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ORIGINAL ARTICLE

Mice Transgenic for Kit^{V620A}: Recapitulation of Piebaldism but not Progressive Depigmentation Seen in Humans with this Mutation

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Piebaldism is an autosomal dominant genetic pigmentary disorder, characterized by congenital white hair and patches located on the forehead, anterior trunk, and extremities. Most piebald patients have a mutation of the *KIT* gene, which encodes a tyrosine kinase receptor involved in pigment cell development. The white hair and patches of such patients are already completely formed at birth and do not usually expand thereafter. This stability of pigmented spots also applies to *Kit*^{W} and *Kitl*^{Sl} mutant mice. However, two novel cases of piebaldism were reported in 2001, in which both mother and daughter having a novel Val620Ala mutation in their *KIT* gene showed progressive depigmentation. To prepare an animal model of this mutation, to explore undefined functions of KIT signaling for maintaining pigmented melanocytes in the skin or more specifically the integrity of the melanocyte stem cell system in the postnatal skin, we produced transgenic mice expressing Val620Ala Kit. These mice well mimicked the white spotting pattern of patients; however, no change in this pattern was observed after birth, even after increasing the transgene expression by various means. Here, we report the unexpectedly extremely stable maintenance of the melanocyte stem cell system under stringent conditions for KIT signaling.

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INTRODUCTION

Piebaldism is a relatively rare autosomal dominant genetic pigmentary disorder, characterized by congenital white hair and patches located on the forehead, anterior trunk, and extremities (Keeler, 1934; Froggat, 1951; Cooke, 1952; Fleischman, 1993; Baxter *et al.*, 2004). In the region of these white patches, melanocytes are almost totally lacking, and therefore piebaldism is thought to be because of defective proliferation, differentiation, or migration of melanocyte precursor or stem cells from the neural crest during development. Melanocytes develop from neural crest cells, and in mice their progenitors migrate from the neural crest to the dermis lateroventrally to cover the whole skin. Melanocytes that migrate into developing bulbs of hair follicles become established there to give rise to melanized hair. The generation of these cells from neural crest stem cells depends on a complex interplay between cell-extrinsic and cell-intrinsic factors (Dorsky *et al.*, 2000; Dupin and Le Douarin, 2003; Hirobe, 2005). Genetic evidence indicates that pigment cell development requires steel factor (SLF, also called KIT-ligand, stem cell factor, or mast cell growth factor), which acts through the tyrosine kinase receptor KIT. The proto-oncogene *Kit/KIT* is a gene encoding a tyrosine kinase receptor and has been mapped to the *dominant white spotting* locus, and the ligand SLF has been mapped to the *Steel* locus (Geissler *et al.*, 1988; Copeland *et al.*, 1990; Kunisada *et al.*, 2001; Yoshida *et al.*, 2001; Wehrle-Haller, 2003). Pigment cell development further depends on endothelin 3, which acts through the G-protein-coupled endothelin receptor B (Baynash *et al.*, 1994; Pla and Larue, 2003) and also on transcription factors that include the SRY-like high mobility group protein SOX10 and the basic helix-loop-helix leucine zipper protein microphthalmia transcription factor (Hodgkinson *et al.*, 1993; Tachibana *et al.*, 1994; Southard-Smith *et al.*, 1998). Most patients with piebaldism have a mutation of their *KIT* gene; and many mutant *KIT* genes including those because of a missense mutation, nonsense mutation, frame-shift mutation, splice-site mutation, in-frame mutation, and various complete and partial large deletions have been reported to exist in individuals with piebaldism (Fleischman *et al.*, 1991; Giebel and Spritz, 1991; Spritz *et al.*, 1992; Fleischman, 1992; Murakami *et al.*, 2004).

Although there seems to be a difference in clinical severity with respect to the location or pattern of the *KIT* gene muta-

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Abbreviations: ACK2, anti-c-kit monoclonal antibody; MT-I, metallothionein-I; SLF, steel factor; Tg, transgenic

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tion, the stability of unpigmented skin regions throughout life is known as one of the common characteristics among patients with piebaldism. In these patients, usually the white hair and patches have already completely formed at birth or within several months after birth. Throughout life thereafter, neither expansion of already formed patches nor the appearance of new ones has been seen. This is also the case in *Kit*^{W} or *Kitl*^{Sl} mutant mice, in some of which once acquired unpigmented spots (in some cases better to be referred as white spots) are stably maintained throughout life, as far as we observed for *Kit*$^{W57/W57}$, *Kit*$^{W/+}$, *Kit*$^{Wv/+}$, *Kitl*$^{Sl/+}$ mice during the maintenance of these lines. In addition, unpigmented spots of other mutant mice such as *Mitf*$^{mi/+}$ and *Sox10*$^{DOM/+}$ do not change. In these mutant mice, genetic lesions are severe enough to prevent cells of the melanocyte lineage from developing properly to cover most of the skin; however, these lesions do not affect the postnatal development of melanocytes in the hair follicles. Clearly, the threshold of signals required for melanocyte development is different in embryonic and postnatal development.

Considering these facts, the novel case, reported in 2001, that a mother and daughter with piebaldism showed progressive depigmentation was quite exceptional (Richards *et al.*, 2001). This paper noted that these patients with piebaldism were born with a white forelock and that their first patch of depigmentation developed within half a year after birth. But surprisingly, their depigmentation gradually progressed during childhood. It should be emphasized that the depigmentation could be distinguished from vitiligo by its congenital nature with whitening of hair as well as skin; however, the pigmented border region around the white spots receded over time. Then a novel Val620Ala (1859T>C) mutation in the cytoplasmic kinase insert region of the *KIT* gene was found in these patients. The mechanism underlying the effect of this exceptional *KIT* mutation may reveal some previously unknown function of KIT signaling to maintain pigmented melanocytes in the skin or more specifically the integrity of the melanocyte stem cell system in the postnatal skin, including self-renewal of melanocyte stem cells during the hair cycle. Aiming to reproduce this phenomenon in an animal model, we produced transgenic (Tg) mice expressing cDNA with the same Val620Ala mutation in the *Kit* gene.

