Suppression of Progressive Loss of Coat Color in Microphthalmia-Vitiligo Mutant Mice

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The coat color of C57BL/6-*Mitf*$^{vit/vit}$ mice whitens with age, because of a one-nucleotide mutation in the DNA-binding region of the microphthalmia-associated transcription factor (MITF), which plays an important role in melanocyte growth and differentiation. To investigate the signals regulating MITF function, we prepared transgenic mice expressing three of the external signals that are important for melanocyte development, i.e., hepatocyte growth factor (HGF), stem cell factor (SCF), and endothelin-3 (ET3), and crossed these mice with *Mitf*$^{vit/vit}$ mice. We found that the age-dependent coat color whitening of the *Mitf*$^{vit/vit}$ mice was completely suppressed by the overexpression of HGF or SCF in the skin, but not by that of ET3. Moreover, HGF, but not ET3, promoted the proliferation of *Mitf*$^{vit/vit}$ mice-derived melanocytes in culture. These results suggest that the signals from exogenous HGF and SCF rescued the *mi-vitiligo* mutation and also that ET3 does not stimulate the common signal transduction pathway for MITF activation shared by HGF and SCF.

Key words: hair/melanocyte/microphthalmia-associated transcription factor/pigmentation disease/vitiligo
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The mutation at the mouse *microphthalmia* (*mi*) locus affects the eye size, pigmentation, inner ears, and the capacity for secondary bone resorption. Homozygous *Mitf*^{vit} mutant mice, C57BL/6-*Mitf*$^{vit/vit}$, are mildly affected as compared with those homozygous for other *Mitf* alleles, and exhibit a lighter coat color and large white spotting. With aging, the pigmented areas of these mice become progressively lighter because of the decrease in the number of melanocytes in their hair follicles, and become almost completely white within 6–12 mo (Lerner *et al*, 1986; Lamoreux *et al*, 1992).

The microphthalmia-associated transcription factor (MITF) contains a basic helix–loop–helix leucine zipper motif. MITF regulates the expression of enzymes necessary for pigmentation, such as tyrosinase, tyrosinase-related proteins 1 and 2 (Yasumoto *et al*, 1994, 1995, 1997, 2002; Fuse *et al*, 1996), the melanosomal membrane protein Pmel17/SILV/GP100 (Du *et al*, 2003), and the anti-apoptotic factor BCL2 (McGill *et al*, 2002). Thus, some of the MITF mutations lead to the loss of viable melanocytes (Hodgkinson *et al*, 1993; Hughes *et al*, 1993).

The *Mitf*$^{vit/vit}$ mice have a spontaneous *mi-vitiligo* mutation of MITF in which a G to A transition at bp 793 of the coding region leads to a D222N substitution (Lamoreux *et al*, 1992; Steingrimsson *et al*, 1994). The *mi-vitiligo* mutant protein was shown to have almost the same DNA-binding activity as compared with that of the wild-type protein; however, the mutant protein caused progressive death of the melanocytes (Hemesath *et al*, 1994).

It is known that hepatocyte growth factor (HGF), stem cell factor (SCF), and endothelins play essential roles in melanocyte maintenance. MET, the receptor for HGF, and KIT, the receptor for SCF, are tyrosine kinase receptors and have been reported to regulate the proliferation, differentiation, and pigmentation of melanocytes (Matsui *et al*, 1990; Matsumoto *et al*, 1991; Halaban *et al*, 1992; Wehrle-Haller and Weston, 1995; Imokawa *et al*, 1998; Kos *et al*, 1999). The endothelins that comprise endothelin -1, -2, and -3 have also been reported to stimulate the proliferation, differentiation, and pigmentation of melanocytes (Yada *et al*, 1991; Lahav *et al*, 1996; Yoshida *et al*, 1996; Opdecamp *et al*, 1998; Shin *et al*, 1999); however, the receptors for endothelins were reported to be G-protein-coupled receptors (Arai *et al*, 1990; Sakurai *et al*, 1990). We reported that transgenic mice expressing HGF, SCF, or endothelin-3 (ET3) in their epidermal keratinocytes were reported to develop prominent epidermal pigmentation[1] (Kunisada *et al*, 1998a, 2000). It is considered that the signals of these three factors regulate the MITF activity and are responsible for the maintenance of melanocytes. In fact, KIT was shown to regulate the MITF function by the phosphorylation of the MITF protein (Hemesath *et al*, 1998; Wu *et al*, 2000).

Here, we aimed at investigating the role of extracellular signals regulating MITF *in vivo* by using transgenic mice

Abbreviations: ET-3, endothelin-3; HGF, hepatocyte growth factor; MITF, microphthalmia-associated transcription factor; PCR, polymerase chain reaction; SCF, stem cell factor

This work was carried out at Odawara, Kanagawa, Japan.

[1]Kunisada T, Motohashi T, Aoki H, *et al*: Melanocyte stem cells induces from ES cells. Pigment Cell Res. 15 (suppl. 9):55–56, 2002 (abstr).

expressing signaling molecules, including SCF, HGF, and ET3, in their skin. The use of double-mutant mice, obtained by crossing *Mitf*$^{vit/vit}$ mice with transgenic mice that singly expressed these factors, allows one to test the effect of each factor on the regulation of MITF; therefore, the vitiligo phenotype, resulting from the cumulative effect of the subtle defect in *Mitf*$^{vit/vit}$, may be altered in response to specifically modulated signalings induced by these exogenous factors.

