Transgene expression of steel factor in the basal layer of epidermis promotes survival, proliferation, differentiation and migration of melanocyte precursors

Takahiro Kunisada1,*, Hisahiro Yoshida2, Hidetoshi Yamazaki1, Akitomo Miyamoto1, Hiroaki Hemmi1, Emi Nishimura2, Leonard D. Shultz2, Shin-Ichi Nishikawa2 and Shin-Ichi Hayashi1

1Department of Immunology, School of Life Science, Faculty of Medicine, Tottori University, Nishi-machi 86, Yonago 683, Japan
2Department of Molecular Genetics, Faculty of Medicine, Kyoto University, Kyoto 606-01, Japan
3The Jackson Laboratory, Bar Harbor, Maine 04609, USA

*Author for correspondence (e-mail: tkunisad@grape.med.tottori-u.ac.jp)

Accepted 22 May; published on WWW 9 July 1998

SUMMARY

Mutations at the murine dominant white spotting (KitW) and steel (MgfSl) loci, encoding c-Kit receptor kinase and its ligand respectively, exert developmental defects on hematopoietic cells, melanocytes, germ cells and interstitial cells of Cajal. The expression patterns of steel factor (SLF) observed in the skin and gonads suggest that SLF mediates a migratory or a chemotactic signal for c-Kit-expressing stem cells (melanocyte precursors and primordial germ cells). By targeting expression of SLF to epidermal keratinocytes in mice, we observed extended distribution of melanocytes in a number of sites including oral epithelium and footpads where neither melanocytes nor their precursors are normally detected. In addition, enlarged pigmented spots of KitW and other spotting mutant mice were observed in the presence of the SLF transgene. These results provide direct evidence that SLF stimulates migration of melanocytes in vivo. We also present data suggesting that SLF does not simply support survival and proliferation of melanocytes but also promotes differentiation of these cells. Unexpectedly, melanocyte stem cells independent of the c-Kit signal were maintained in the skin of the SLF transgenic mice. After the elimination of c-Kit-dependent melanoblasts by function-blocking anti-c-Kit antibody, these stem cells continued to proliferate and differentiate into mature melanocytes. These melanoblasts are able to migrate to cover most of the epidermis after several months. The SLF transgenic mice described in this report will be useful in the study of melanocyte biology.

Key words: Kit, steel factor, Melanocyte development, Melanoblast, Transgenic mouse, c-Kit

INTRODUCTION

The c-Kit receptor tyrosine kinase of the mouse and its ligand, steel factor (SLF), are encoded by the dominant white spotting (KitW), hereafter abbreviated as W, and steel (MgfSl), hereafter abbreviated as Sl, loci, respectively. Mutations in these loci result in pleiotropic developmental defects affecting pigment cells, hematopoietic cells, germ cells and interstitial cells of Cajal in the intestine (Reith and Bernstein, 1991; Williams et al., 1992; Maeda et al., 1992; Besmer et al., 1993; Galli et al., 1993; Ward et al., 1994). Most of the defects in these cells are displayed as a loss of individual cell lineages in homozygous W or Sl mutant mice and therefore it is hard to delineate what types of biological processes regulated by c-kit and SLF are impaired in these cell lineages. For example, the complete loss of hair pigmentation observed in homozygous W or Sl mutant mice could be caused by any one of a number of possible events occurring in melanocytes such as decreased survival, impaired proliferation, defective differentiation/maturation or impaired migration. In fact, the biological processes listed above are all known to be influenced by the stimulation of c-Kit-expressing target cells by SLF (Galli et al., 1993; Broudy, 1997).

Three of the four known targets of Sl and W mutations originate from highly migratory stem cells. It has been speculated that the absence of these cell lineages at their final destination in Sl and W mice may result from inability of their precursors to migrate properly as well as from their failure to survive or proliferate (Benett, 1956; Mintz and Russel, 1957). Although a significant part of the reduction of the number of germ cells in the mutant embryos is attributed to diminished proliferation and/or excessive cell death (McCoshen and McCallion, 1975), it was reported that 30% of W/W germ cells are found in ectopic sites, suggesting impaired migration of the mutant germ cells (Bueher et al., 1993). SLF expression along the migratory pathway of primordial germ cells, from the dorsal aorta to the genital ridge, and its disappearance after colonization except in the developing gonad also suggest that a defect in migratory/chemotactic function occurs in Sl and W mice (Matsui et al., 1990; Orr-Urtreger et al., 1990; Keshet et al., 1991). A role of SLF in supporting migration is also suggested for precursors of melanocytes called melanoblasts. SLF is expressed in the mesenchyme underlying developing skin of mouse embryos while melanoblasts migrate dorsolaterally and then colonize the skin (Matsui et al., 1990; Manova and Bachvarova,
2916 T. Kunisada and others

1991; Wehrle-Haller and Weston, 1995). After melanoblast colonization in the skin, SLF expression is thought to be confined to the dermal papillae of hair follicles, where melanoblasts mature into melanocytes and provide pigment granules to the hair keratinocytes (Yoshida et al., 1996b).