RESULTS

Rationale for constructing mouse version of KIT^{V620A} mutant

To create a phenotype as close to that of the human piebaldism because of the KIT^{V620A} mutation in a mouse model, we used the mouse Kit cDNA sequence rather than the human KIT cDNA for the following reasons: Firstly, overall ligand specificity of KIT molecules might be different between mouse and human, because the amino-acid sequence diversity of the ligand-binding extracellular region of KIT is prominent in these species (Marklund *et al.*, 1999). In fact, although mouse Slf stimulates human KIT as effectively as it does mouse Kit, human SLF is known to act less effectively when stimulating mouse Kit (Zsebo *et al.*, 1990; Lev *et al.*, 1992; Majumdar *et al.*, 1996). Secondly, the region containing the KIT^{V620A} mutation site is extremely well preserved in human and mouse; that is, among the 114 amino-acid residues from 549 to 662 of the cytoplasmic tyrosine kinase domain of the two species containing the Val620Ala mutation, only one isoleucine to valine alteration was found. Therefore, if the KIT^{V620A} mutation itself represents an intrinsic trait for the specific phenotype of progressive human piebaldism, the same amino-acid change in the extremely well-conserved sequence in the mouse Kit cDNA would be likely to express the same phenotype in the mouse model. Actually a previous report showed that the phenotype produced by the same *Kit/KIT* mutation in two different species, human and mouse, was very similar (Fleischman, 1992). As the dominant-negative effects of mutant KIT molecules was previously well established (Ray *et al.*, 1991; Spritz *et al.*, 1992), we sought to express a significant amount of Kit^{V620A} cDNA in mouse melanocytes by using the metallothionein-I (MT-I) promoter sequence (Palmiter *et al.*, 1993).

Establishment of mt-Kit^{V620A} Tg mice recapitulating human piebaldism and expression of transgene mRNAs in their skin

Seven independent Tg mice expressing the mutant *Kit* (mt-*Kit*) transgene driven by the MT-I promoter were identified by PCR. Four of them stably transmitted their white hair color phenotypes and were named mt-Kit^{V620A} Tg-1 to 4. Two lines, mt- Kit^{V620A} Tg-1 and mt-Kit^{V620A} Tg-2, showed a similar coat color pattern during postnatal development; namely, they displayed white-spotted areas on their forehead, the back along the posterior median line, and the ventral side (Figure 1a). The shape of the white spot in each area was very close, that is, a diamond-shaped white spot on the forehead,

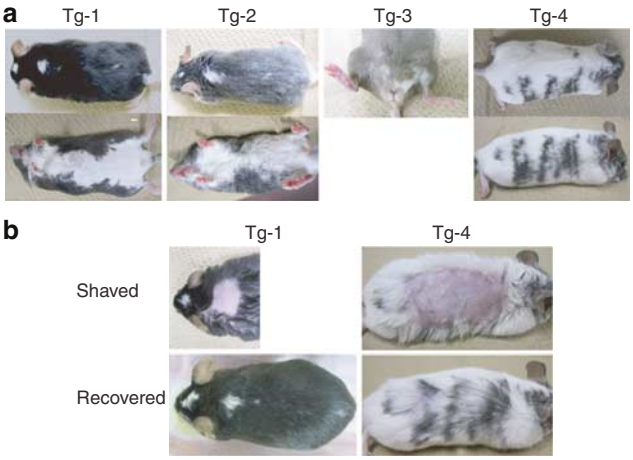

Figure 1. Coat color phenotypes of 4-month-old mt-Kit^{V620A} Tg mice and the stability of the coat color pattern. (**a**) Founders of Tg mouse lines mt-Kit^{V620A} Tg-1 to -4 are shown. The mt-Kit^{V620A} Tg-1 F_0 and Tg-2 F_0 had a few white spots on their forehead and back, and a wide range of white region on their ventral side, whereas the mt-Kit^{V620A} Tg-3 F_0 had only a narrow white spot around its external genitalia. The fourth founder, mt-Kit^{V620A} Tg-4 F_0, had a wide region of white hair, with the remaining normal black hair extending asymmetrically and zonally from the dorsal midline toward the ventral side. (**b**) These photographed mice, mt-Kit^{V620A} Tg-1 and Tg-4, were partially shaved; and their coat color pattern recovered precisely to their respective original ones shown in (**a**) after 3 weeks.

larger diamond-shaped and small-size white spots on the back, and a large white spot covering most of the ventral side. This coat color pattern well corresponds to the typical pigmentation pattern of *W* mutant mice (Silvers, 1979; Nocka *et al.*, 1990; Besmer *et al.*, 1993) and patients with piebaldism (Keeler, 1934; Froggat, 1951; Cooke, 1952), thus suggesting that the pattern is likely a consequence of impaired Kit signaling by the transgene. All progenies produced by mating these mice with C57BL/6 displayed a similar white spot pattern even after the fourth generation, indicating that this pattern is a genetically transmitted characteristic as in *W* mutant mice. Another line, mt-Kit^{V620A} Tg-3, showed only a narrow white spot around the external genitalia (Figure 1a). The Tg mouse that expressed the most abnormal phenotype for coat color was line 4, mt- Kit^{V620A} Tg-4. The white-spotted area of this mouse reached 70% or more of the body surface. Areas of pigmented hair appeared on the right side of the body as did black stripes that extended from the dorsal midline toward the ventral side, as shown in Figure 1a. Progenies produced by mating Tg-4 with C57BL/6 showed similar patterns varying in different width, length, and number of the pigmented areas: but all maintained the common phenotype of asymmetrical and narrow lines of pigmented hairs extending from the back to the abdomen. Even after four or more generations, these line 4 mice had a diamond-shaped white spot on their forehead, and the specific coat pattern of the founder. Thus, we also regarded the line 4 phenotype as a stably transmitted genetical trait. All mice of mt-Kit^{V620A} Tg-1–4 had black eyes, and none showed any symptoms of anemia or infertility (data not shown).

Expression of the transgene mRNA in the skin of the mt-Kit^{V620A} Tg-1 and mt- Kit^{V620A} Tg-4 mice was examined at 4 weeks after birth. Reverse transcription-PCR analysis was performed using total RNAs extracted from the shaved skin and liver. Transgene mRNA expression was detected in tissues of Tg mice, not in wild-type littermates (Figure 2a). Of note, the transgene mRNA expression level of mt-Kit^{V620A} Tg-4 was obviously higher than that of mt-Kit^{V620A} Tg-1. This difference may well correlate with the fact that the white spot region was eminently wider in mt-Kit^{V620A} Tg-4 than that in mt-Kit^{V620A} Tg-1, given that the dominant-negative effect of the mt-Kit^{V620A} transgene should increase with the increase in the amount of the mt-Kit^{V620A} message. Next, we examined whether there was a difference in the level of transgene expression between the white and colored parts of the skin of mt-Kit^{V620A} Tg-1. Slightly more transgene expression was observed in the colored skin area (Figure 2b). However, the almost comparable expression in the white skin area indicates that expression of the transgene in melanocytes of the pigmented region was overwhelmed by the vast majority of skin keratinocytes that might also express the MT-I-driven transgene. Thus, we successfully established a mouse model of the reported human piebaldism with a Val620Ala mutation in the *KIT* gene (Richards *et al.*, 2001).