Results

Age-dependent modulation of the coat color of *Mitf*$^{vit/vit}$ mice expressing growth factor transgenes in their skin We prepared mice expressing HGF and SCF in the *Mitf*$^{vit/vit}$ skin (*Mitf*$^{vit/vit}$; *HGF*$^{Tg/+}$, and *Mitf*$^{vit/vit}$; *SCF*$^{Tg/+}$, respectively), as described in "Materials and Methods." *Mitf*$^{vit/vit}$ mice exhibited a slightly lighter coat color on the C57BL/6 background at 2 mo after birth (Fig 1*D*). At the same age, *HGF*$^{Tg/+}$ (Fig 1*A*) and *SCF*$^{Tg/+}$ (Fig 1*B*) mice exhibited darker coat colors with no unpigmented regions and pigmented interfollicular skin, including that of the nose and ears. In double-mutant *Mitf*$^{vit/vit}$; *HGF*$^{Tg/+}$ (Fig 1*E*) and *Mitf*$^{vit/vit}$; *SCF*$^{Tg/+}$ (Fig 1*F*) mice, darkened coat color in the pigmented skin region and large ventral white spots were observed in all mice at 2 mo after birth. Along with the progression in age, *Mitf*$^{vit/vit}$ mice lose their coat color in regions previously covered with pigmented hair (Fig 1*H*). This progressive whitening of the coat color, observed in every *Mitf*$^{vit/vit}$ mouse, however, was completely suppressed in *Mitf*$^{vit/vit}$; *HGF*$^{Tg/+}$ (Fig 1*I*) and *Mitf*$^{vit/vit}$; *SCF*$^{Tg/+}$ (Fig 1*J*) double-mutant mice even after 6 mo. In *HGF*$^{Tg/+}$ and *SCF*$^{Tg/+}$ mice, whitening of the coat color was not observed after 10 mo (data not shown). Thus, the progressive loss of hair pigmentation observed in homozygous *mi-vitiligo* mice was completely suppressed by the exogenously supplied HGF or SCF in their skin.

ET3$^{Tg/+}$ mice were also extensively covered with pigmented hair, and their interfollicular skin, including that of the nose and ears, was also heavily pigmented (Fig 1*C*). The coat color of *Mitf*$^{vit/vit}$; *ET3*$^{Tg/+}$ double-mutant mice was similar to that of *Mitf*$^{vit/vit}$; *HGF*$^{Tg/+}$, and *Mitf*$^{vit/vit}$; *SCF*$^{Tg/+}$ mice, i.e., they both exhibited darkened coat color in the pigmented region and a ventral white spot after 2 mo (Fig 1*G*). Within 6 mo, however, pigmentation of the dorsal hair was apparently lost in *Mitf*$^{vit/vit}$; *ET3*$^{Tg/+}$ mice (Fig 1*K*), and the extent of the progression of coat color whitening was similar to that in *Mitf*$^{vit/vit}$ mice (Fig 1*H*). Clearly, the progressive loss of hair pigmentation observed in homozygous *mi-vitiligo* mice was not prevented by the exogenously supplied ET3.

The color of the non-hairy skin, such as that on the nose and ears, was different among the different types of double-mutant mice, i.e., the color of the non-hairy skin of the *Mitf*$^{vit/vit}$; *SCF*$^{Tg/+}$ mice was darkest and that of the *Mitf*$^{vit/vit}$; *HGF*$^{Tg/+}$ mice was of almost the same density as that of the *Mitf*$^{vit/vit}$; *ET3*$^{Tg/+}$ mice (Fig 1*E–G*). The color of the non-hairy skins in these double-transgenic mice, including the *Mitf*$^{vit/vit}$; *ET3*$^{Tg/+}$ mice, did not change with age (Fig 1*I–K*).

Quantitative evaluation of coat color whitening after induced hair regeneration In order to test whether the suppression of coat color whitening by HGF and SCF transgenes is directly related to the growth and differentiation of follicular melanocytes, we induced hair regeneration by plucking. As shown in Fig 2*A*, the amount of hair melanin measured at optical density $(OD)_{400}$ was reduced after plucking the pigmented hair observed in 4-mo-old *Mitf*$^{vit/vit}$ mice. As expected, no significant loss of hair melanin was observed in *Mitf*$^{vit/vit}$; *HGF*$^{Tg/+}$, and *Mitf*$^{vit/vit}$; *SCF*$^{Tg/+}$ double-mutant mice. In *Mitf*$^{vit/vit}$; *ET3*$^{Tg/+}$ mice, the loss of hair melanin was comparable with that of *Mitf*$^{vit/vit}$ mice after