To further address the migratory/chemotactic roles of SLF, we have produced transgenic mice expressing SLF in the basal layer of the skin. In normal mice, the numbers of epidermal melanoblasts or melanocytes decrease from day 4 after birth and reach extremely low levels after one month of age (Hirobe, 1984). A 5′ regulatory sequence of the human cytokeratin 14 (hk14) gene was used to express murine SLF cDNA in the basal layer of stratified squamous epithelia including skin, tongue and esophagus (Vassar et al., 1989). Distribution pattern of melanoblasts and melanocytes of these transgenic mice indicated that ectopically expressed SLF can promote migration of melanocytes in vivo. This was further supported by genetic crosses of transgenic mice with W mutant mice. In W/W* or W*/W* transgenic progeny, the coats were mostly pigmented or the size of the pigmented regions were increased. The increase of the pigmented region was also observed by crossing with bt/bt spotting mutant mice.

The effect of SLF on melanoblasts was also assessed by using the function-blocking anti-c-Kit monoclonal antibody ACK2 (Nishikawa et al., 1991; Yoshida et al., 1993). As expected, ACK2 treatment completely prevented pigmentation of postnatal hk14-SLF transgenic mice. Unexpectedly, a month after ceasing ACK2 treatment, recovery of skin pigmentation was observed in the transgenic mice. This clearly indicates that c-Kit-independent stem cells of melanocytes, which are present only in the hair follicles of normal mice (Yoshida et al., 1996a) are maintained in the interfollicular skin of the transgenic mice.

MATERIALS AND METHODS

Mice

Original inbred colonies of mice containing the W, W* or bt mutation were obtained from the Jackson Laboratory and maintained in our animal colony to perform genetic crosses with transgenic mice described in this report. B6 and BDF1 mice were purchased from Japan SLC Inc. (Shizuoka, Japan). Appropriate pairs of animals positive for vaginal plugs were scored as 0.5 dpc at noon on that day.

Generation of transgenic animals

Full-length mouse SLF cDNA that could produce both soluble and membrane-bound forms of SLF (Yasunaga et al., 1995) was cloned into constructs containing the human cytokeratin 14 upstream region (Vassar et al., 1989), rabbit β-globin intron and poly(A) signal of human cytokeratin 14. Transgene excised from the plasmid sequence was injected into fertilized oocytes collected from (C57BL×SJL)F1 mice as described (Kimura et al., 1995). Integration of transgenes was verified by PCR of genomic DNA with transgene-specific primers. The restriction map of the transgene was described previously (Kunisada et al., 1998).

Genetic crosses

WB/ReJ/C57BL/6J→W/W* double homozygotes were crossed with SLFTg1-1 transgenic mice. The W+/+; SLFTg1-1 or W+/+; SLFTg1-

![](image)

**Fig. 1.** Phenotype of hk14-SLF transgenic mice. Transgenic mouse line SLFTg1-1, heterozygous for the transgene, was used. Newborn (A), 4-day-old (B), 14-day-old (C) and 2-month-old (D,E) transgenic mice were photographed with their littermates. Transgenic mice are readily distinguished by their hyper-pigmented area as shown by arrowheads in A. Arrowheads in E show shaved area of back skin (note that haired skin of the transgenic mouse is pigmented). Expression of melanocyte-specific TRP2 protein was examined by immunohistochemistry for 2-month-old (F) and littermate (G). TRP2-positive cells (arrowheads) were detected in the whole epidermis in transgenic skin but were restricted to dermal papillae in littermates. Asterisks show dermoeipidermal junction and an arrow in F points to a melanin granule responsible for pigmentation of the transgenic skin. Coat phenotypes of different SLF transgenic animals, SLFTg2-1 (H), SLFTg4-1 (I), SLFTg4-2 (J), SLFTg4-3 (K), were photographed at their age of 2 month old. Unpigmented ventral skin region is indicated by an arrow in H.
Development of melanocytes and steel factor 2917