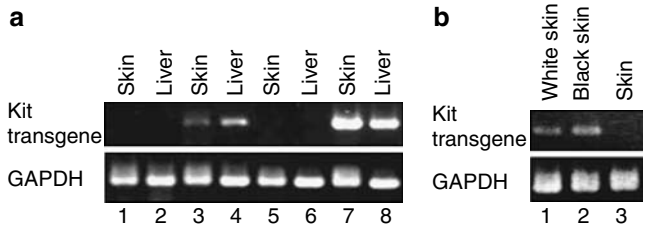

Figure 2. Reverse transcription-PCR assay for the expression of mutant Kit mRNA of mt-Kit^{V620A} Tg-1 and mt-Kit^{V620A} Tg-4 mice. Total RNAs extracted from the mouse skin and liver were used with the primer set MT-F and MT-R to amplify Tg KIT mRNA. (**a**) Lanes 1, 2: non-Tg littermate mouse of mt-Kit^{V620A} Tg-1; lanes 3, 4: mt-Kit^{V620A} Tg-1; lanes 5, 6: non-Tg littermate mouse of mt-Kit^{V620A} Tg-4; lanes 7, 8: mt-Kit^{V620A} Tg-4. All mice were analyzed at 4 weeks of age. The mutant Kit mRNA expression level, normalized with respect to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), in mt-Kit^{V620A} Tg-4 (lanes 7 and 8) was obviously higher than that in mt-Kit^{V620A} Tg-1 (lanes 3 and 4) in both tissues. (**b**) RNA was extracted from a white area (lane 1) and colored area (lane 2) of a 6-week-old mt-Kit^{V620A} Tg-1. In lane 3, skin of a non-Tg littermate was used.

Stable maintenance of coat color phenotypes of mt-Kit^{V620A} Tg mice

We next determined whether the change in the pattern of the coat color would take place during the postnatal life of the Tg mice, because clinical study had reported this Val620Ala mutation to cause piebaldism with progressive depigmentation (Richards *et al.*, 2001). However, none of the founders of mt-Kit^{V620A} Tg-1–4 lines exhibited expansion of their white-spotted areas throughout life (data not shown). Then, all progenies resulting from mt-Kit^{V620A} Tg-1 and -4 founder Tg animals were crossed with C57BL/6 mice and were also observed. As early as 4 days after birth, when the first cycles of hair formation begin, the characteristic coat color patterns were visible in both lines; but progressive depigmentation was not seen thereafter throughout their entire life (Figure 3).

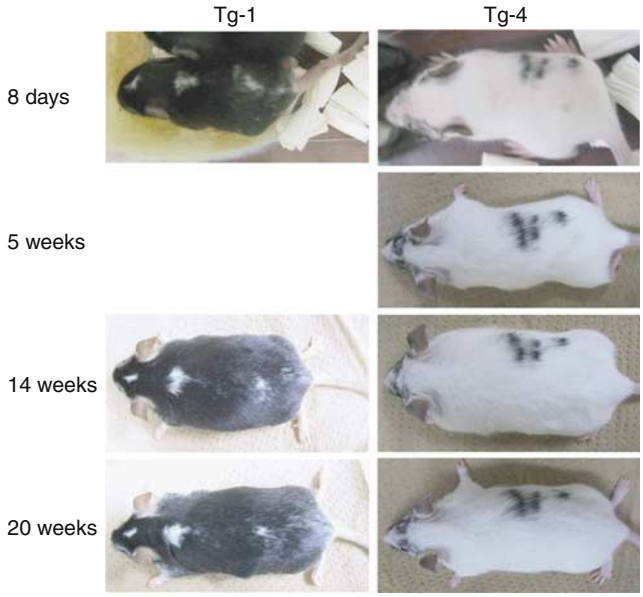

Figure 3. Stability of coat color phenotypes of mt-Kit^{V620A} Tg mice throughout life. The coat color of mt-Kit^{V620A} Tg-1 F_1 and mt-Kit^{V620A} Tg-4 F_1 mice at the various ages indicated is shown. For both lines, no noticeable change in the coat color pattern was observed after birth.

It is conceivable that depigmentation of the skin is accompanied by the loss of melanocytes caused by the reduced self-renewal of melanocyte stem cells in the hair follicles. To facilitate self-renewal of melanocyte stem cells and thus increase the chance to visualize depigmentation induced by the loss of melanocytes, we shaved the hair of mt-Kit^{V620A} Tg-1 and -4 mice (Figure 1b). By this stimulation and after the entry into a new hair cycle (Hattori and Ogawa, 1983; Ogawa and Hattori, 1983), the hair of all of the Tg mice maintained exactly the same pattern as seen before shaving (Figure 1b). Even after a second or third shaving of these mice, we could not see any change in the pigmentation patterns (data not shown).

Upregulation of transgene expression by Zn intake still insufficient to induce postnatal induction of depigmentation

No spreading of white spot regions in Kit^{V620A} Tg lines even after several rounds of stimulation of melanocyte self-renewal might still be attributed to an insufficient amount of dominant-negative Kit^{V620A} messages from the transgene. So we then took advantage of the fact that Zn ions are able to upregulate MT-I promoter activity, as was demonstrated in previous studies (Palmiter *et al.*, 1993; Bottinger *et al.*, 1997; Kindy *et al.*, 2000). Induction of the transgene was attained by the addition of $ZnSO_4$ to the drinking water (25 mM) for 2 weeks. Nine-week-old mt-Kit^{V620A} Tg-1 and -4 mice with littermates were thus administered Zn. After 2 weeks, a significant increase in transgene expression was observed in the skin and liver (Figure 4a). In either Tg-1 or -4 Zn-treated mice, however, there was no expansion of the white spot regions thereafter (Figure 4b). In addition, we explored the effect of Zn-induced upregulation of the transgene continuously from newborn to young adult of mt-Kit^{V620A} Tg-1 and -4 mice; and in both cases, no hair color pattern change was observed, regardless of the shaving of the hair (data not shown). Any increase in transgene expression attained by the zinc administration did not result in extension of the white hair regions in Kit^{V620A} Tg mice after birth.

To increase the total expression level of the transgene two-fold, we produced homozygous mt-Kit^{V620A} Tg-1 mice. As expected, these mice had a greatly increased white spot region (Figure 4c) from neonatal stages; nevertheless, these homozygous mice did not show any depigmentation in postnatal life.