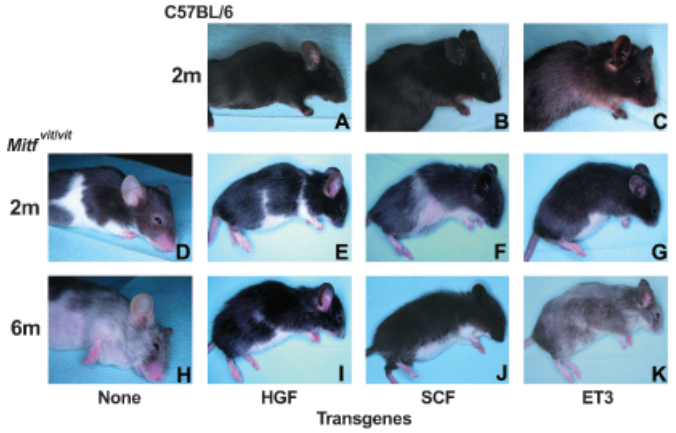

Figure 1
Coat color changes in the *Mitf*$^{vit/vit}$ mice by the overexpression of hepatocyte growth factor (HGF), stem cell factor (SCF), and endothelin-3 (ET3). Each growth factor transgene were expressed in C57BL/6 (*A–C*) and *Mitf*$^{vit/vit}$ (*D–K*) mice. The *Mitf*$^{vit/vit}$ mice have a natural ventral white spot (*D*). The remaining pigmented hair in *Mitf*$^{vit/vit}$ mice turned white with aging. The coat color whitening becomes remarkable at 6 mo (*H*). In all the three double-mutant mice, i.e., *Mitf*$^{vit/vit}$; *HGF*$^{Tg/+}$ (*E*), *Mitf*$^{vit/vit}$; *SCF*$^{Tg/+}$ (*F*), and *Mitf*$^{vit/vit}$; *ET3*$^{Tg/+}$ (*G*), the size and location of the white spots observed at 2 mo are the same as those observed in *Mitf*$^{vit/vit}$ mice. Six months or later, the progress of the coat color whitening as observed in *Mitf*$^{vit/vit}$ mice (*H*) was suppressed in *Mitf*$^{vit/vit}$; *HGF*$^{Tg/+}$ (*I*) and *Mitf*$^{vit/vit}$; *SCF*$^{Tg/+}$ (*J*) mice (photographs in "*H*," "*I*," and "*J*" were taken at 6 mo), but no suppression was observed in *Mitf*$^{vit/vit}$; *ET3*$^{Tg/+}$ mice at 6 mo (*K*) or later. MITF, microphthalmia-associated transcription factor.

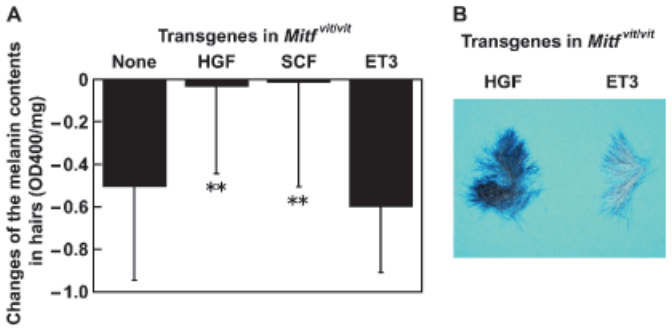

Figure 2
Quantitation of the melanin content in the hair before and after depilation. (*A*) The change in the melanin content of hair (OD_{400}/mg) is demonstrated by the reduction in absorbance before and after the depilation of hair in 4-mo-old mice. None: *Mitf*$^{vit/vit}$ (N = 21), hepatocyte growth factor (HGF): *Mitf*$^{vit/vit}$; *HGF*$^{Tg/+}$ (N = 10), stem cell factor (SCF): *Mitf*$^{vit/vit}$; *SCF*$^{Tg/+}$ (N = 10), endothelin 3 (ET3): *Mitf*$^{vit/vit}$; *ET3*$^{Tg/+}$ (N = 9). Mean ± SD, $^{**}p < 0.01$ (*t* test) compared with the melanin content in *Mitf*$^{vit/vit}$ mice. (*B*) Photographs of the regenerated hair in *Mitf*$^{vit/vit}$; *HGF*$^{Tg/+}$ (*left*) and *Mitf*$^{vit/vit}$; *ET3*$^{Tg/+}$ (*right*) mice used for quantification of the melanin content. MITF, microphthalmia-associated transcription factor; OD, optical density.

plucking. We also demonstrated the appearance of regenerated hair before melanin quantitation in *Mitf*$^{vit/vit}$; *HGF*$^{Tg/+}$ (Fig 2*B*, *left*) and *Mitf*$^{vit/vit}$; *ET3*$^{Tg/+}$ (Fig 2*B*, *right*) mice. Therefore, the suppression of coat color whitening of homozygous *mi-vitiligo* mice by transgenic HGF and SCF is likely to be attained by promoting melanocyte regeneration coupled with hair regeneration.

