanocyte developmental pathways as well as disease processes in case of their deficiency. Generally, coat color pigmentation depends on the amount of melanin produced by melanocytes derived from neural crest cells (Tomas and Erickson, 2008). Therefore, the hooded phenotype is considered to be a defect in melanocyte migration, development, and/or melanin production. In this study, I attempted to identify a gene responsible for hooded phenotype. To this end, I confined the hooded locus by generating fine linkage map for the hooded locus using 795 backcrossed progenies from non-hooded inbred strain, IS and hooded

inbred strain, LEA. From the fine linkage map generated, I concluded that the *Kit* gene is a strong candidate responsible for the hooded locus. In fact, it has been reported that many alleles of *Kit* gene affect coat color phenotype in mice and rats, because the *Kit* gene regulates melanoblast development (Aoki et al., 2005; Cable et al., 1995; Geissler et al, 1981; Mackenzie et al., 1997; Niwa et al., 1991). Moreover, it has been reported that there is relationship between *Kit* mutation and pigmentation, coat color or white spotting in other vertebrates from zebrafish to human (Hasse et al., 2007; Parichy et al., 1999; Richards et al., 2001). Nucleotide sequence of the *Kit* gene of the LEA rat revealed a nucleotide transversion in exon 2; however, it was synonymous one. The rat *Kit* genomic DNA spans ~80 Kbp consisting of ~3 Kbp for the coding region. As I speculated that a mutation might be present in the promoter region so that the expression of the *Kit* mRNA might be altered, I sequenced ~2 Kbp upstream of the exon 1 and compared sequences in the region showing homology among rat, mouse, and human, which were assumed to contain promoter region. However, I could not find any mutation between IS and LEA rats (data not shown). These results are consistent with the result that I could not find difference in the *Kit* mRNA expression between IS and LEA rats. However, it has been reported that the necessity of Kit signaling depends on embryonic stage by the study with *Kit* antibody both *in vivo* and *in vitro* (Hou et al., 2000;

Yoshida et al., 1996). Furthermore, it has been reported that gene expressions are regulated in some genes by locus control region where locates far upstream of the gene (Moon and Ley, 1990). Thus, I cannot exclude a possibility that a mutation occurs in more upstream region of the *Kit* gene in hooded rats, which may regulate fine expression of the *Kit* gene at the embryonic stages or during periodic hair cycles after birth.

In the previous studies, non-pigmented pattern of hooded rats is due to the delay of melanoblast migration (Wendt-Wagener et al., 1961) and both melanin and melanocytes were not observed in the white spot of skin (Baynash et al., 1994). As shown in Figure 1, HE staining data of non-pigmented skin of LEA rats were consistent with the previous reports. However, quantitative analysis showed that the *Kit* mRNA was expressed even in the non-pigmented skin. Because the Kit is one of the melanocyte markers, this result suggests that melanocytes may be present in the non-pigmented skin in hooded rats. This was confirmed by the result that the expression of the *Trp2* mRNA, another marker of the melanocyte, was also detected in the non-pigmented skin. Therefore, I analyzed expression of Kit and the presence of melanin in the adjacent tissue sections. This result shows that Kit-positive cells, but not melanin, are present in the same non-pigmented hair follicles. Thus, results in this study provide a mysterious conclusion. Genetic analysis suggests strongly that

the *Kit* gene is responsible for the hooded locus. However, no mutation was found in the coding region of the *Kit* gene. Further, expression of the *Kit* mRNA was normal from embryo through adult tissues in hooded rats. Furthermore, Kit-positive melanocytes were found even in non-pigmented hair follicles of hooded rats. There are three conceivable hypotheses. 1) A truncated Kit product or a soluble form of Kit is synthesized and plays a role as decoy by binding Kit ligands, because it is reported that exon skipping in the *Kit* gene and production of soluble form of the Kit influences the melanogenesis in the horse and human, respectively (Brooks et al., 2005; Kasamatsu et al., 2008). However, this hypothesis is unlikely, because there were no quantitative and qualitative differences in each RT-PCR product, when Kit cDNA was amplified as 6-devided fragments (data not shown). 2) Although bulk expression of the *Kit* mRNA in the non-pigmented skin of LEA rats is not different from that of IS rats, timing of the *Kit* expression might be disordered in the periodic hair regenerating cycles due to a hidden mutation in the intron, far upstream or downstream of the *Kit* gene. 3) The timing of *Kit* expression might also be disordered in early embryonic stages due to the some reasons as in 2). This disorder might be compensated by other factors promoting the migration of neural crest cells and development of melanocytes such as endothelin 3, type B endothelin receptor, Ret, Sox10 etc. However, a disorder of the timing of *Kit* gene expression at

embryonic stages might remain mal-effect on the function of melanocytes even after development of melanocytes. Disorder in the *Kit* expression and substantial mutation of the *Kit* gene remain unknown, but should be resolved in future investigation.

Summary

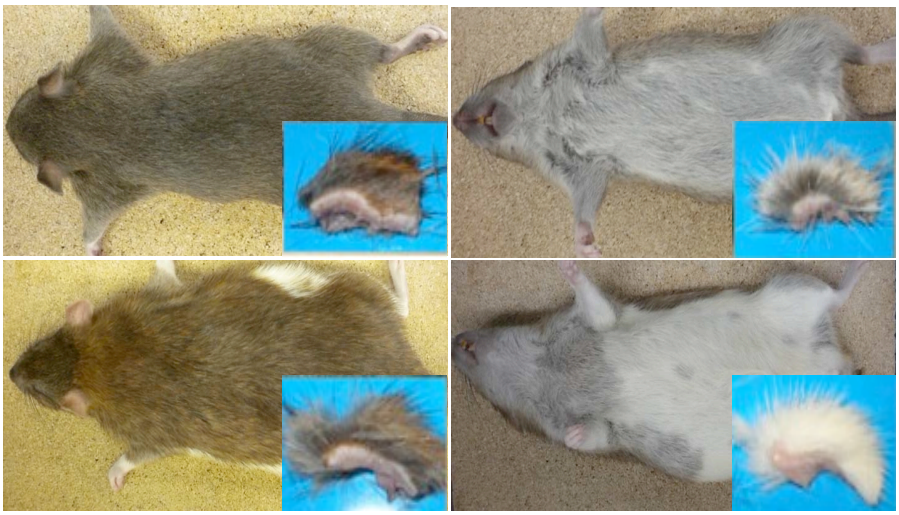
The hooded phenotype showing non-pigmented hairs in the abdominal skin is one of the coat color phenotypes seen peculiarly in the laboratory rat. The hooded locus showing autosomal recessive inheritance has been mapped on Chr 14 in previous investigations. Through genetic fine mapping using feral rat-derived inbred strain, IS and hooded phenotype inbred strain, LEA, I narrowed critical region of the hooded locus and revealed that only *Kit* gene, known as a marker of melanocytes, existed in this region, suggesting strongly that the *Kit* is a gene responsible for the hooded locus. Nucleotide sequence analysis of the coding region of the *Kit* gene revealed a G to C transversion in exon 2, which was, however, a synonymous substitution. Further, the expressions of *Kit* mRNA were not different in fetal neural tubes and both neonatal and adult skins between IS and LEA rats. I then examined Kit-positive cells and melanin granules in the same hair follicles histochemically. The result showed that Kit-positive cells, possibly melanocytes, were observed in non-pigmented hair follicles in the LEA rat abdominal skin, although no melanin granules were observed in the same non-pigmented hair follicles. These results suggest that the synthesis of melanin is impaired in the Kit-positive melanocytes residing in the non-pigmented hair follicles of

hooded phenotype rats. Molecular mechanisms in the appearance of hooded phenotype and substantial mutation of the *Kit* gene remain unknown, but should be resolved in future investigation.