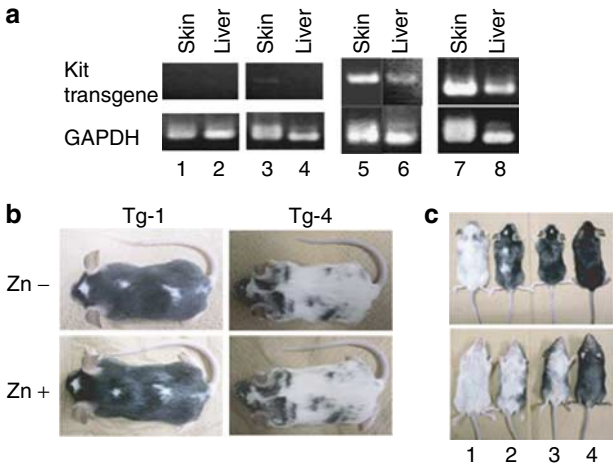

Figure 4. Upregulation of transgene expression by Zn intake and production of homozygous Tg mice. For 2 weeks 9-month-old mt-Kit^{V620A} Tg-1 and Tg-4 and their littermates were provided drinking water containing $ZnSO_4$ (25 mM). (**a**) RNA samples extracted from the Tg-1 mouse skin and liver before and after Zn treatment were used to amplify the *Kit* transgene. Lanes 1, 2: non-Tg littermate mouse before Zn treatment; lanes 3, 4: non-Tg littermate mouse after Zn treatment; lanes 5, 6: mt-Kit^{V620A} Tg-1 before Zn treatment; lanes 7, 8: mt-Kit^{V620A} Tg-1 after Zn treatment. Increased transgene expression in the skin and liver of mt-Kit^{V620A} Tg-1 mice was observed. (**b**) In Zn-treated mt-Kit^{V620A} Tg-1 and Tg-4 mice, the white spot patterns were the same before (indicated as Zn−) and after (indicated as Zn+) Zn treatment. (**c**) Homozygous mt-Kit^{V620A} Tg-1 mice were produced. Genetic crossing of heterozygous Tg mice produced homozygous (column 1), heterozygous (columns 2 and 3), and wild-type (column 4) mice.

Pigmentation pattern of SLF and mt-Kit^{V620A} double Tg mice with pigmented interfollicular skin

Unlike human ones, skin melanocytes in mice survive only in the hair follicles after birth (Hirobe, 1984); and, therefore, we may not simply compare the size of the pigmented area in these two species. To convert the mouse skin so as to permit melanocyte survival in the interfollicular skin as occurs in human skin, we previously established Tg mice expressing SLF, the ligand for KIT, in skin keratinocytes by using the cytokeratin 14 promoter sequence (SLFTg, Kunisada *et al.*, 1998). These Tg mice were covered with pigmented keratinocytes, and melanocytes were maintained in the epidermis of the interfollicular skin throughout life. Our double Tg mice, SLFTg/+/mt-Kit^{V620A} Tg-1/+ and SLFTg/+/mt-Kit^{V620A} Tg-4/+, had a characteristic hair pigmentation pattern comparable to that of each Kit^{V620A} Tg line, with the pigmented interfollicular skin strictly restricted to the region covered with pigmented hair (Figure 5a). We observed a significant increase in the size of the pigmented area (Figure 5b), as had been observed in double Tg mice of SLFTg and W^{57}/W^{57}, W/W^{57}, or *bt/bt* mutants, which might have occurred during embryogenesis (Kunisada *et al.*, 1998). Also in this case, even in human-like skin with interfollicular melanocytes in these SLF and mt-Kit^{V620A} double Tg mice, we could not detect postnatal changes in pigmentation patterns in either the interfollicular skin or hair, even after shaving of the pigmented hair or Zn administration (data not shown).

Requirement of KIT signaling to maintain melanocyte stem cells in pigmented skin areas

In previous sections, all attempts to increase dominant-negative Kit^{V620A} expression in Tg mice to recapitulate the progressive depigmentation observed in the patients with this mutation resulted in rather stable maintenance of the pigment pattern formed during development, even in the mice that had human-like interfollicular skin melanocytes. We therefore speculated that these Tg mice somehow lost their requirement for KIT signals for the survival of melanocyte lineage cells. To test this possibility, we administered anti-c-kit monoclonal antibody (ACK2) to neonatal Kit^{V620A} Tg mice to completely block the KIT signal (Nishikawa *et al.*, 1991).

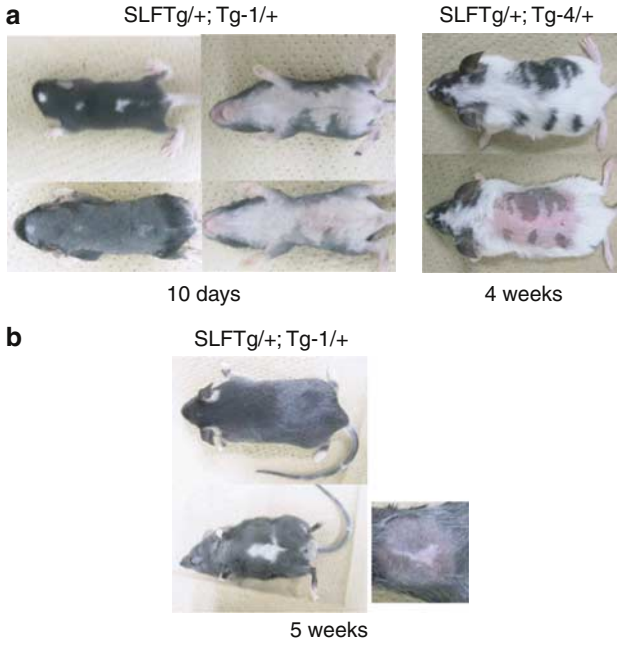

Figure 5. Pigmentation pattern of SLF and mt-Kit^{V620A} double Tg mice. (**a**) Our double Tg mice, SLFTg/+/mt-Kit^{V620A} Tg-1/+ and SLFTg/+/mt-Kit^{V620A} Tg-4/+, had a characteristic hair pigmentation pattern comparable to that of each Kit^{V620A} Tg line, with pigmented interfollicular skin strictly restricted to the region covered with pigmented hair. Part of the coat hair was shaved to visualize the interfollicular area of melanization, as shown in the lower panels. (**b**) In some SLFTg/+/mt-Kit^{V620A} Tg-1/+ mice, the formation of an apparently wider pigmented area was observed (compare with SLFTg/+/mt-Kit^{V620A} Tg-1/+ in (**a**)), although the coat color pattern after birth did not change as indicated in the lower panel. Part of the coat hair was shaved in the right panel.