Histological analysis of transgenic *Mitf*$^{vit/vit}$ mice In order to elucidate whether the suppression or progression of coat color whitening of *Mitf*$^{vit/vit}$; *HGF*$^{Tg/+}$, *Mitf*$^{vit/vit}$; *SCF*$^{Tg/+}$, and *Mitf*$^{vit/vit}$; *ET3*$^{Tg/+}$ mice is related to the decrease or increase in the number of melanocytes, we performed a histological analysis of the skin of these mutant mice. Two months after birth, melanocytes were observed in the dermis of *Mitf*$^{vit/vit}$; *HGF*$^{Tg/+}$ mice (Fig 3*D*), similar to *HGF*$^{Tg/+}$ mice (Fig 3*A*) that we previously reported (Kunisada *et al*, 2000). The number and location of melanocytes were maintained in *Mitf*$^{vit/vit}$; *HGF*$^{Tg/+}$ mice at 4 mo (Fig 3*G*) and 6 mo (Fig 3*J*) after birth. The melanocytes of *SCF*$^{Tg/+}$ mice were observed only in the basal layer of the epidermis (Fig 3*B*), as we reported previously (Kunisada *et al*, 1998a, b). On the other hand, the melanocytes of *Mitf*$^{vit/vit}$; *SCF*$^{Tg/+}$ mice were observed not only in the basal layer of the epidermis but also in dermis (Fig 3*E*, *H*, and *K*). The number of melanocytes in 2-mo-old *Mitf*$^{vit/vit}$; *SCF*$^{Tg/+}$ mice (Fig 3*E*) was not less than that in *SCF*$^{Tg/+}$ mice (Fig 3*B*), and the number was still maintained in 4-mo-old (Fig 3*H*) and 6-mo-old mice (Fig 3*K*). In 2-mo-old *ET3*$^{Tg/+}$ mice (Fig 3*C*), numerous melanocytes were observed in the dermis. The number of melanocytes in *ET3*$^{Tg/+}$ mice (Fig 3*C*) was obviously larger than that in *HGF*$^{Tg/+}$ and *SCF*$^{Tg/+}$ mice (Fig 3*A* and *B*, respectively). the number of melanocytes in 2-mo-old *Mitf*$^{vit/vit}$; *ET3*$^{Tg/+}$ mice (Fig 3*F*), however, was obviously smaller than that in *ET3*$^{Tg/+}$ mice (Fig 3*C*), whereas the location of the melanocytes *Mitf*$^{vit/vit}$; *ET3*$^{Tg/+}$ mice was the same as that in *ET3*$^{Tg/+}$ mice (Fig 3*C* and *F*). The number of melanocytes in *Mitf*$^{vit/vit}$; *ET3*$^{Tg/+}$ mice decreased with age (4-mo-old (Fig 3*I*) and 6-mo-old (Fig 3*L*)) and mostly disappeared at 6 mo after birth (Fig 3*L*).

We quantitatively compared the number of interfollicular melanocytes among three double-mutation mice (Fig 4). The number of melanocytes in *Mitf*$^{vit/vit}$; *HGF*$^{Tg/+}$, and *Mitf*$^{vit/vit}$; *SCF*$^{Tg/+}$ mice remained unchanged from 2 to 6 mo after birth. In contrast, although the number of melanocytes in *Mitf*$^{vit/vit}$; *ET3*$^{Tg/+}$ mice was constantly maintained from 3 wk to 2 mo after birth, a significant decrease was observed in the number of melanocytes at 4 mo after birth, and few melanocytes survived until 6 mo. Thus, the gradual loss of interfollicular melanocytes started as early as 2 mo after birth in *Mitf*$^{vit/vit}$; *ET3*$^{Tg/+}$ mice.

To elucidate whether the progressive loss of melanocytes in the *Mitf*$^{vit/vit}$; *ET3*$^{Tg/+}$ mice is caused by a sudden decrease in the ET3 transgene expression level, we analyzed the ET3 gene expression level in the *Mitf*$^{vit/vit}$; *ET3*$^{Tg/+}$ mice aged 2 and 6 mo by using the quantitative reverse-transcriptase polymerase chain reaction (PCR) method. The relative ET3 expression levels against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the *Mitf*$^{vit/vit}$ mice aged 2 and 6 mo were 0.0011 and 0.0013, respectively, and those in the *Mitf*$^{vit/vit}$; *ET3*$^{Tg/+}$ mice aged 2 and 6 mo were 0.30 and 0.21, respectively. At 2 mo of age, the ET3 expression level in the *Mitf*$^{vit/vit}$; *ET3*$^{Tg/+}$ mice was clearly higher than that in the *Mitf*$^{vit/vit}$ mice, and was even higher at 6 mo of age. These results indicated that the progressive loss of melanocytes in *Mitf*$^{vit/vit}$; *ET3*$^{Tg/+}$ mice was not because of a decrease in the ET3 transgene expression level with age.