A

IS (H/H)

LEA(h/h)

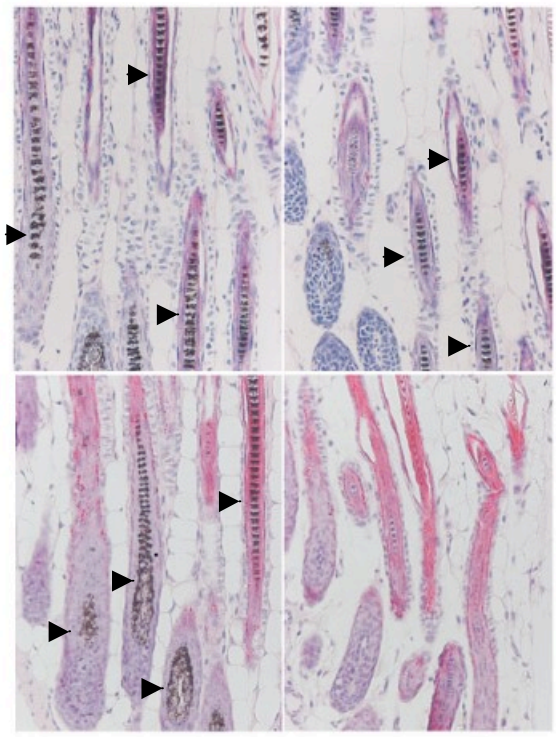


B

dorsal abdominal

IS

LEA



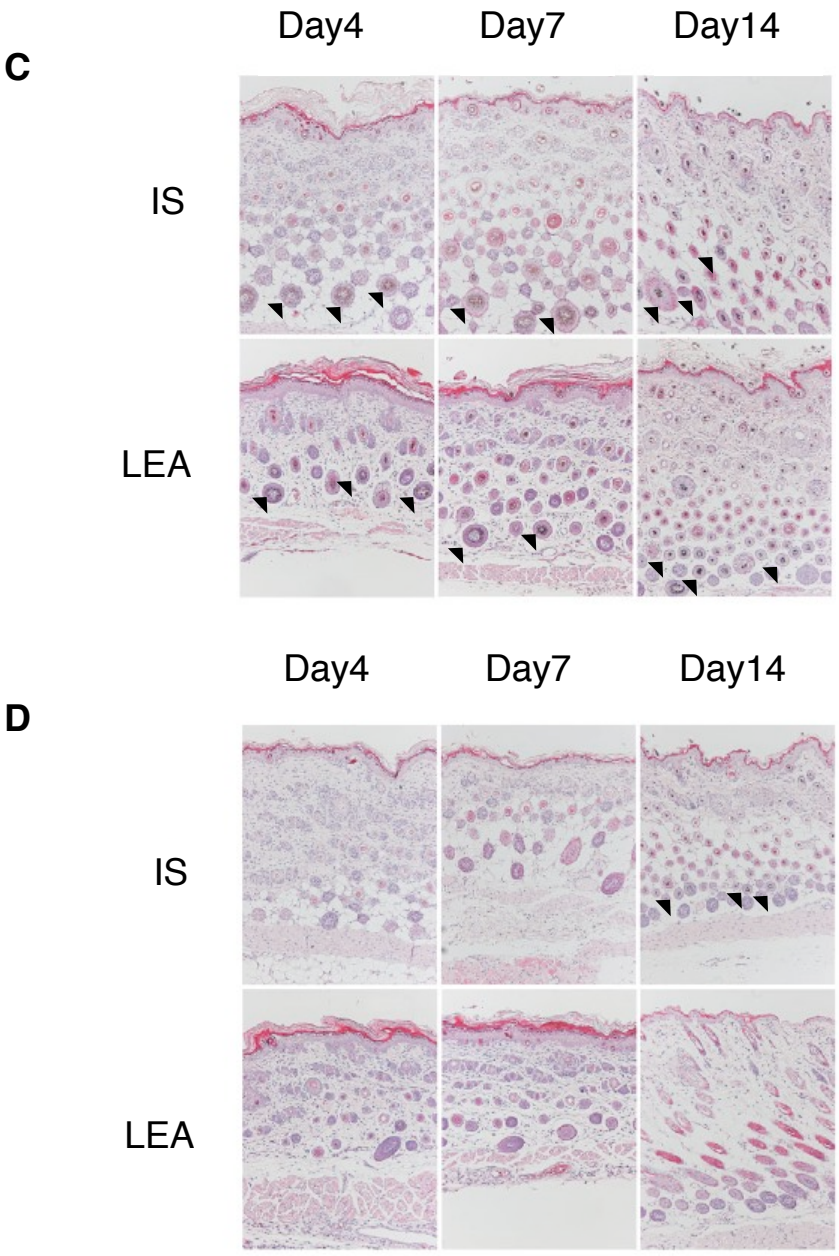


Figure 1 Characteristics of the hooded phenotype. A; Photographs of dorsal (left panels) and abdominal (right panels) hairs of IS (upper panels) and LEA (lower panels) rats. Right lower insets show cross-section of the skin. B; HE staining of adult IS (upper) and LEA (lower) rat skins. C; HE staining of dorsal skin of IS (upper) and LEA (lower) rats at postnatal day 4 (left), 7 (middle), and 14 (right). D; HE staining of abdominal skin of IS (upper panels) and LEA (lower panels) rats at postnatal day 4 (left), 7 (middle), and 14 (right). Arrowheads indicate pigmented hairs.

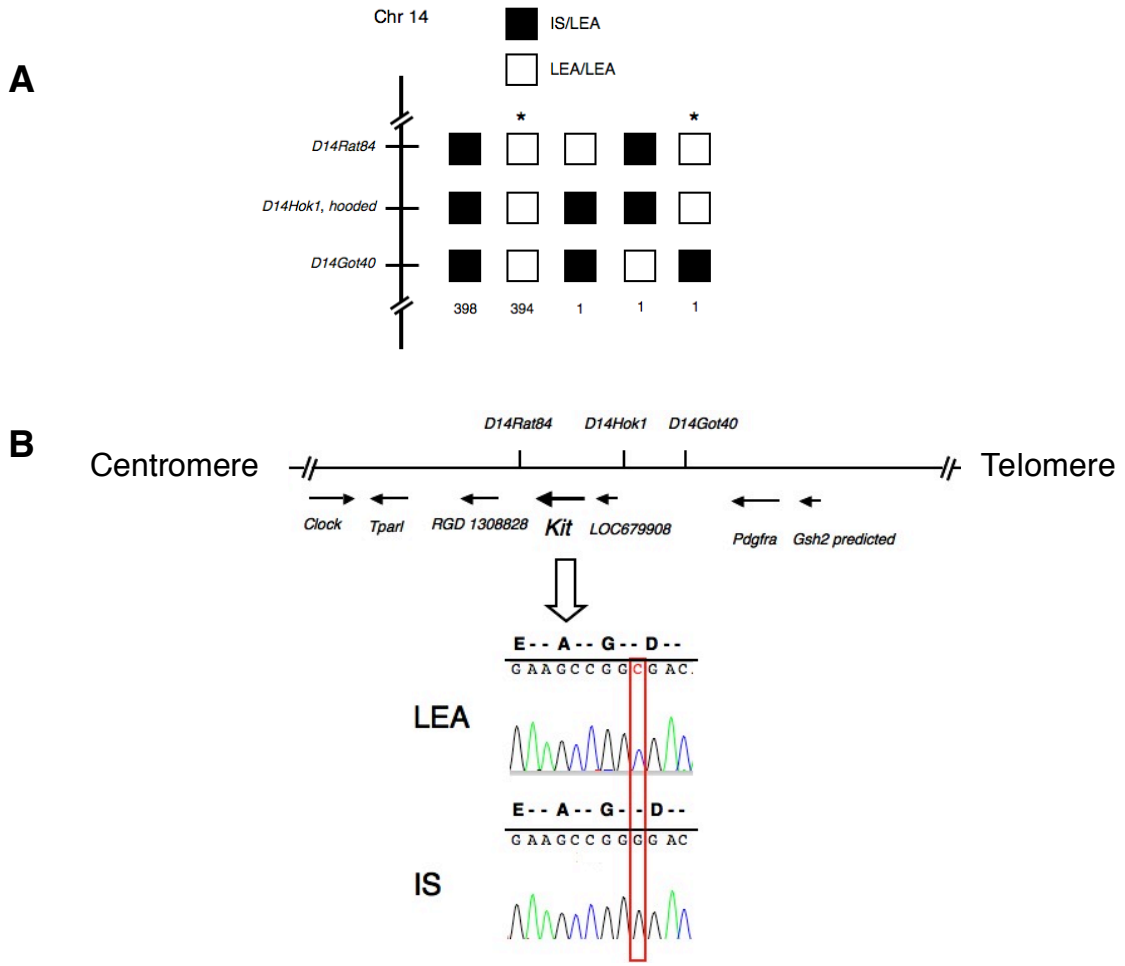


Figure 2 Identification of a gene responsible for the hooded locus. A; Haplotype map of backcrossed progenies, LEA x (LEA x IS)F_1. Open and filled squares represent the homozygous for the LEA allele and the heterozygous for IS and LEA alleles, respectively. The asterisks indicate the progenies showing hooded phenotype. Values at the bottom are the number of progenies. B; Upper panel, transcription map of the critical region for the hooded locus. Transcriptional orientations are shown by arrows. Only *Kit* is protein-coding gene located between *D14Rat84* and *D14Got40* loci. Lower panel, comparison of the nucleotide sequence of the *Kit* cDNA between IS and LEA rats. Amino acid sequence is shown over the nucleotide sequence. A transversion of G to C, which is synonymous mutation, is observed at nucleotide position 197.