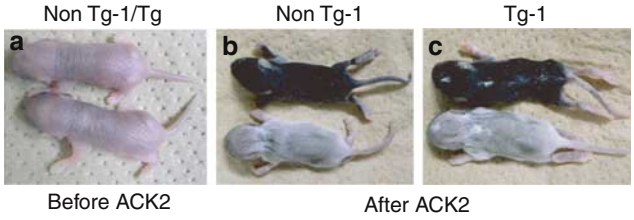

Figure 6. Effect of ACK2 on the coat color of mt-Kit^{V620A} Tg mice. Kit^{V620A} Tg mice and non-Tg littermates were subcutaneously injected with 0.5 mg ACK2 (anti-Kit) on days 3, 5, 7, 9 after birth. (**a**) A 3-day-old non-Tg (upper mouse) and an mt-Kit^{V620A} Tg-1 (lower mouse) before ACK2 administration. (**b**) A non-Tg without injection (upper) and a non-Tg with ACK2 injection (lower) on day 13 after birth. (**c**) A mt-Kit^{V620A} Tg-1 without injection (upper) and a mt-Kit^{V620A} Tg-1 with ACK2-injection (lower) on day 13 after birth. As in the case of the non-Tg mice, the pigmented areas of mt-Kit^{V620A} Tg-1 mice showed a prompt loss of their pigmented areas by day 10 after the first injection.

After administration of the antibody, the pigmented areas of mt-Kit^{V620A} Tg-1/+ or mt-Kit^{V620A} Tg-4/+ mice were promptly lost (Figure 6), indicating that requirement of KIT signals for the development of melanocytes from their precursors or stem cells was not canceled in these Tg mice.

Influence of the mt-Kit^{V620A} transgene on mast cell numbers – implication that the phenotype of Tg mice was a direct effect of the transgene

The hematopoietic system in mice with a mutation at the *KIT^{W}* locus is affected in several ways. Effects are seen on the stem cell compartment, the erythroid cell lineage, and mast cells (Geissler *et al.*, 1981; Lyon and Glenister, 1982). It was reported that ectopic expression of a *KIT^{W42}* minigene in Tg mice affects mast cell development (Ray *et al.*, 1991). To evaluate whether transgene expression affected mast cells in mt-Kit^{V620A} Tg, we determined the number of mast cells in skin sections prepared from these mice. Skin specimens of mt-Kit^{V620A} Tg-1 and mt-Kit^{V620A} Tg-4 mice contained 38% (20.0 + 2.6/cm) and 69% (36.4 + 3.1/cm), respectively, of the number of mast cells found in the non-Tg littermates (50.8 + 17.8/cm). Thus, we can conclude that the mt-Kit^{V620A} Tg transgene also affected mast cell development. Collectively with the coat color changes, these phenotypic changes indicate a dominant-negative effect of the transgene on KIT signaling.

DISCUSSION

A human piebaldism due to a Val620Ala mutation in the *KIT* gene was recapitulated in mice by introducing the same mutation in the mouse *Kit* gene followed by forced expression in melanocytes. Four independent depigmented Tg lines also showed the loss of mast cells, indicating that loss of KIT activities induced by Val620Ala is the cause of this phenotype.

The variability of the coat color phenotype appears to be the result of clonal variation of the expression of the transgene. In all four of the Tg lines, mt-Kit^{V620A} Tg-1–4, both the site of chromosomal insertion and the number of copies of the transgene, are presumed to be random. But we did not test to see whether different copy numbers of the transgene had been inserted, because there is no general relationship between the copy number and the level of expression (Hammer *et al.*, 1987). However, it is very well established that expression of transgenes is influenced by position effects imposed by *cis*-acting factors at the integration sites and by strain-specific *trans*-acting factors that may induce epigenetic modification of these loci (Lacy *et al.*, 1983; Allen *et al.*, 1988, 1990; Kothary *et al.*, 1988; Engler *et al.*, 1991). Thus, position effects and/or differences in genetic background (Lamoreux, 1999) may determine the variations in penetrance and expressivity observed in mt-Kit^{V620A} Tg-1–4 mice.

We then tried to find in these mice the progression of depigmentation observed specifically in the human Val620Ala mutation in postnatal life. However, we did not see any changes in the pigmentation pattern in any of the Tg lines throughout life, even after forced hair cycle progression by shaving. Furthermore, neither Zn-mediated augmentation of the transgene regulated by the metallothionein promoter sequence nor a doubling of the gene dosage by creating homozygous Tg mice led to any increase in the depigmented area after birth in any of the individual mice examined.

We next hypothesized that the difference in phenotypes of Val620Ala mutations in mice and human might be attributed

to the difference in the distribution of the melanocytes in the skin in these two species, as there are fewer interfollicular melanocytes in the adult mouse skin than in human skin. Therefore, we crossed mt-Kit^{V620A} Tg mice with Tg mice expressing SLF in which pigmented keratinocytes and melanocytes were maintained in the epidermis of the interfollicular skin. Also in these double Tg animals, we never observed any increase in the white spotted region after birth. Under the condition in which melanocytes in the interfollicular skin were stably maintained as in human skin, the Val620Ala mutation in the mouse *Kit* gene did not induce progression of depigmentation as observed specifically for the same mutation in humans.

Our study with Tg mice provides evidence that the *KIT* Val620Ala mutation itself is not sufficient to give the very unusual phenotype of progressive loss of pigmented areas. The most obvious alternative explanation for the piebald mother and daughter, as pointed out by the authors of the original report, is that a mutation in a second, unknown gene might have led to the increased susceptibility for progressive pigment loss. This presumption can be supported by the finding that some other family members showed minor postnatal pigmentary loss without having the *KIT* mutation (Richards *et al.*, 2001). Thus, this particular *KIT* mutation would then be no different from others discovered nearby in the coding sequence. As other possible alternative explanations for our negative observations, we may speculate that there is some fundamental difference between human and mouse melanocyte maintenance, that the use of the MT-I promoter instead of the KIT promoter contributed to our results, or that the lifespan of mice is just too short to allow the expansion of the depigmented areas observed in the human kindred over many years. But in order to distinguish among these different explanations and specify the mechanism for appearance of the human novel phenotype, more additional analysis concerning the reported family as well as further clarification of many unknown aspects of the generation, differentiation, maintenance, and survival of melanocytes in both human and mice is necessary.