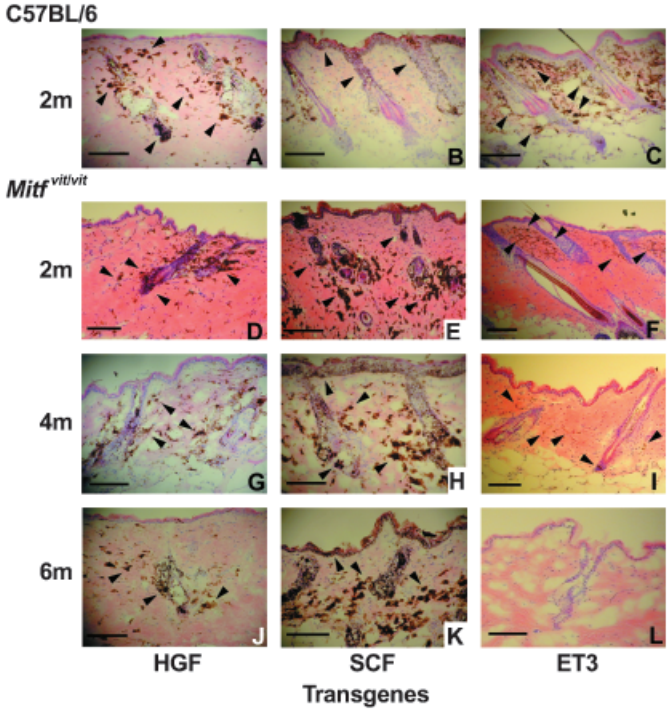

Figure 3
Age-dependent changes in the number of melanocytes in the double-mutant mice. The double-mutation mice, *Mitf*$^{vit/vit}$; *HGF*$^{Tg/+}$, *Mitf*$^{vit/vit}$; *SCF*$^{Tg/+}$, and *Mitf*$^{vit/vit}$; *ET3*$^{Tg/+}$, were generated by crossing the C57BL/6-*HGF*$^{Tg/+}$, C57BL/6-*SCF*$^{Tg/+}$, and C57BL/6-*ET3*$^{Tg/+}$ transgenic mice with *Mitf*$^{vit/vit}$. Hematoxylin and eosin-stained sections of 2-mo-old *HGF*$^{Tg/+}$ (*A*), *SCF*$^{Tg/+}$ (*B*), and *ET3*$^{Tg/+}$ (*C*) mice revealed pigmented melanocytes in the dermis (*HGF*$^{Tg/+}$ (*A*) and *ET3*$^{Tg/+}$ (*C*)) or the epidermis (*SCF*$^{Tg/+}$ (*B*)). The number of pigmented melanocytes in *ET3*$^{Tg/+}$ (*C*) mice was apparently more than that in *HGF*$^{Tg/+}$ (*A*) and *SCF*$^{Tg/+}$ (*B*) mice. The melanocytes in 2-mo-old *Mitf*$^{vit/vit}$; *HGF*$^{Tg/+}$ mice (*D*) were located around hair follicles, similar to *HGF*$^{Tg/+}$ mice of the same age (*A*). The melanocytes in 2-mo-old *Mitf*$^{vit/vit}$; *SCF*$^{Tg/+}$ mice (*E*) were observed not only in the basal layer of the epidermis but also in the dermis, unlike in *SCF*$^{Tg/+}$ mice of the same age (*B*). The melanocytes in 2-mo-old *Mitf*$^{vit/vit}$; *ET3*$^{Tg/+}$ mice (*F*) were observed in the dermis, but the number was less than that in *ET3*$^{Tg/+}$ mice of the same age (*C*). The sections of 4- and 6-mo-old *Mitf*$^{vit/vit}$; *HGF*$^{Tg/+}$ (*G* and *J*, respectively), *Mitf*$^{vit/vit}$; *SCF*$^{Tg/+}$ (*H* and *K*, respectively), and *Mitf*$^{vit/vit}$; *ET3*$^{Tg/+}$ (*I* and *L*, respectively) mice skins were also indicated. The number and location of the melanocytes in *Mitf*$^{vit/vit}$; *HGF*$^{Tg/+}$ (*D*, *G*, and *J*), *Mitf*$^{vit/vit}$; *SCF*$^{Tg/+}$ (*E*, *H*, and *K*) mice were stably maintained through 6 mo of age. The number of melanocytes in *Mitf*$^{vit/vit}$; *ET3*$^{Tg/+}$ mice decreased with age (*F*, *I*, and *L*) and mostly disappeared at 6 mo after birth (*L*). Arrowheads indicate the pigmented melanocytes in each figure. *Scale bar* = 50 μm. MITF, microphthalmia-associated transcription factor; HGF, hepatocyte growth factor; SCF, stem cell factor; ET3, endothelin 3.

ET3 is not effective for the proliferation of *Mitf*$^{vit/vit}$ mice-derived melanocytes in culture In order to elucidate the effects of ET3 on the proliferation of melanocytes derived from *Mitf*$^{vit/vit}$ mice, we compared the number of cells of the *Mitf*$^{vit/vit}$ mice-derived melanocytes with those from C57BL/6 mice 7 d after the cultures were initiated in the presence of

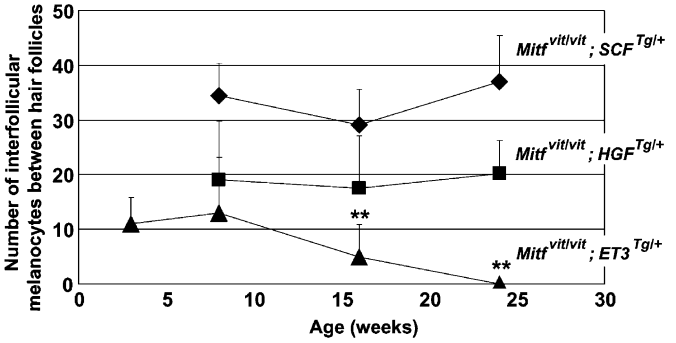

Figure 4
Measurement of the number of pigmented interfollicular melanocytes in the double-mutant mice. At 2 mo of age, the number of pigmented interfollicular melanocytes in *Mitf*$^{vit/vit}$; *SCF*$^{Tg/+}$ mice (◆) was more than that in *Mitf*$^{vit/vit}$; *HGF*$^{Tg/+}$ (■) and in *Mitf*$^{vit/vit}$; *ET3*$^{Tg/+}$ (▲) mice. The number of melanocytes in *Mitf*$^{vit/vit}$; *HGF*$^{Tg/+}$ and *Mitf*$^{vit/vit}$; *SCF*$^{Tg/+}$ mice was maintained through 6 mo of age, but that of *Mitf*$^{vit/vit}$; *ET3*$^{Tg/+}$ was continuously decreased after 4 mo of age. Mean ± SD, ** $p<0.01$ (*t* test) compared with each 2-mo-old mice. MITF, microphthalmia-associated transcription factor; HGF, hepatocyte growth factor; SCF, stem cell factor; ET3, endothelin 3.