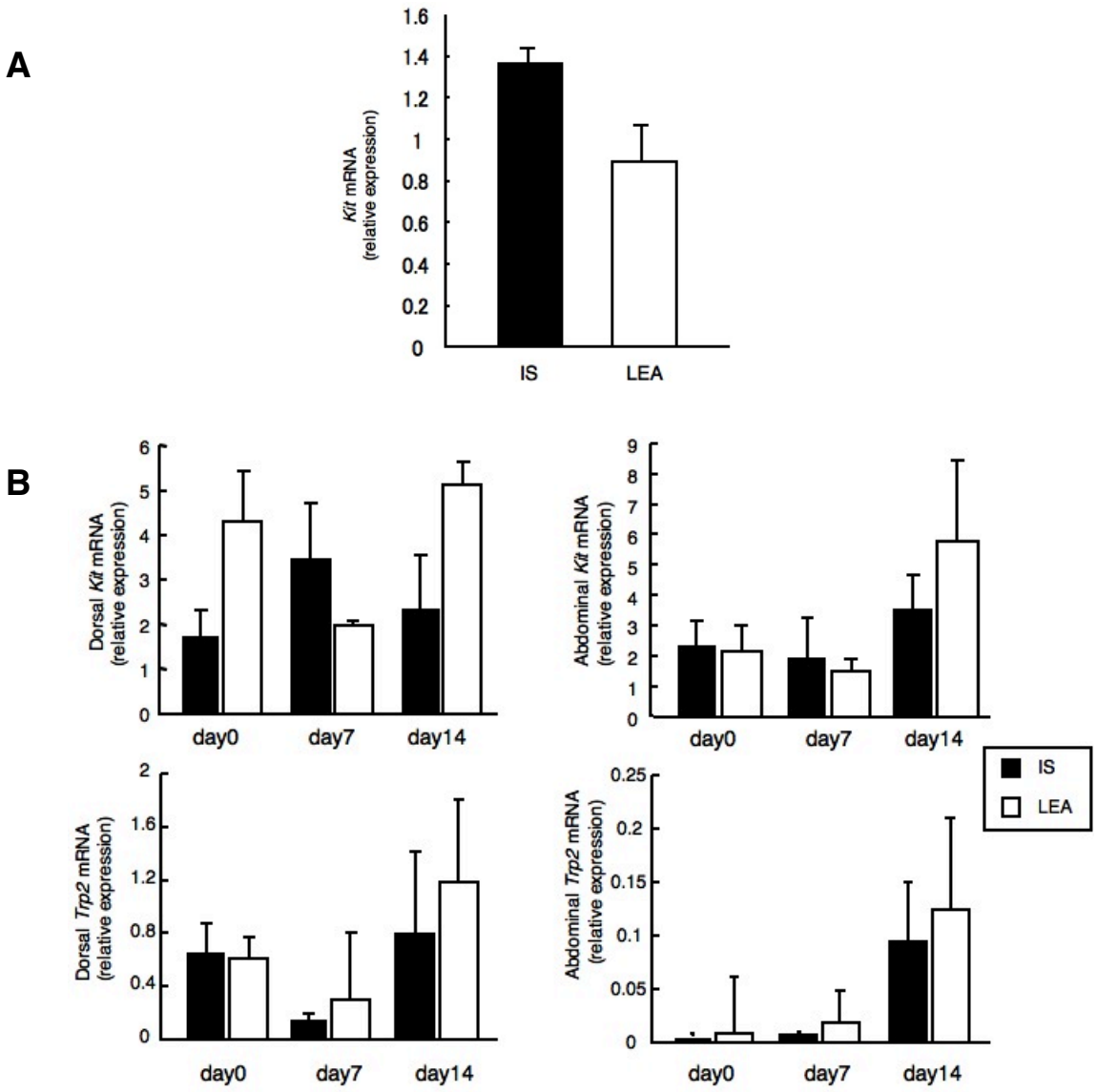


Figure 3 Quantitative expression analysis of *Kit* and *Trp2* mRNAs at postnatal periods. A; The expression of *Kit* at embryonic day 10.5. B; The expression of *Kit* (upper) and *Trp2* (lower) genes in dorsal (left) and abdominal (right) skins. Filled and open bars represent the data for IS and LEA rats, respectively. The mRNA expression levels of target genes were normalized to the expression of beta-actin (*Actb*). The data represent means ± SEM (n = 3).

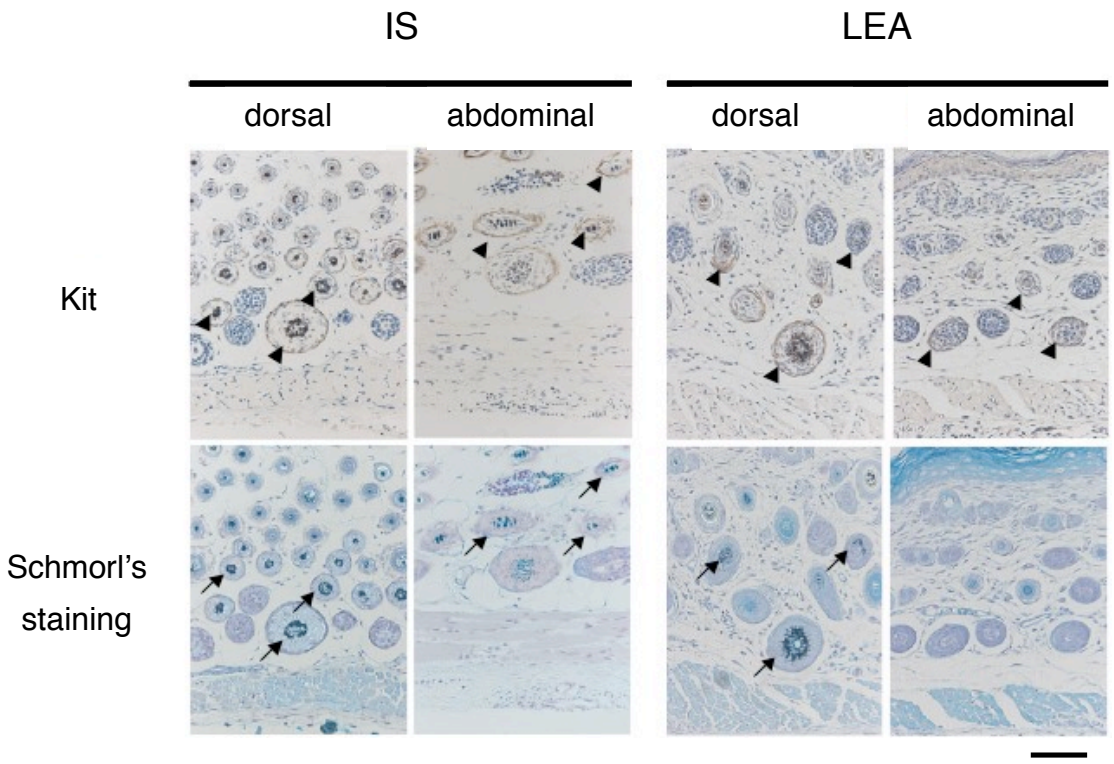


Figure 4 Immunohistochemical staining with anti-Kit antibody (upper panels) and Shmorl's staining for melanin (lower panels) in the adjacent tissue sections prepared from the dorsal and abdominal skins at day 14. Melanin was detected all samples except for the abdominal skin of LEA rats. Arrowheads and arrows indicate hair follicles containing Kit- and melanin-positive cells, respectively. The bar shown in the lower right corner indicates the length of 100 μm.

Part2

Genetic analysis of modifiers for the hooded phenotype in the rat

Introduction

As described in part 1, the hooded phenotype, which is peculiar in the rat, shows that colored coat covers head and mid-dorsal regions. Hooded phenotype has, however, many alleles such as hooded (*h*), hooded Irish (h^i), hooded notch (h^n), hooded extreme (h^e), and hooded restricted (H^{re}), which cause different extents of pigmented coat area (Gumbreck et al., 1971; Palmer et al., 1974; Robinson, 1965; Robinson, 1989). Homozygotes with hooded alleles show the quantitative order of colored coat area as follows; $h^i > h > h^n > h^e > H^{re}$. The h^i allele shows pigmented coat covering almost whole surface except for a ventral white spot between or behind front legs (Curtis and Dunning, 1937). The h^n allele resembles *h* allele except that the area of pigmentation is further restricted so that the hooded region in the head is smaller than that of *h* allele without mid-dorsal pigmented stripe. All these three alleles are completely recessive to the wild type and the *h* is also recessive to the h^i allele (Castle, 1951). As described in part 1, I found a strong candidate gene, *Kit*, responsible for the hooded locus.