In mt-Kit^{V620A} Tg-1 and mt-Kit^{V620A} Tg-4 mice, the number of mast cells was 38 and 69%, respectively, of that for each control mice. Although this result is in contrast to the pigmentary aspects of the mt-Kit^{V620A} Tg-1 and mt-Kit^{V620A} Tg-4 mice, it agrees with a previous report on *Kit* Tg mice (Ray *et al.*, 1991). As the previous report has also described, these results may indicate different levels of transgene expression in mast cells and melanoblasts of the two lines; and they may reflect the different microenvironments of connective tissue mast cells and melanocytes in the skin. For example, whereas mast cells are found only in the dermis, melanocytes are found in the dermis and in the epidermis (hair follicles; Nakayama *et al.*, 1988).

There exist mainly two phases requiring KIT signals in melanocyte development: firstly in the early development of melanocyte stem cells soon after their specification from neural crest cells in the embryonic trunk up to their entry into developing hair follicles in the skin, and secondly in the cyclic activation of melanocyte stem cells in the hair follicles throughout postnatal life. In the first phase, the pattern of the final melanocyte distribution in the skin are sensitively affected by KIT activities and the characteristic white spottings are indicative of individual *Kit* mutations that result in different amounts of KIT tyrosine kinase activity (Yoshida *et al.*, 1993). In other words, the extent of white spotted area is completely determined in this first developmental stage by the amount of available KIT activities in melanocyte precursors. In contrast, after birth the melanocytes are stably maintained in the pigmented area; and the proportions of their existing area characteristic to individual spotting patterns are strictly conserved along with the growth of the body. In the second phase, melanocytes or their precursors seem to require the least amount of KIT signaling. In the case of mt-Kit^{V620A} Tg-4/+ mice, only several bands of pigmented hair were formed in the trunk region, indicating that almost all of the KIT activities were blocked by the expression of the Val620Ala mutant *Kit* gene in the embryonic skin. In this extreme case and even after shaving the hair of the Tg mice to stimulate KIT-dependent growth of melanocyte stem cells in the hair follicles, no indication of progressive loss of pigmented area including hair graying was observed. Therefore, unexpectedly we demonstrated an extremely stable maintenance of the melanocyte stem cell system under stringent conditions for KIT signaling. However, as previously indicated (Nishikawa *et al.*, 1991; Okura *et al.*, 1995; Kunisada *et al.*, 1998; Botchkareva *et al.*, 2001), complete blockade of KIT signals by the administration of anti-KIT antibody faded the once established pigmented areas in these Tg mice, indicating that at least a small level of KIT signaling was required for their survival.

MATERIALS AND METHODS

Transgene construct

The parent expression vector 2999 (Palmiter *et al.*, 1993) was obtained from Dr Richard Palmiter (University of Washington, Seattle). This vector has 814 bp of the mouse MT-I promoter fused via an *Nru*I linker to 650 bp of the human growth hormone 3′ untranslated region and polyadenylation signal, and has 10 kb of MT locus containing a 5′ DNase hypersensitive site and 7 kb of it containing a 3′ DNase hypersensitive site. Unique *Sal*I (or *Cla*I and *Sst*I) sites flanking the mouse sequences were used to remove the Bluescript-based vector sequences. Because we had difficulty in cloning a cDNA directly into the *Nru*I-digested blunt ends of this large plasmid, we used the following strategy: Vector 2999 was digested with *Not*I to isolate a 1.4-kb fragment containing 814 bp of the MT-I promoter, the *Nru*I cloning site, and 650 bp of the human growth hormone 3′ untranslated region. This fragment was then inserted into the *Not*I site of pBluescript II KS(+). After the mouse Val620Ala Kit cDNA fragment, described later, had been inserted into the *Nru*I site of this plasmid, the plasmid was then digested with *Not*I. The isolated *Not*I fragment was ligated with the *Not*I-digested 20-kb fragment of 2,999 from which the MT-I promoter, the *Nru*I cloning site, and the human growth hormone 3′ untranslated region had been removed, thus creating 2,999MT− mVal620Ala Kit.

The mouse Val620Ala Kit cDNA fragment was prepared as follows: pBluescript II KS(+) lacking an *Apa*I site (pBSδ*Apa*I) was prepared by digestion of pBluescript II KS(+) with *Apa*I and

was blunt ended and self-ligated. The mouse Kit cDNA cloned into the *Eco*RI and *Hind*III site of pUC19 was digested with *Eco*RI and *Hind*III, and the resulting 2.9-kb Kit cDNA fragment was then inserted into the *Eco*RI and *Hind*III site of pBSδApaI and designated as pBSδApaI/mKit. A 196-bp DNA fragment containing a Val620Ala (corresponding to Val619Ala, 1,856 T→C in the mouse *Kit* gene) mutation was synthesized by PCR using pBSδ*Apa*I/mKit as a template with the following primer set: KitVal620Ala-Sense (5′-CTGCATATGGCTTGATTAAGTCGGATGCTGCCATGACAGCTG CCG-3′; containing an *Nde*I site and 1,856 T→C mutation), and KitVal620Ala-Antisense (5′-TATTCTGTAATGACCAGGGTGGGCCC TCC-3′; containing an *Apa*I site). Presence of the mutation was confirmed by direct sequencing after cloning the fragment by use of the pGEM-T Easy Vector System (Promega, Madison, WI). Then, the 168-bp fragment was cut from the vector with *Nde*I and *Apa*I, and ligated into the pBSδ*Apa*I/mKit plasmid digested with *Nde*I and *Apa*I to obtain pBSδ*Apa*I/mVal620Ala Kit. This plasmid was digested with *Pst*I and blunt ended to prepare the mouse Val620Ala Kit cDNA fragment.

Generation and identification of Tg mice

2,999MT-mVal620Ala Kit was digested with *Cla*I and *Sst*II (Figure 7), and the transgene DNA fragment separated from the vector sequence was isolated by using agarose gel electrophoresis and cleaned with a Geneclean Kit (Bio 101, Carlsbad, CA). The purified DNA fragment was microinjected into pronuclei of day 0.5 Slc/BDF1. Injected embryos were implanted into the oviducts of day –0.5 pseudopregnant female Slc/ICR mice. Tg mice were identified by PCR by using the following primer set: MT-F (5′-GAGTGCAGTGGCACAA TCTTGGCTC-3′) and MT-R (5′-GCATGAGAGGACAGTGCCAAGC AAG-3′), both corresponding to the human growth hormone 3′ untranslated region. The Tg mice were maintained within C57BL/6 mice afterwards. The medical ethical committee of the Gifu University approved all described studies.