ET3 (Fig 5). The number of C57BL/6 mice-derived melanocytes significantly increased in the culture with ET3, and did not change in the culture with HGF. In contrast, the number of *Mitf*$^{vit/vit}$ mice-derived melanocytes was not affected in the presence of ET3; instead, significant proliferation was observed in the culture supplemented with HGF.

Thus, it is conceivable that reduction in the melanocytes in *Mitf*$^{vit/vit}$; *ET3*$^{Tg/+}$ skin (Fig 3*F*) compared with numerous melanocytes in *ET3*$^{Tg/+}$ skin (Fig 3*C*) implies the lack of stimulatory effect of exogenous ET3 produced in the skin on the melanocytes with *Mitf*$^{vit/vit}$ genetic background.

Discussion

The dependence of melanocyte development on MITF is obvious in the loss-of-function mutants of the *Mitf* gene. Forced expression of MITF in Swiss NIH3T3 cells is known to induce differentiation of this cell line into melanocyte lineages (Tachibana *et al*, 1996).

Our results demonstrate that the progressive coat color whitening of *Mitf*$^{vit/vit}$ mice was suppressed by the overexpression of SCF or HGF in their skin, both of which are known to be growth and differentiation factors for melanocytes. It was reported that SCF signaling, through its receptor KIT tyrosine kinase, finally triggers MITF activation via the mitogen-activated protein kinase/extracellular signal-regulated kinase-mediated phosphorylation of Ser73 and via the p90 Rsk-1-mediated phosphorylation of Ser409 of the MITF protein (Hemesath *et al*, 1998; Wu *et al*, 2000). On the other hand, it was not known whether HGF signaling by MET receptor tyrosine kinase also activates MITF. Based on our genetic analysis, we believe that it is likely that HGF signaling activates the proliferation of melanocytes through MITF stimulation.

It was clear that the *mi-vitiligo* mutation, D222N, affects coat color whitening with age, but the *mi-vitiligo* mutant protein did not have a measurable effect on the DNA-binding activity, the production of homodimer, and the production of the heterodimeric complex with TFE3 or TFEB, the basic helix-loop-helix zipper type transcription factors (Hemasath *et al*, 1994). This suggested two possibilities. First, a small difference in the DNA-binding activities because of *mi-vitiligo* mutation was sufficient to induce the age-dependent whitening of coat color. Second, the modulation of the tetrameric forms with TFE3 or TFEB because of *mi-vitiligo* mutation prevented the DNA binding. We believe that the induction of MITF phosphorylation by the overexpression of HGF or SCF resulted in the recovery of the DNA-binding activities of the *mi-vitiligo* mutant protein and rescued the progressive loss of melanocytes in the *Mitf*$^{vit/vit}$ mice.

On the other hand, overexpression of ET3 in the skin did not rescue the mice from this *mi-vitiligo* phenotypic defect. It is known that all the three endothelins, ET1, ET2, and ET3, stimulate the generation of melanoblasts from neural crest cells (Opdecamp *et al*, 1998; Shin *et al*, 1999). It is clear that ET3 could stimulate the development of the melanocyte lineage, because the extent of pigmentation observed in the transgenic skin and hair was not less than that observed in HGF and SCF transgenic mice (Fig 3*A–C*). On the other hand, our histological analysis demonstrated that the

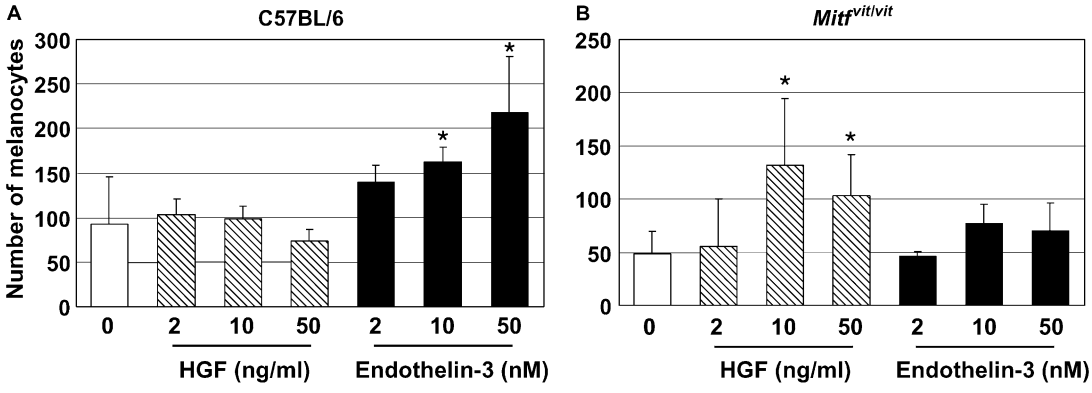

Figure 5
Effects of growth factors on the maintenance of melanocytes derived from C57BL/6 and *Mitf*$^{vit/vit}$ mice. The number of melanocytes derived from C57BL/6 (*A*) or *Mitf*$^{vit/vit}$ (*B*) mice after 7 d of culture with hepatocyte growth factor (HGF) or endothelin-3 (ET3) was indicated. ET3 increased the number of C57BL/6-derived melanocytes in culture, but not that of *Mitf*$^{vit/vit}$-derived melanocytes. Proliferative effect of HGF was observed in *Mitf*$^{vit/vit}$-derived melanocytes. Mean ± SD, * $p<0.05$ (*t* test) compared with corresponding control.