Furthermore, hooded modifier locus, such as Hm^l for a long dorsal hooded pattern and Hm^s for a short dorsal hooded pattern, is reported to influence the hooded phenotype. The *Hm* locus is linked to the *h* locus, but a gene responsible for *Hm* is not yet identified (Stolc, 1984).

In the course of pathogenetic study of *Erysipelothrix rhusiopathiae* in rats, I found strain difference in resistance or susceptibility to the pathogen. Namely, the LEA rat, possessing *h* allele for the hooded phenotype, is resistant to the *Erysipelothrix rhusiopathiae* infection, whereas the BN rat, possessing h^i allele, is sensitive. I, then, generated F_2 progenies from both strains to be used for the genetic study of the resistant to the *Erysipelothrix rhusiopathiae* infection. In this study, I found that the hooded phenotype was not segregated into 3:1 ratio for the h^i and *h* phenotypes as following the Mendel's law, but distributed various and consecutive extents of pigmented regions. Thus, this attractive phenomenon led me to elucidate the genetic mechanism of it. In part 2, I describe the presence of genetic modifiers for the hooded phenotype in the rat as a consequence of extensive QTL analysis.

Materials and Methods

Animals

BN rats were purchased from Japan SLC (Shizuoka, Japan). LEA rats were provided from the National Bio Resource Project for Rat (Kyoto, Japan). F_1 progenies were obtained from female LEA rats mated with male BN rats. F_2 progenies were produced by mating F_1 progenies randomly. Animals were maintained in specific pathogen-free conditions with feeding and drinking *ad libitum*. In the experimental animal care and handling, the author adhered to the Regulation for the Care and Use of Laboratory Animals, Hokkaido University.

Measurement of pigmented coat ratio

Photographs of both dorsal and ventral sides of rats were taken with COOLPIX 4500 digital camera (Nikon, Tokyo, Japan). Pigmented and non-pigmented areas were traced manually, separated with two colors (Figure 5A right panel of each photograph), and calculated as pixels using histogram function of Photoshop Elements 4.0 (Adobe Systems, California, USA). Area of four foots was excluded from the calculation. To control for variations in size among animals, percentage of pigmented area (pigmented area/total area x 100) was calculated separately for dorsal, ventral, and total body surfaces of each animal. These values were used for quantitative traits. For pigmented area measurements, data were presented as means ± SEM, compared each other using the Student's *t*-test, and considered to

be significantly different at $p < 0.05$.

Genotyping of microsatellite markers

Extraction of genomic DNA from tail clips was performed by the standard methods. A total of 115 microsatellite markers showing polymorphisms between BN and LEA rats were used for genetic study (Table 1). The average interval of adjacent microsatellite markers was 15.1 cM. The map positions of microsatellite loci were based on information from the NCBI. PCR was carried out on a Bio-Rad PCR thermal cycler (iCycler, California, USA) with the cycling sequence of 95°C for 1 min (one cycle), followed by 35 cycles consisting of denaturation at 95°C for 30 sec, primer annealing at 58°C for 30 sec, and extension at 72°C for 30 sec. PCR mixture and enzymes were purchased from TaKaRa (Ex Taq DNA Polymerase, Otsu, Japan). The amplified samples were electrophoresed with 10-15% polyacrylamide gel (Wako, Osaka, Japan), stained with ethidium bromide, and then photographed under an ultraviolet lamp.

QTL analysis

QTL analysis was performed with Map Manager QTXb20 software program (Manly et

al., 2001). In this program, linkage probability was examined by interval mapping. Genome-wide significance thresholds were set, as suggested previously, at the 37th ("suggestive"), 95th ("significant"), and 99.9th ("highly significant") percentiles, which correspond to the chance of finding 1 false-positive linkage 0.63, 0.05, and 0.001 times, respectively (Lander and Kruglyak, 1995; Manly and Olson, 1999). For each chromosome, the likelihood ratio statistic (LRS) values were calculated by 5,000 random permutations of the trait values relative to genotypes of the maker loci. For the quantitative trait of dorsal region, suggestive, significant, and highly significant values were 10.0, 17.2, and 27.1, respectively. For the quantitative trait of ventral region, suggestive, significant, and highly significant values were 10.1, 17.1, and 27.0, respectively. For the quantitative trait of total region, suggestive, significant, and highly significant values were 10.0, 16.7, and 24.5, respectively. Confidence intervals (CI) were estimated by bootstrap analysis (Visscher et al., 1996; Walling et al., 1998) instead of the classic 1-LOD supports interval (Lander and Kruglyak, 1995), since it has been shown to be more reliable over all QTL strengths (Dravasi and Sokker, 1997).

Possible interactions between all pairs of marker loci were primarily screened with QTXb20 software program. This program searches for digenic epistasis by testing all pairs of

marker loci for both main effects and interaction effects using regression. Pairs of loci must pass two tests in order to be reported as having a significant interaction effect. First, the total effect of the two loci must have a *p*-value less than 10^{-5} (software suggestion). Second, the interaction effect itself must have a *p*-value less than 0.01. For each significant two-locus interaction, QTX software calculates LRS for each main effect, the interaction effect, and the total effect. In addition, this software allows me to run permutation tests for two-locus interactions for given data set to determine threshold LRS for declaring significance. I used 2,000 permutation tests of my data set for declaring a significant two-locus interaction. After screening epistatic interactions with QTX software program, statistic analysis of detected interactions of microsatellite loci including near loci was performed with ANOVA program to confirm substantial epistatic interactions.

Results

Phenotyping of parental strains and their F_1 and F_2 progenies

Initially, I measured pigmented area for dorsal, ventral, and total regions in parental and F_1 rats (Figure 5A). The ratios for dorsal region were 1.00 in BN rats (n = 5), 0.960 ± 0.009 in

LEA rats (n = 7), and 0.997 ± 0.0004 in F_1 rats (n = 8) (Figure 5B upper panel). The ratios for ventral region were 0.997 ± 0.002 in BN rats, 0.395 ± 0.02 in LEA rats, and 0.987 ± 0.003 in F_1 rats (Figure 5C upper panel). The ratios for total region were 0.998 ± 0.001 in BN rats , 0.678 ± 0.012 in LEA rats, and 0.992 ± 0.002 in F_1 rats (Figure 5D upper panel). The ratios in F_1 rats were similar to those of BN rats in all three regions. There was significant difference between BN and LEA rats and between F_1 and LEA rats ($p < 0.01$). These results were consistent with previous study that the h^i allele is dominant to the h allele (Curtis and Dunning, 1937). Next, I measured phenotype of F_2 (n = 152) progenies (Figure 5A and 5B-D lower panel). These values were not segregated into two groups such as BN and LEA types with 3:1 ratio, but showed consecutive values in all three regions. These data suggest that the extent of pigmented area of the hooded phenotype was under multigenic control.

Genome-wide scan for mapping hooded-modifier loci in F_2 progenies

To clarify the association between the variety of hooded phenotype in F_2 rats, I performed genome-wide scan using 115 microsatellite markers found polymorphisms between BN and LEA rats with Map Manager QTXb20 software program. In this QTL analysis, bootstrap analysis was performed to detect CI of the QTL. Only peak located in the

CI was recognized as a substantial QTL. Two highly significant chromosomal regions regulating hooded phenotype for dorsal region were detected on Chr 14 and 17 (Figure 6A). A highly significant chromosomal region regulating hooded phenotype for ventral and total regions was also detected on Chr 14 and two suggestive chromosomal regions were detected on Chr 15 and 17 (Figure 6B and C). Table 2 summarizes microsatellite markers linked to the phenotype, showing LRS values, genetic effects, CIs, and phenotypic values in each genotype. The highly significant QTL (*D14Got40*) on Chr 14 was detected as a common locus with fur exceeding LRS threshold value and BN-dominant effect in QTL analyses using quantitative traits of dorsal, ventral, and total regions. This QTL (*D14Got40*) contributed 70-90% variance for hooded phenotype of dorsal, ventral, and total regions (Table 2). Two suggestive QTLs detected on Chr 15 and 17 had slightly exceeding threshold LRS value in ventral and total regions. A QTL on Chr 15 showed non-additive and non-dominant trait, whereas a QTL on Chr 17 showed LEA-dominant trait.