RNA extraction and reverse transcription-PCR analysis

Total RNA was extracted from the mouse skin by using ISOGEN (Nippon Gene, Tokyo, Japan) as described by the manufacturer. The extracted total RNA (5 μg) was reverse transcribed in a 20-μl reaction mixture by using a SuperScript First Strand Synthesis System (Invitrogen, Carlsbad, CA). One microliter of the resulting first-strand cDNA mixture was used for PCR with *Taq* polymerase (Takara, Tokyo, Japan), which amplification was performed in a 50-μl mixture. Ten microliters of each PCR product was subjected to electrophoresis on a 2.0% agarose gel and visualized with ethidium bromide. Primers used for PCR were as follow: MT-F, MT-R, glyceraldehyde-3-phosphate dehydrogenase-F (5′-CTTCACCAC CATGGAGAAGGC-3′), and glyceraldehyde-3-phosphate dehydrogenase-R (5′-GGCATGGACTGTGGTCATGAG-3′).

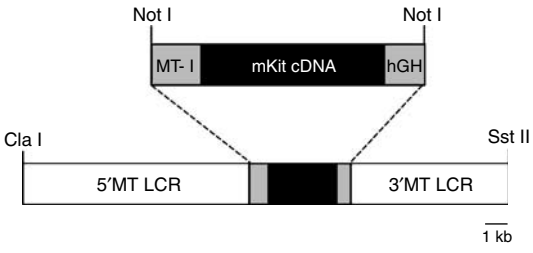

Figure 7. Transgene construction: schematic representation of the 21.5-kb *Cla*I/*Sst*II DNA fragment used to generate mt-Kit^{V620A} Tg mice. This expression construct contained the mutated mouse mutant Kit cDNA (black box), the mouse MT-1 promoter, human growth hormone gene polyadenylation site (hGH), and the 5′ and 3′ flanking sequences including the locus control region (LCR) of the mouse MT gene (white boxes).

Staining and counting of mast cell

Mice at 6–7 weeks of age were killed after anesthesia. Pieces of the dorsal skin were removed with a 6 mm diameter punch and were embedded in paraffin. Sections (4-μm thickness) were stained with alcian blue according to standard procedures (Kunisada *et al.*, 1998). Mast cells lying between the epithelium and panniculus carnosus in five specimens for each Tg mouse were counted under a microscope, and the numbers were converted into the number per cm of skin specimen.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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REFERENCES

Allen ND, Gran DG, Barton SC, Hettle S, Reik R, Surani MAH (1988) Transgene as probes for active chromosomal domains in mouse development. *Nature* 333:852–5

Allen ND, Norris ML, Surani MAH (1990) Epigenetic control of transgene expression and imprinting by genotype-specific modifiers. *Cell* 61:853–61

Baxter LL, Hou L, Loftus SK, Pavan WJ (2004) Spotlight on spotted mice: a review of white spotting mouse mutants and associated human pigmentation disorders. *Pigment Cell Res* 17:215–24

Baynash AG, Hosoda K, Giaid A, Richardson JA, Emoto N, Hammer RE *et al.* (1994) Interaction of endothelin-3 with endothelin-B receptor is essential for development of epidermal melanocytes and enteric neurons. *Cell* 79:1277–85

Besmer P, Manova K, Duttlinger R, Huang EJ, Packer A, Gyssler C *et al.* (1993) The *kit*-ligand (steel factor) and its receptor c-*kit*/W: pleiotropic roles in gametogenesis and melanogenesis. *Development* (Suppl.):125–37

Botchkareva NV, Khlgatian M, Longrey BJ, Botchkarev VA, Gilchrest BA (2001) SCF/c-kit signaling is required for cyclic regeneration of the hair pigmentation unit. *FASEB J* 15:645–58

Bottinger EP, Jakubczak JL, Roberts ISD *et al.* (1997) Expression of a dominant-negative mutant TGF-beta type II receptor in transgenic mice reveals essential roles for TGF-beta in regulation of growth and differentiation in the exocrine pancreas. *EMBO J* 16:2621–33

Cooke JV (1952) Familial white skin spotting (piebaldness) ('partial albinism') with white forelock. *J Pediatr* 41:1–12

Copeland NG, Gilbert DJ, Cho BC *et al.* (1990) Mast cell growth factor maps near the steel locus on mouse chromosome 10 and is deleted in a number of steel alleles. *Cell* 63:175–83

Dorsky RI, Moon RT, Raible DW (2000) Environmental signals and cell fate specification in premigratory neural crest. *BioEssays* 22:708–816

Dupin E, Le Douarin NM (2003) Development of melanocyte precursors from the vertebrate neural crest. *Oncogene* 22:3016–23

Engler P, Haasch D, Pinkert CA, Doglio L, Glymour M, Brinster R *et al.* (1991) A strain-specific modifier on mouse chromosome 4 controls the methylation of independent transgene loci. *Cell* 65:939–47

Fleischman RA (1992) Human piebaldism trait resulting from a dominant negative mutant allele of the c-kit membrane receptor gene. *J Clin Invest* 89:1713–7

Fleischman RA (1993) From white spots to stem cells: the role of the Kit receptor in mammalian development. *Trends Genet* 9:285–90

Fleischman RA, Saltman DL, Stastny V, Zneimer S (1991) Deletion of the c-kit protooncogene in the human developmental defect piebald trait. *Proc Natl Acad Sci USA* 88:10885–9

Froggat P (1951) An outline with bibliography of human piebaldism and white forelock. *Ir J Med Sci* 398:86–94

Geissler EN, McFarland EC, Russell ES (1981) Analysis of pleiotropism at the dominant white-spotting (W) locus of the house mouse: a description of ten new W alleles. *Genetics* 97:337–61

Geissler EN, Ryan MA, Housman DE (1988) The dominant-white spotting (W) locus of the mouse encodes the c-kit proto-oncogene. *Cell* 55:185–92

Giebel LB, Spritz RA (1991) Mutation of the KIT (mast/stem cell growth factor receptor) protooncogene in human piebaldism. *Proc Natl Acad Sci USA* 88:8696–9

Hammer RE, Krumlauf R, Camper SA, Brinster RL, Tilghman SM (1987) Diversity of alpha-fetoprotein gene expression in mice is generated by a combination of separate enhancer elements. *Science* 235:53–8

Hattori M, Ogawa H (1983) Biochemical analysis of hair growth from the aspects of aging and enzyme activities. *J Dermatol* 10:45–54

Hirobe T (1984) Histochemical survey of the distribution of the epidermal melanoblasts and melanocytes in the mouse during fetal and postnatal periods. *Anat Rec* 208:589–94