number of melanocytes in the skin of *Mitf*$^{vit/vit}$; *ET3*$^{Tg/+}$ mice was obviously less than that of *ET3*$^{Tg/+}$ mice at just 2 mo after birth (Fig 3*C* and *F*). Moreover, these melanocytes in *Mitf*$^{vit/vit}$; *ET3*$^{Tg/+}$ mice were not maintained with age and lead to the coat color whitening in *Mitf*$^{vit/vit}$; *ET3*$^{Tg/+}$ mice to the same extent as that in *Mitf*$^{vit/vit}$ mice. We also showed that the ET3 expression level in the *Mitf*$^{vit/vit}$; *ET3*$^{Tg/+}$ mice was several 100-fold higher than that in the *Mitf*$^{vit/vit}$ mice, and the level was maintained until the age of 6 mo. Apparently, the overexpression of ET3 was unable to rescue the progressive loss of melanocytes that occurred because of *mi-vitiligo* mutation.

These *in vivo* observations were supported by the *in vitro* proliferation assay using the *Mitf*$^{vit/vit}$ mice-derived melanocytes. It was demonstrated that ET3 promoted the proliferation of the C57BL/6 mice-derived melanocytes in culture, but not *Mitf*$^{vit/vit}$ mice-derived melanocytes (Fig 5). In contrast, the addition of HGF increased the number of the *Mitf*$^{vit/vit}$ mice-derived melanocytes (Fig 5). It has been observed that HGF induces the proliferation of normal epidermal melanocytes (Matsumoto *et al*, 1991); an increase in the melanocytes in the skin of *HGF*$^{Tg/+}$ mice was observed (Kunisada *et al*, 2000). This indicated that HGF was an inducer of melanocyte proliferation. Our results, however, showed that HGF promoted the proliferation of melanocytes that were derived from *Mitf*$^{vit/vit}$ mice, but not of those derived from C57BL/6 mice. It was suggested that the effect of HGF in inducing melanocyte proliferation was prominent in more stringent medium. In fact, HGF was shown to induce melanocyte proliferation in bovine pituitary extract-free melanocyte growth medium (Matsumoto *et al*, 1991). We believed that the limited effect of HGF on the melanocytes from C57BL/6 mice observed in this study was because of the coculture of melanocytes with keratinocytes in a nutrient-rich medium—Dulbecco's modified eagle's medium (DMEM) supplemented with 15% fetal bovine serum (FBS). In contrast, melanocytes from *Mitf*$^{vit/vit}$ mice were stimulated by HGF. This cannot be explained well with the available information. But there is a possibility that the induction of the MITF phosphorylation by HGF rescued the *mi-vitiligo* mutation; this rescued MITF acts differently when compared with the normal MITF. The melanocytes from the *Mitf*$^{vit/vit}$ mice were able to proliferate in DMEM supplemented with 15% FBS. Nevertheless, our results showed that ET3 did not induce the melanocyte proliferation in the *Mitf*$^{vit/vit}$ mice. It was suggested that the *mi-vitiligo* mutation was crucial for the signaling of ET3 through its G-protein-coupled receptor, Endothelin receptor B, and acts differently from that of SCF and HGF. Although the precise signaling pathways that start from ET3 have not been revealed completely, the *mi-vitiligo* mutation may provide the key to a more complete understanding of the signals regulating the biological function of MITF.

It was demonstrated that MITF regulated the transcription of anti-apoptotic BCL2 and the *BCL2*$^{+/-}$; *Mitf*$^{vit/+}$ double heterozygote mutant mice exhibited an age-related whitening of coat color similar to that exhibited by the *Mitf*$^{vit/vit}$ homozygote mice (McGill *et al*, 2002). Interestingly, they mentioned that the survival of melanocytes from KIT-deficient mice was not affected by the transgenic expression of BCL2. This suggests that receptors of tyrosine kinases act independent of the BCL2 expression to rescue the MITF defect. Based on our observation, it is predicted that the overexpression of SCF and HGF may rescue the age-related whitening phenotype of BCL2 and *mi-vitiligo* double heterozygotes mutant mice.

Recently, patients receiving an oral tyrosine kinase inhibitor which targets the KIT receptor as well as other kinase receptors (Abrams *et al*, 2003) exhibited loss of hair pigmentation which recovered in the intervals between drug treatment (Moss *et al*, 2003). These observations indicate that the loss of MITF activity, possibly induced by the inhibition of KIT signaling, leads to the loss of hair pigmentation in humans. Moreover, the mutations of *MITF*, the human homologue of mouse *Mitf*, occur in patients with Waardenburg syndrome type 2, a disorder associated with melanocyte abnormalities, including those of head hair (Waardenburg, 1951; Tassabehji *et al,* 1994, 1995; Nobukuni *et al*, 1996). Based on these observations in humans and this experiment on mice, it is conceivable that graying of hair in humans may be because of the age-dependent attenuation of DNA-binding abilities of MITF. Earlier, we confirmed the existence of melanocytes in the outer root sheath of gray hair in humans (Takada *et al*, 1992). Repigmentation of gray hair might be attained through the proliferation of the remaining melanocytes or their progenitors in the outer root sheath through MITF activation.