Identification of epistatic interactions involved in the hooded phenotype

To find epistatic interactions among microsatellite loci, I performed analysis using the interaction function of Map Manager QTX. Permutation tests in this software program for the

interaction analysis in dorsal region showed that LRS values of 33.4, 45.1, and 59.4 were necessary for suggestive, significant, and highly significant interactions, respectively. Similarly, LRS values of 31.0, 45.1, and 59.4 in ventral region, and 31.3, 40.9, and 50.1 in total region are necessary for suggestive, significant, and highly significant interactions, respectively. Pair-wise testing across all 115 markers revealed significant interaction between Chr 14 (*D14Rat36*) and Chr 20 (*D20Mit1*) and suggestive interaction between Chr 14 (*D14Rat17*) and Chr 17 (*D17Rat102*) in dorsal region. In total region, suggestive interaction between Chr 14 (*D14Rat17*) and Chr 17 (*D17Rat2*) was detected. Next, I performed ANOVA analysis with those microsatellite loci including other loci locating in the vicinity to confirm substantial epistatic interaction. The ANOVA analysis revealed that several microsatellite loci locating in the vicinity of the above microsatellite loci also showed significant interaction. Thus, I considered that *D14Got40* locus could be representative of maicrosatellite loci showing significant interaction on Chr14, because it locates in a peak position of QTL on Chr 14 by bootstrap analysis. Similarly, *D17Rat2* locus was considered to be representative of microsatellite loci showing significant interaction on Chr 17. Thus, I showed epistatic interaction between *D14Got40* and *D20Mit1* loci in Figure 7A, and between *D14Got40* and *D17Rat2* loci in Figure 7B. Both *D20Mit1* and *D17Rat2* loci show significant effect, only

when the genotype of *D14Got40* locus is homozygous for LEA. It is noteworthy that the extent of pigmented coat is maximal, when the genotype of *D17Rat2* locus is homozygous for LEA.

Discussion

The hooded phenotype is peculiarly expressed in the rat and there are many alleles reported. A typical hooded phenotype, *h* allele, shows that pigmented coat covers head and mid-dorsal region. However, *h* allele shows the variation in the extent of pigmented area, for example, the LEA rat possessing *h* allele shows that pigmented coat covers more widely in the dorsal region than that of the typical *h* allele rat strain. In the BN rat, on the other hand, pigmented coat covers almost whole surface except for a ventral small white spot between or behind front legs, which is categorized as h^i allele. When BN and LEA rats were mated, F_1 rats showed the same phenotype as the BN rat, consistent with previous observation that the h^i allele is dominant to the *h* allele. However, phenotypes of F_2 progenies did not follow the Mendel's law. The extent of pigmented coat was not segregated into 3:1 ratio, but varied with consecutive values. This suggests that the extent of coat color pigmentation receives

multigenetic control, which led me to perform QTL analysis.

QTL analysis revealed a main QTL located closely to *D14Got40* with extremely high LRS value, which is possibly corresponding to the hooded locus. Possibly h^i and *h* alleles cause polymorphisms on the hooded gene, which should be identified in future. In this study I succeeded to identify several other QTLs located closely to *D15Rat5*, *D17Rat2,* and *D17Arb7*, all of which possibly affect the extent of pigmented coat cooperatively. Among these QTLs, the *D17Rat2* locus is of interest. The *D17Rat2* locus provided highly significant LRS value by itself in the QTL analysis in the dorsal region phenotype (Figure 6A). Further, the *D17Rat2* locus showed epistatic interaction with the hooded locus, *D14Got40* (Figure 7B). It is noteworthy that the ability of increasing the extent of pigmented coat in the dorsal region depends on the genotypes of the *D17Rat2* locus, when the hooded locus, *D14Got40*, is homozygous for LEA allele. The extent of pigmented coat is the highest in the F_2 rats possessing LEA-homozygous genotype of the *D17Rat2* locus followed by LEA/BN heterozygous and BN homozygous genotypes. This result proposes the reason why the LEA rat, which possesses the *h* allele, shows the wider pigmented coat area in the dorsal region than that of the typical hooded rat strain possessing the same *h* allele. The *D20Mit1* is another locus showing significant epistatic interaction with the *D14Got40* locus (Figure 7A). The

D20Mit1 locus by itself never affects the extent of pigmented coat area because of not being detected in QTL analysis, whereas it affects the extent of pigmented coat area, only when the hooded locus, *D14Got40*, is homozygous for LEA allele. The effect of *D20Mit1* locus is maximal in BN-homozygous genotype followed by heterozygous and LEA-homozygous genotypes. Both *D17Rat2* and *D20Mit1* loci may be relating to the *Hm* locus reported previously (Stolc, 1984).

Until now, more than 150 different loci have been identified as coat color mutants in mice. Furthermore, it has been reported that genetic interactions additively or synergistically affected melanocyte development (Potterf et al., 2000; Stanchina et al., 2006). In part 1, I speculate that the *Kit* gene is a strong candidate responsible for the hooded locus. In fact, there are some reports that show genetic interaction between *Kit* and other genes using mutant mice (Aoki et al., 2005; Hou et al., 2000; Silver et al., 2008). However, these genes reported in previous studies were not mapped to either Chr 17 or Chr 20, where genetic loci showing epistatic interaction with *D14Got40* were detected. Because the molecular mechanisms and signaling pathway regulating the development of melanocyte are complicated, further study is necessary for the identification of genes affecting the hooded phenotype.

Summary

The hooded phenotype is one of the coat color phenotype seen peculiarily in the rat. The hooded locus showing autosomal recessive inheritance is mapped to Chr 14 and that the hooded phenotype receives modification by hooded-modifier gene showing the linkage to the hooded locus. However, a gene responsible for the hooded-modifier is not yet identified. To clarify genetic control of the hooded phenotype, I carried out genetic linkage studies using BN and LEA rats. For determination of phenotypic variation, I measured ratio of pigmented coat area in parental and their F_1 and F_2 rats. I, then, conducted a genome-wide scan on 152 F_2 rats for linkage with ratio of pigmented coat area for the dorsal, ventral, and total regions. A major QTL, *D14Got40*, showing highly significant linkage and contributing 70-90% of the variance for the hooded phenotype was detected on Chr 14, which may be corresponding to the hooded locus. In addition, another QTL, *D17Rat2* showing highly significant linkage was also detected on Chr 17 in dorsal region phenotype as well as a QTL showing suggestive linkage on Chr15 in ventral region phenotype. I, further, investigated a genome-wide scan for epistatic interactions and detected significant interactions between *D14Got40* and *D20Mit1*, and between *D14Got40* and *D17Rat2* in the dorsal region phenotype. These results suggest

that a major QTL in Chr 14, which is possibly correspondent to the hooded locus, mainly regulates the hooded phenotype with some modifier loci, two of which show epistatic interactions with the hooded locus.

Table 1 Microsatellite markers used for the genotyping F_2 progenies.

Microsatellite Markers	Posision (Mbp)	Microsatellite Markers	Posision (Mbp)	Microsatellite Markers	Posision (Mbp)	Microsatellite Markers	Posision (Mbp)	Microsatellite Markers	Posision (Mbp)
D1Rat250	13	*D3Got34*	86	*D6Rat3*	145	*D11Rat43*	85	*D16Rat55*	76
D1Rat403	41	*D3Rat21*	110	*D7Mgh11*	2	*D12Rat58*	4	*D17Rat2*	10
D1Mit1	59	*D3Mit4*	131	*D7Rat31*	28	*D12Rat14*	29	*D17Rat102*	27
D1Rat344	116	*D3Rat8*	132	*D7Rat51*	50	*D12Rat22*	46	*D17Rat14*	33
D1Mgh9	155	*D3Mit3*	136	*D7Rat112*	90	*D13Rat59*	31	*D17Rat13*	34
D1Arb29	180	*D3Mgh3*	155	*D7Rat128*	121	*D13Mgh4*	38	*D17Rat15*	37
D1Rat169	229	*D3Arb15*	170	*D8Rat164*	28	*D13Mit2*	62	*D17Arb7*	71
D1Rat90	267	*D4Rat5*	9	*D8Rat188*	44	*D13Mit4*	90	*D17Rat47*	85
D2Rat309	21	*D4Rat231*	88	*D8Mgh4*	86	*D14Mit2*	18	*D18Mit1*	12
D2Rat184	40	*D4Rat78*	105	*D8Rat90*	114	*D14Rat36*	33	*D18Rat132*	25
D2Arb7	57	*D4Rat273*	133	*D8Rat3*	126	*D14Got40*	35	*D18Rat91*	61
D2Rat19	58	*D4Arb28*	154	*D9Rat46*	6	*D14Rat12*	42	*D18Rat5*	77
D2Rat74	70	*D4Rat203*	161	*D0Rat30*	20	*D14Rat15*	44	*D19Rat17*	12
D2Rat203	76	*D5Rat121*	9	*D9Mit3*	55	*D14Rat17*	59	*D19Arb1*	14
D2Mit17	109	*D5Rat190*	25	*D9Rat90*	73	*D14Rat94*	89	*D19Rat68*	43
D2Mit8	148	*D5Mit10*	57	*D9Rat4*	90	*D14Rat38*	99	*D19Rat107*	48
D2Rat40	163	*D5Rat107*	94	*D9Rat108*	103	*D15Rat5*	22	*D20Mgh5*	9
D2Mit13	182	*D5Rat95*	130	*D10Rat51*	11	*D15Rat6*	32	*D20Rat37*	32
D2Rat52	202	*D5Rat33*	143	*D10Mgh6*	67	*D15Rat11*	51	*D20Rat54*	41
D2Rat61	227	*D5Rat99*	157	*D10Mgh3*	100	*D15Mgh4*	83	*D20Mit1*	48
D3Rat52	14	*D6Rat105*	18	*D10Rat7*	105	*D15Rat106*	106	*DXRat82*	19
D3Mgh7	36	*D6Rat136*	48	*D11Rat21*	18	*D16Mgh4*	18	*DXRat5*	25
D3Mgh6	68	*D6Rat11*	115	*D11Rat5*	37	*D16Rat65*	61	*DXRat102*	133