Among nine independent transgenic mice expressing SLF driven by skin-specific cytokeratin 14 promoter, two stably transmitted a black epidermal color phenotype (SLFTg1-1 and SLFTg1-2). In these two lines, the overall characters of skin pigmentation during postnatal development were similar. At birth, nose, pawpads, and external genitalia were already pigmented (Fig. 1A, arrowheads) while most parts of the skin appeared to be normal. As early as 4 days of age, pigmentation of the entire epidermis was visible (Fig. 1B). By 14 days of age, most parts of the skin, including tail, were heavily pigmented (Fig. 1C). Pigmentation of the unhaired skin such as mouth and paws (Fig. 1D) as well as haired skin (Fig. 1E) increased progressively until adulthood. In the skin of SLFTg1 transgenic mice, melanocytes expressing TRP2 protein were observed in the basal layer of the epidermis including hair follicles (Fig. 1F, arrowheads). Since TRP2 is the first melanocyte lineage-specific protein to be expressed in melanoblasts (Steel et al., 1992; Pavan and Tilghman, 1994; Wehrle-Haller and Weston, 1995, Kunisada et al., 1996), it appears that production of SLF in the keratinocytes induced the proliferation of melanocytes in the transgenic mice. In contrast, TRP2-positive cells of skin in normal nontransgenic control mice were restricted to the dermal papillae (Fig. 1G, arrowheads). Four of the transgenic mice showed slightly pigmented skin at birth and, in these mice, the entire skin was heavily pigmented by 4 days after birth (SLFTg2-1 to SLFTg2-4; the analysis of these mice was reported in Kunisada et al., 1998). Pigmentation in these mice was apparently more severe than in SLFTg1 strains. One of these heavily pigmented strains, SLFTg2-1, showed an unpigmented dorsal region (Fig. 1H, shown by an arrow). All of these severe phenotypes suffered growth arrest after 2 weeks of age and died within a few months after birth. A total of 20 F1 of SLFTg2-1 and SLFTg2-2 mice reproduced by ovary transfer showed similar phenotype. One transgenic mouse was heavily pigmented in...

Histology and immunohistochemistry

Tissues from transgenic and littermate mice were fixed, embedded in polyester wax and sectioned (7 μm) as described (Yoshida et al., 1993). After removing wax with ethanol and rehydrating twice with PBS, the sections were preincubated for 20 minutes in 5% normal goat serum in PBS and incubated overnight with the primary antibody. For the primary antibody, rabbit anti-mouse TRP2 polyclonal antibody provided by V. Hearing (Tsukamoto et al., 1992) diluted 300-fold with 5% goat serum was used. After three washes in PBS/0.02% Tween20 (PBST), the sections were incubated with biotin-conjugated goat anti-rabbit IgG antibody solution (Vector) for 2 hours. After two washes with PBST and one wash with 0.1 M Tris (pH 8.0), the sections were incubated with HRP-conjugated avidin (Vector) for 1 hour, washed three times with 0.1 M Tris (pH 8.0) and then mounted for fluorescence microscopy using Vectashield mounting solution (Vector). Sections stained with secondary antibody alone were used as a control.

RESULTS

Pigmentation of SLF transgenic mice skin during postnatal development

Among nine independent transgenic mice expressing SLF driven by skin-specific cytokeratin 14 promoter, two stably transmitted a black epidermal color phenotype (SLFTg1-1 and SLFTg1-2). In these two lines, the overall characters of skin pigmentation during postnatal development were similar. At birth, nose, pawpads, and external genitalia were already pigmented (Fig. 1A, arrowheads) while most parts of the skin appeared to be normal. As early as 4 days of age, pigmentation of the entire epidermis was visible (Fig. 1B). By 14 days of age, most parts of the skin, including tail, were heavily pigmented (Fig. 1C). Pigmentation of the unhaired skin such as mouth and paws (Fig. 1D) as well as haired skin (Fig. 1E) increased progressively until adulthood. In the skin of SLFTg1 transgenic mice, melanocytes expressing TRP2 protein were observed in the basal layer of the epidermis including hair follicles (Fig. 1F, arrowheads). Since TRP2 is the first melanocyte lineage-specific protein to be expressed in melanoblasts (Steel et al., 1992; Pavan and Tilghman, 1994; Wehrle-Haller and Weston, 1995, Kunisada et al., 1996), it appears that production of SLF in the keratinocytes induced the proliferation of melanocytes in the transgenic mice. In contrast, TRP2-positive cells of skin in normal nontransgenic control mice were restricted to the dermal papillae (Fig. 1G, arrowheads). Four of the transgenic mice showed slightly pigmented skin at birth and, in these mice, the entire skin was heavily pigmented by 4 days after birth (SLFTg2-1 to SLFTg2-4; the analysis of these mice was reported in Kunisada et al., 1998). Pigmentation in these mice was apparently more severe than in SLFTg1 strains. One of these heavily pigmented strains, SLFTg2-1, showed an unpigmented dorsal region (Fig. 1H, shown by an arrow). All of these severe phenotypes suffered growth arrest after 2 weeks of age and died within a few months after birth. A total of 20 F1 of SLFTg2-1 and SLFTg2-2 mice reproduced by ovary transfer showed similar phenotype. One transgenic mouse was heavily pigmented in...
the entire skin region at birth and died within 12 hours (SLFTg3, data not shown). The remaining three transgenic mice were lightly pigmented in ears, external genitalia and tail, and not pigmented in the haired skin. In SLFTg4-1, only the tail was pigmented (Fig. 1I). In SLFTg4-2, tail and external genitalia were pigmented (Fig. 1J). In SLFTg4-3, tail, external genitalia and ears, but not haired skin, were pigmented (Fig. 1K). Unless otherwise mentioned, the SLFTg1-1 strain was used throughout the experiments in this paper.