Materials and Methods

Animals *Mitf*$^{vit/vit}$ mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). Transgenic HGF, SCF, and ET3 mice were prepared in our laboratory (Kunisada *et al*, 1998a, 2000). In brief, the complementary DNA of each growth factor were cloned into constructs containing the human cytokeratin 14 upstream region, rabbit β-globin intron, and poly(A) signal of human cytokeratin 14 and injected into fertilized oocytes. *Mitf*$^{vit/vit}$ and heterozygous HGF, SCF, and ET3 transgenic mice were crossed to obtain double-mutant mice, i.e., *Mitf*$^{vit/+}$; *HGF*$^{Tg/+}$, *Mitf*$^{vit/+}$; *SCF*$^{Tg/+}$, and *Mitf*$^{vit/+}$; *ET3*$^{Tg/+}$, respectively. These transgenic mice were further crossed to produce double-mutant mice with the *Mitf*$^{vit/vit}$ genotype. Double-mutant mice, with *Mitf*$^{vit/vit}$ expressing each growth factor transgene, were identified as mice with large white spots that are characteristic of *Mitf*^{vit} homozygotes, and pigmented nose and ears that resulted from the action of each growth factor transgene. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of LION Corporation, Japan.

Evaluation of suppression of hair graying and measurement of hair melanin The pelages of the double-mutant mice were plucked at 4 mo after birth to force regeneration. This manipulation turned the coat color of *Mitf*$^{vit/vit}$ mice into gray, earlier than the normal 6 mo after birth. The amount of hair melanin was measured as OD_{400} per mg of the hair after overnight digestion with protease Esperase 8.0L (SIGMA, St Louis, Missouri) at 37°C.

Counts of the interfollicular melanocytes in the transgenic mice We prepared the 8 μm cryosections of the mouse skin, hematoxylin and eosin stained, and counted the number of pigmented melanocytes in interfollicular dermal skin.

Quantitative real-time PCR Real-time PCR was performed using a GeneAmp 5700 sequence detection system (Applied Biosystems, Foster City, California). The reactions were performed using SYBR Green PCR Master Mix (Applied Biosystems). The PCR cycling conditions were as follows: 95°C for 10 min, 40 cycles at

94°C for 15 s, and 60°C for 1 min. The following primers were used: GAPDH: TTGTCAAGCTCATTTCCTGGTATG and TCCACCACCCTGTTGCTGTA, and ET3: CGAGCTTACTGTGAGTGTGGAGATG and CCCACTCAAATGCCGTTTCC.

Culture of the melanocytes derived from C57BL/6 and *Mitf*$^{vit/vit}$ mice Dorsal skins of 2-mo-old C57BL/6 and *Mitf*$^{vit/vit}$ mice were dissected 3 d after depilation, in order to activate the proliferation of hair follicular melanocytes by facilitating the entry of the anagen phase of the hair cycle. The obtained skins were cut into small pieces and incubated in a 0.5% trypsin solution (Invitrogen, Carlsbad, California) for 6 h at 4°C, followed by 37°C for 15 min. The skins were cut more finely and repeatedly pipetted. In order to remove the debris, they were filtered with a 40 μm pore-size filter (Becton Dickinson, Franklin Lakes, New Jersey). The filtered cells were pelleted by centrifugation for 5 min at 170 × *g*. The collected cells were re-suspended in DMEM (Sigma, St Louis, Missouri) containing 15% fetal bovine serum (Invitrogen, Carlsbad, California) and plated equally onto the culture dishes (Sumitomo Bakelite, Tokyo, Japan). The plated dishes were divided into three groups. HGF (PeproTech, Rocky Hill, New Jersey) was added to the first group, ET3 (Sigma) was added to the second group, and phosphate-buffered saline was added as a negative control to the third group. The culture medium supplemented with each growth factor was first changed 24 h after the culture was started; subsequently, it was changed every 2 d.

Immunohistochemistry for cultured cells After 7 d of culture, the melanocytes of each group were immunohistochemically detected. The cells were fixed by 1% acetic acid/99% ethanol at 4°C for 10 min, and were incubated for 60 min at 4°C by the blocking solution, that is, 1% skim milk (Becton Dickinson, Franklin Lakes, New Jersey) in tris-buffered saline. They were incubated overnight at 4°C with the primary antibody of anti-PEP8 (provided by Dr Vincent J Hearing), which recognized tyrosinase-related protein 2 as the melanocyte-specific protein. Subsequently, they were incubated with an anti-rabbit immunoglobulin G antibody/horse radish peroxidase (HRP)-conjugated (Santa Cruz Biotechnology, Santa Cruz, California) as a secondary antibody, for 3 h at room temperature. The HRP-conjugated secondary antibody was visualized using the AEC Peroxidase Substrate Kit (Vector Laboratories, Burlingame, California). Control experiments without primary antibodies were carried out for each of the antibodies tested.

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