Table 2 Characteristics of QTLs detected with Map Manager QTX for extent of pigmented coat areas.

Regions	Markers	Posision[a)]	LRS	%[b)]	CL[c)]	BN/BN[d)]	BN/LEA[d)]	LEA/LEA[d)]
Dorsal	*D14Got40*	22.5	183.5	70	5	100	99.6±0.2	$75.8\pm2.0^{***\dagger\dagger\dagger}$
Dorsal	*D17Rat2*	6.0	30.1	18	19	83.4±3.4	$94.6\pm1.1^{***}$	$97.9\pm0.8^{***\dagger}$
Ventral	*D14Got40*	22.5	367.0	91	4	99.9±0.05	$93.0\pm1.4^{***}$	$32.1\pm1.6^{***\dagger\dagger\dagger}$
Ventral	*D15Rat5*	15.4	11.0	7	50	86.5±3.7	$67.3\pm4.4^{**}$	$79.9\pm4.3^{\dagger}$
Ventral	*D17Arb7*	54.9	13.7	9	41	75.9±5.2	67.9±4.2	$88.3\pm3.7^{\dagger\dagger\dagger}$
Total	*D14Got40*	22.5	355.3	90	4	99.9±0.02	$96.3\pm0.8^{***}$	$54.0\pm1.5^{***\dagger\dagger\dagger}$
Total	*D15Rat5*	15.4	10.4	6	56	91.5±2.5	$78.8\pm3.0^{**}$	86.1±3.1
Total	*D17Rat2*	6.0	10.6	7	52	76.0±4.8	$86.7\pm2.2^{*}$	$90.2\pm2.4^{**}$

[a)]Expressed in cM according to recombination fraction by Map Manager QTX software.

[b)]Percentage of total variance attributable to locus.

[c)]95% confidence interval of QTL location as caluculated by QTX software.

[d)]Mean phenotypic value ± SEM for rats homozygotes for the BN allele (BN/BN), heterozygotes (BN/LEA) and homozygotes for the LEA allele (LEA/LEA).

$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, vs. BN/BN

$^{\dagger}p < 0.05$,$^{\dagger\dagger}p < 0.01$,$^{\dagger\dagger\dagger}p < 0.001$, vs. BN/LEA

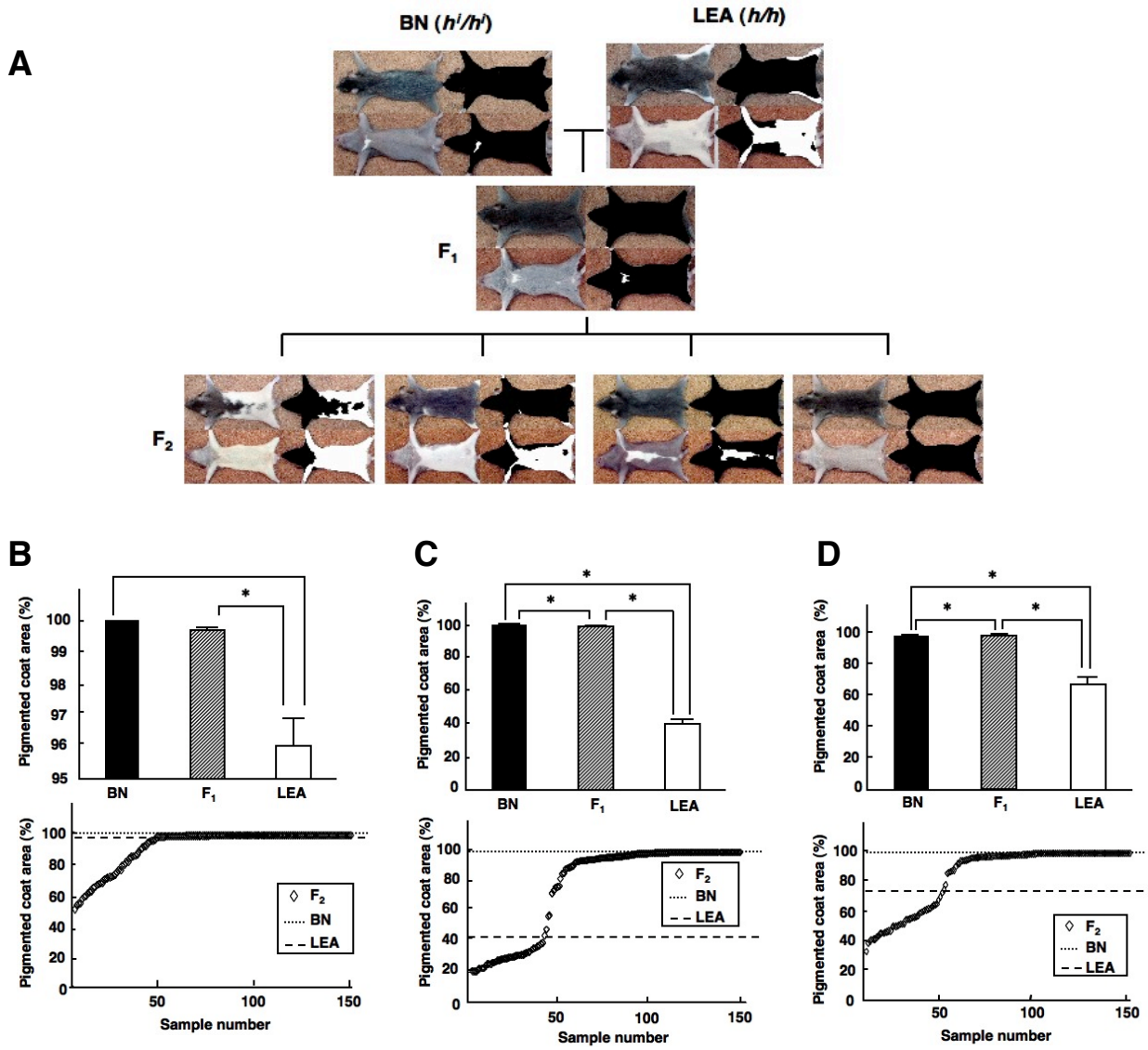
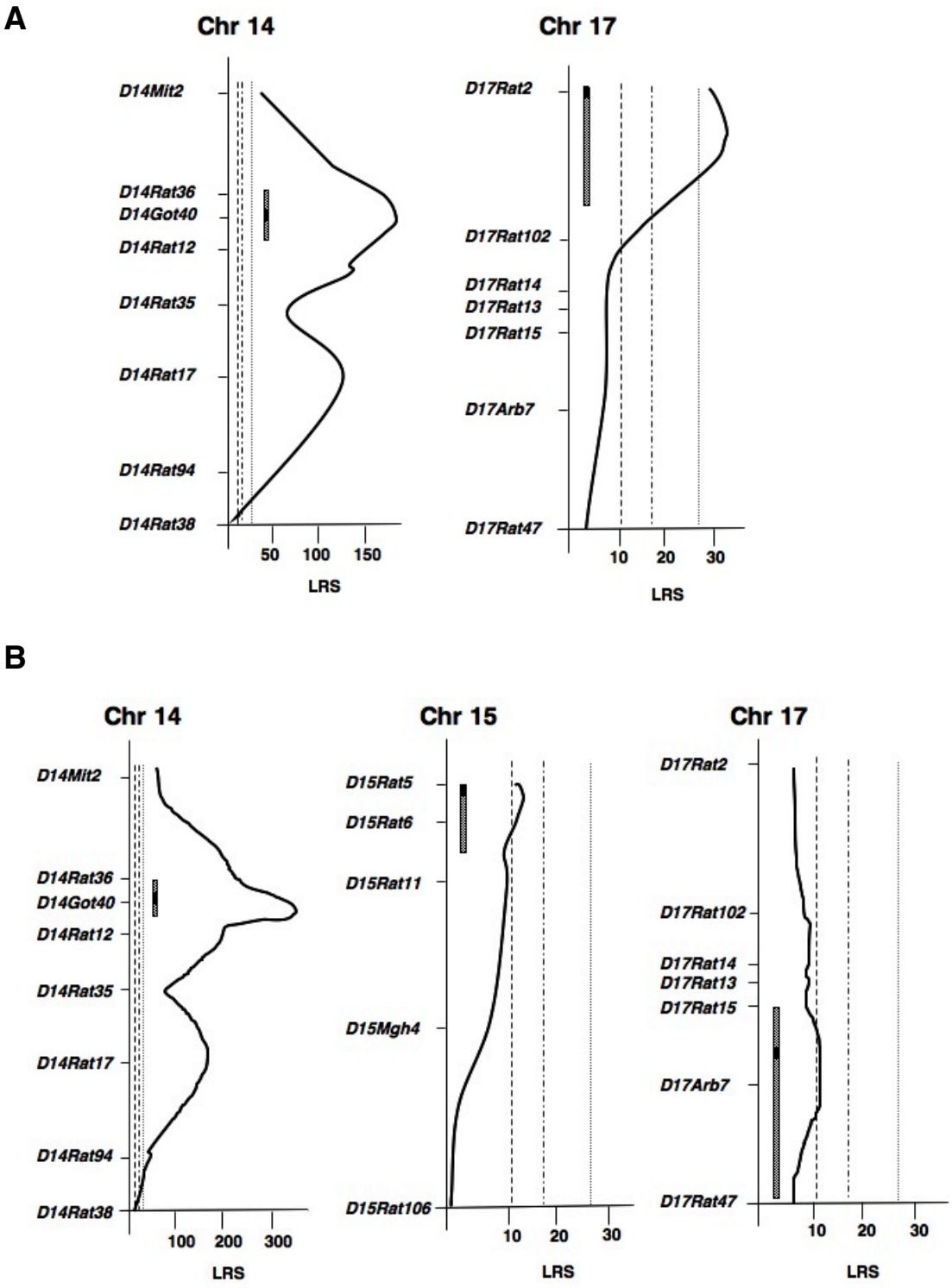