Hirobe T (2005) Role of keratinocyte-derived factors involved in regulating the proliferation and differentiation of mammalian epidermal melanocytes. *Pigment Cell Res* 18:2–12

Hodgkinson CA, Moore KJ, Nakayama A, Steingrimsson E, Copeland NG, Jenkins NA *et al.* (1993) Mutations at the mouse microphthalmia locus are associated with defects in a gene encoding a novel basic-helix-loop-helix-zipper protein. *Cell* 74:395–404

Keeler CE (1934) The heredity of a congenital white spotting in Negroes. *J Am Med Assoc* 103:179–80

Kindy MS, de Beer MC, Yu J, de Beer FC (2000) Expression of mouse acute-phase (SAA1.1) and constitutive (SAA4) serum amyloid A isotypes: influence on lipoprotein profiles. *Arterioscler Thromb Vasc Biol* 20:1543–50

Kothary R, Clapoff S, Brown A, Campbell R, Peterson A, Rossant J (1988) A transgen containing *lacZ* inserted into the *dystonia* locus is expressed in neural tube. *Nature* 335:435–7

Kunisada T, Yamazaki H, Hayashi SI (2001) Ligands for receptor tyrosine kinases expressed in the skin as environmental factors for melanocyte development. *J Investig Dermatol Symp Proc* 6:6–9

Kunisada T, Yoshida H, Yamazaki H *et al.* (1998) Transgene expression of steel factor in the basal layer of epidermis promotes survival, proliferation, differentiation and migration of melanocyte precursors. *Development* 125:2915–23

Lacy E, Roberts S, Evans EP, Burtenshaw MD, Copnstantini FD (1983) A foreign beta-globin in transgenic mice: integration at abnormal chromosomal positions and expression in inappropriate tissues. *Cell* 34:343–58

Lamoreux ML (1999) Strain-specific white-spotting patterns in laboratory mice. *Pigment Cell Res* 12:383–90

Lev S, Yarden Y, Givol D (1992) Dimerization and activation of the kit receptor by monovalent and bivalent binding of the stem cell factor. *J Biol Chem* 267:15970–7

Lyon MF, Glenister PH (1982) A new allele sash (Wsh) at the W-locus and a spontaneous recessive lethal in mice. *Genet Res* 39:315–22

Majumdar MK, Everett ET, Xiao X, Cooper R, Langley K, Kapur R *et al.* (1996) Xenogeneic expression of human stem cell factor in transgenic mice mimics codominant c-kit mutations. *Blood* 87:3203–11

Marklund S, Moller M, Sandberg K, Andersson L (1999) Close association between sequence polymorphism in the KIT gene and the roan coat color in horses. *Mamm Genome* 10:283–8

Murakami T, Fukai K, Oiso N *et al.* (2004) New *KIT* mutations in patients with piebaldism. *J Dermatol Sci* 35:29–33

Nakayama H, Kuroda H, Fujita J, Kitamura Y (1988) Studies of *Sl/Sl*^{d}-+/+ mouse aggregation chimeras. I. Different distribution patterns between melanocytes and mast cells in the skin. *Development* 102:107–16

Nishikawa S, Kusakabe M, Yoshinaga K *et al.* (1991) *In utero* manipulation of coat color formation by a monoclonal anti-*c-kit* antibody: two distinct waves of *c-kit*-dependency during melanocyte development. *EMBO J* 10:2111–8

Nocka K, Tan JC, Chiu E, Chu TY, Ray P, Traktman P *et al.* (1990) Molecular bases of dominant negative and loss of function mutations at the murine c-*kit*/white spotting locus: W^{37}, W^{v}, W^{41} and W. *EMBO J* 9:1805–13

Ogawa H, Hattori M (1983) Regulation mechanisms of hair growth. In: *Normal and abnormal epidermal differentiation* (Seiji M, Bernstein IA, eds), Tokyo: Tokyo University Press, 159–70

Okura M, Maeda H, Nishikawa S, Mizoguchi M (1995) Effects of monoclonal anti-c-kit antibody (ACK2) on melanocytes in newborn mice. *J Invest Dermatol* 105:322–8

Palmiter RD, Sandgren EP, Koeller DM, Brinster RL (1993) Distal regulatory elements from the mouse metallothionein locus stimulate gene expression in transgenic mice. *Mol Cell Biol* 13:5266–75

Pla P, Larue L (2003) Involvement of endothelin receptors in normal and pathological development of neural crest cells. *Int J Dev Biol* 47:315–25

Ray P, Higgins KM, Tan JC *et al.* (1991) Ectopic expression of a c-*kit*^{W42} minigene in transgenic mice: recapitulation of W phenotypes and evidence for c-*kit* function in melanoblast progenitors. *Genes Dev* 5:2265–73

Richards KA, Fukai K, Oiso N, Paller AS (2001) A novel KIT mutation results in piebaldism with progressive depigmentation. *J Am Acad Dermatol* 44:288–92

Silvers WK (1979) *The coat colors of mice: a model for mammalian gene action and interaction*. New York: Springer-Verlag

Southard-Smith EM, Kos L, Pavan WJ (1998) Sox 10 mutation disrupts neural crest development in Dom Hirschsprung mouse model. *Nat Genet* 18:60–4

Spritz RA, Giebel LB, Holmes SA (1992) Dominant negative and loss of function mutations of the c-kit (mast/stem cell growth factor eceptor) proto-oncogene in human piebaldism. *Am J Hum Genet* 50: 261–9

Tachibana M, Perez-Jurado LA, Nakayama A *et al.* (1994) Cloning of MITF, the human homolog of the mouse microphthalmia gene and assignment to chromosome 3p14.1-p12.3. *Hum Mol Genet* 3:553–7

Wehrle-Haller B (2003) The role of Kit-ligand in melanocyte development and epidermal homeostasis. *Pigment Cell Res* 16:287–96

Yoshida H, Kunisada T, Grimm T, Nishimura EK, Nishioka E, Nishikawa SI (2001) Melanocyte migration and survival controlled by SCF/c-kit expression. *J Investig Dermatol Symp Proc* 6:1–5

Yoshida H, Nishikawa S-I, Okamura H, Sakakura T, Kusakabe M (1993) The role of c-kit proto-oncogene during melanocyte development in mouse. *In vivo* approach by the *in utero* microinjection of anti-c-kit antibody. *Dev Growth Differ* 35:209–20

Zsebo KM, Williams DA, Geissler EN *et al.* (1990) Stem cell factor is encoded at the Sl locus of the mouse and is the ligand for the c-kit tyrosine kinase receptor. *Cell* 5:213–24