Figure 5 Ratio of pigmented coat area of parental strains, F_1, and F_2 progenies. A; Photographs of representatives of each generation. Each right side panel shows the tracing patterns for pigmented area. B, C, and D show the ratios of pigmented coat area for dorsal, ventral, and total regions, respectively. The upper panels show the ratio of pigmented area in parental and F_1 rats, and the lower panels show the ratio of pigmented area in F_2 rats. The number of rats for BN, LEA, F_1, and F_2 rats are 5, 7, 8, and 152, respectively. Data represent the mean ± SEM and asterisks indicate $p < 0.01$ in the upper panels.


A
Chr 14
D14Mit2
D14Rat36
D14Got40
D14Rat12
D14Rat35
D14Rat17
D14Rat94
D14Rat38
50
100
150
LRS
Chr 17
D17Rat2
D17Rat102
D17Rat14
D17Rat13
D17Rat15
D17Arb7
D17Rat47
10
20
30
LRS
B
Chr 14
D14Mit2
D14Rat36
D14Got40
D14Rat12
D14Rat35
D14Rat17
D14Rat94
D14Rat38
100
200
300
LRS
Chr 15
D15Rat5
D15Rat6
D15Rat11
D15Mgh4
D15Rat106
10
20
30
LRS
Chr 17
D17Rat2
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D17Rat14
D17Rat13
D17Rat15
D17Arb7
D17Rat47
10
20
30
LRS

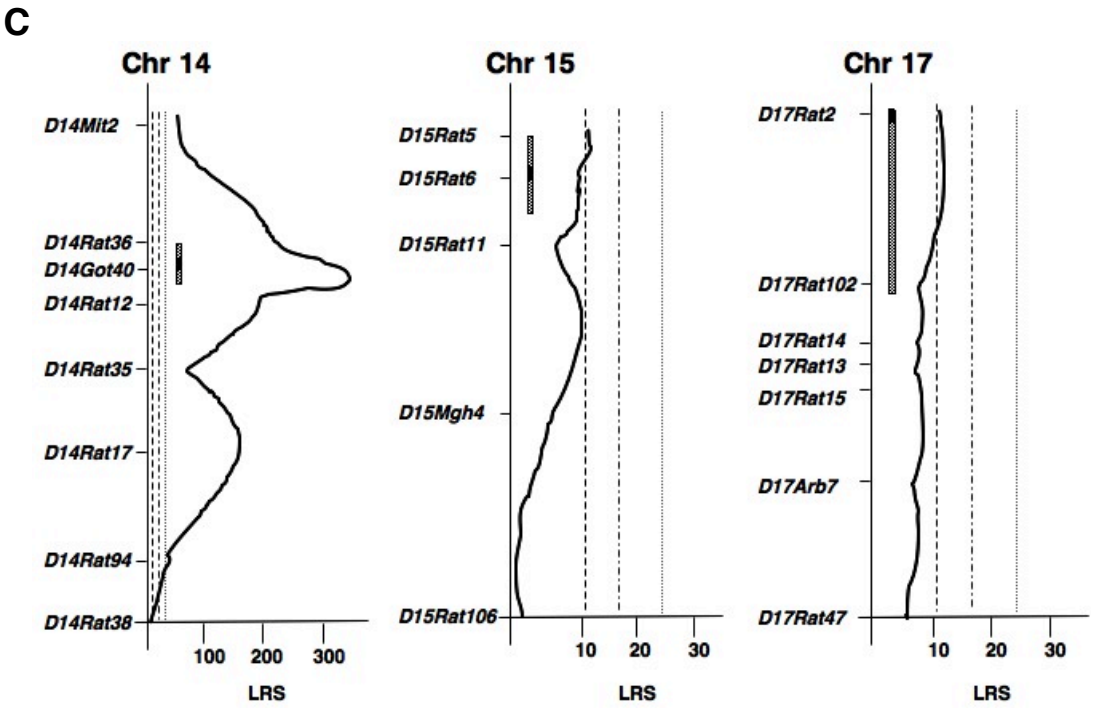

Figure 6 QTLs detected to influence the extent of pigmented coat area in F_2 progenies. Each vertical axis represents the genetic map for the rat chromosome and markers in F_2 rats. A, dorsal region; B, ventral region; C, total region. Dashed, dashed-dotted, and dotted lines indicate suggestive, significant, and highly significant thresholds, respectively. Shaded bars represent CI calculated by bootstrap analysis with the black bars at the peak position of each QTL.

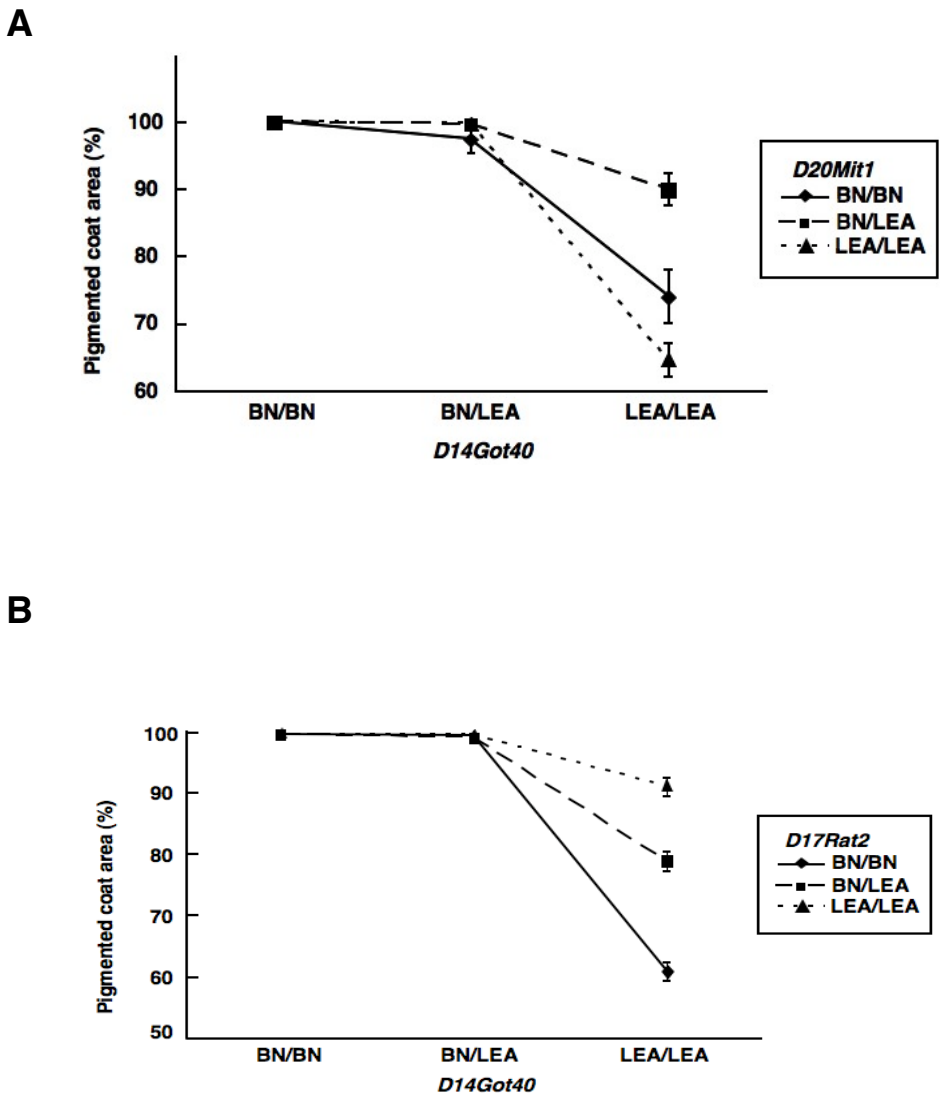

Figure 7 Significant epistatic interactions detected to influence the extent of pigmented coat area in F_2 progenies. Two significant epistatic interactions were detected in the dorsal region phenotype between *D14Got40* and *D20Mit1* loci (A); and between *D14Got40* and *D17Rat2* loci (B). Data represent the mean ± SEM.

Conclusion

The hooded phenotype showing non-pigmented hairs in the abdominal skin is one of the coat color phenotypes seen peculiarly in the laboratory rat. The hooded locus showing autosomal recessive inheritance has been mapped on Chr 14 and that the hooded phenotype receives modification by hooded-modifier gene showing linkage to the hooded locus. Thus, I have conducted genetic studies to identify a gene responsible for the hooded locus and genes responsible for hooded-modifiers.

In part 1 of this study, I narrowed critical region of the hooded locus and revealed that only *Kit* gene, known as a marker of melanocyte and one of coat color genes, exists in this region through genetic fine mapping using backcrosses from feral rat-derived inbred strain IS and hooded phenotype strain, LEA. Although a G to C transversion was observed in exon 2 of the *Kit* gene, it was synonymous substitution. Further, the expressions of *Kit* mRNA were not different in fetal neural tubes and neonatal and adult skins between IS and LEA rats. Furthermore, Kit-positive cells were observed in LEA rat abdominal skin in spite of the absence of melanin in this region. These results suggest that the synthesis of melanin is impaired possibly due to the malfunction of *Kit*-expressed melanocytes residing in the

non-pigmented hair follicles of hooded phenotype rats. However, substantial mutation of the *Kit* gene and the mechanisms by which Kit impairs the function of melanocytes in non-pigmented hair follicle remain unknown.

In part 2 of this study, I carried out genetic linkage studies using BN and LEA rats to clarify genetic control in the extent of the hooded phenotype. A genome-wide scan was conducted on 152 F_2 rats for linkage with ratio of pigmented coat area for the dorsal, ventral, and total regions. The result indicated that a major QTL was mapped to *D14Got40*, which is the microsatellite marker closely present to the hooded locus. In addition, another QTL, *D17Rat2* showing highly significant linkage was also detected on Chr 17 in dorsal region phenotype as well as a QTL showing suggestive linkage on Chr15 in ventral region phenotype. I further investigated a genome-wide scan for epistatic interactions and detected significant interactions between *D14Got40* and *D20Mit1*, and between *D14Got40* and *D17Rat2* in dorsal region phenotype. These results suggest that the hooded locus regulates the extent of the hooded phenotype with some modifier genes.

This study proposes that *Kit* is a strong candidate gene responsible for the hooded locus and some genetic loci modify the extent of the hooded phenotype, although the precise mechanisms of them are still unclear. Thus, further study is necessary to understand the

mechanisms by which the hooded phenotype appears with various extent of pigmented ratio in the rat.

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Summary in Japanese

げっ歯類における被毛色研究は、その明らかな表現型故に古くから研究がなされており、現在までに約 150 個の遺伝子座と約 1,000 個の対立遺伝子の存在が明らかとなっている。一般的に被毛色は、発生初期段階において神経堤より派生する神経堤細胞から分化、誘導されるメラノサイトが産生するメラニンによって規定される。神経堤細胞はメラノサイト以外にも骨細胞や軟骨細胞、末梢神経細胞、内分泌細胞など様々な系列に分化できる多能性を持っており、ワーデンブルグ症候群に代表される神経堤細胞の分化異常の疾患では、被毛色異常と同時に難聴や腸管神経節欠損など、皮膚以外の臓器にも異常が見られることが報告されている。

頭巾斑表現型はラット特有の被毛色表現型で、その遺伝子座は第 14 染色体に座位し常染色体劣性遺伝様式を示すことが明らかとなっている。頭巾斑遺伝子座には複数の対立遺伝子が存在することや、頭巾斑の表現型を修飾する遺伝子座が存在することが報告されているが、それらの責任遺伝子は未だに明らかとなっていない。また、頭巾斑表現型は被毛色のみに現れ、その他の神経堤細胞由来の細胞系列は正常であると考えられる。従って、頭巾斑遺伝子座の解析は被毛色異常のメカニズムを明らかにするだけでなく、神経堤細胞の分化、遊走のメカニズム解明にも有用であると考えら

れる。よって本研究では、頭巾斑表現型ならびに頭巾斑表現型修飾因子の遺伝学的解析を試みた。

第一章では野生型の IS ラットと頭巾斑表現型の LEA ラットの戻し交配個体 795 匹を用いて詳細マッピングを行った。その結果、頭巾斑遺伝子座はマイクロサテライトマーカー*D14Hok1* と強く連鎖し、*D14Rat84* から *D14Got40* までの約 0.4 Mbp に存在することを明らかにした。この領域に含まれるタンパク質をコードする遺伝子は *Kit* のみであり、ゼブラフィッシュからヒトに至るまで多くの脊椎動物において *Kit* 遺伝子の変異により色素産生異常を呈することが報告されている。このことから *Kit* が頭巾斑遺伝子座の有力な原因遺伝子であると考えられた。しかしながら *Kit* のコーディング領域には野生型と頭巾斑表現型の間で 1 つの同義変異が存在するのみで、胎齢期から新生子期、成体まで *Kit* の mRNA の発現量に有意な差は見られなかった。さらに、免疫染色によるとメラニンが存在しない LEA ラットの白色被毛部位においてもメラノサイトマーカーである Kit 陽性の細胞が検出された。このことから頭巾斑表現型は *Kit* の単純な発現量の変化ではなく、何らかのメカニズムによるメラノサイトの機能異常が原因であると推測された。

次に、頭巾斑表現型の修飾遺伝子座を同定するために、野生型に類似した表現型を示す頭巾斑の対立遺伝子 h^i を保持する BN ラットと LEA ラットを用いて遺伝解析

を行った。F_2 個体において体表面積に占める有色被毛で覆われた面積を背側、腹側、全体についてそれぞれ調べたところ、その値は全て明らかな 2 群に分離せず連続的な値を示したことから複数の遺伝子座による制御が示唆された。そこで QTL 解析を行ったところ、全ての場合において *D14Got40* 近傍に非常に強い QTL が検出された。また背側における第 17 染色体に強い QTL が、ならびに腹側における第 15 染色体に弱い QTL が検出された。次に遺伝子座間の相互作用、エピスタシス解析を行ったところ、背側の表現型に関して *D14Got40* と *D20Mit1* 間、および *D14Got40* と *D17Rat2* 間において強い相互作用があることが明らかとなった。*D14Got40* は頭巾斑遺伝子座のまさに近傍であり、これらの結果から頭巾斑遺伝子座そのものがその他の遺伝子座と相互作用して頭巾斑表現型を調節している事、つまり *Kit* と他の遺伝子座にコードされるタンパク質の相互作用が頭巾斑表現型の調節に重要である事が示唆された。