**Hyperpigmentation of transgenic mice is SLF dependent**

Skin-specific expression of SLF from transgene was first tested by northern analysis (SLFTg1-1, SLFTg1-2 and SLFTg2-1, data not shown) or RT-PCR with transgene-specific primers (SLFTg4-1 to SLFTg4-3, data not shown). To confirm that interfollicular skin pigmentation of the transgenic mice was dependent on SLF produced by the transgene, we administrated anti-c-Kit monoclonal antibody (ACK2) to the newborn mice. ACK2 is known to eliminate proliferating melanocyte lineage cells by the blockage of SLF induced c-Kit function (Nishikawa et al., 1991; Yoshida et al., 1993). Intraperitoneal injection of the antibody at 0, 2 and 4 days after birth completely abolished the pigmentation of normal mice (compare +/+ control and +/+ ACK2-treated mice of Fig. 2). This complete depigmentation was also maintained in antibody-treated SLFTg1-1 mice for at least 1 week after birth (Fig. 2, tg/+ cont and tg/+ACK2). Thus, increased epidermal pigmentation in SLF transgenic mice is absolutely dependent on expression of transgenic SLF.

**Extension of the pigmented skin region in SLF transgenic mice**

The appearance of heavily pigmented regions in transgenic mice such as nose, ears, lip, gums, paws, nails and external genitalia, which are normally unpigmented or hypopigmented in the wild-type mice was a common feature of hkl4-SLF transgenic mice as shown in Fig. 1. Among these tissues, nose, ears, lip and external genitalia were not completely unpigmented even in the adult wild-type mice: histological examination of these tissues always indicated the presence of mature dendritic melanocytes although they were low in number (data not shown). Therefore, hyperpigmentation in these regions in SLFTg mice could be readily explained by the ability of exogenous SLF to promote the c-Kit-dependent proliferation of melanocyte precursors that had previously migrated to these areas.

However, in gums, paws and nails, we could not detect mature melanocytes in normal adult mice (data not shown). At 3 days after birth, gums for the incisor teeth and molar teeth of SLF transgenic mice were heavily pigmented and the entire oral epithelium including lips was also lightly pigmented (Fig. 3A). In a wild-type litter, no pigmentation was observed in the entire region of the oral epithelium although the haired skin region outside the lips was lightly pigmented (Fig. 3B). At day 11 after birth, no pigmentation was seen in the oral epithelium of the wild-type litter (Fig. 3C); however, most of the oral epithelium including lips and gums was heavily pigmented in SLF transgenic mice (Fig. 3D). In some cases, the inside of the incisor tooth was pigmented (data not shown). In paws of transgenic mice, the pads of the palmar surface of the forelimb were heavily pigmented at day 3 (Fig. 3E) and the pigmentation over the paws became extensive, including on the inside of the nails (Fig. 3F). In contrast, no pigmentation was observed in the unhaired pads and nails of the wild-type litter (Fig. 3G). These observations raise the possibility that melanocytes of the transgenic mice responsible for the pigmentation of the normally unpigmented areas such as gums or pads were derived from melanocyte precursors (melanoblasts) that migrated out from the limit of their normal distribution area where melanocytes in normal mice eventually reside. Thus, the SLF transgene might have promoted the further migration of melanocyte lineage cells.

**Distribution of melanocyte precursors in SLF transgenic mice**

If extended migration of melanoblasts promoted by exogenous SLF is responsible for pigmentation of gums and pads of the transgenic mice, then melanoblasts should be located in those areas prior to pigmentation in situ. To test this, we performed immunohistological analysis by using antibody for melanocyte lineage-specific TRP2 enzyme. In 14.5 dpc embryos, no melanoblasts stained by anti-TRP2 antibody were found in the oral epithelium of gums or orpharinx region of either SLF transgenic or wild-type litter mice (data not shown). In 16.5 dpc embryos, melanoblasts expressing TRP2 were found in the basal cell layer of the oral epithelium of transgenic mice (Fig. 4A). However, melanoblasts were not detected in the corresponding part of the wild-type litters (Fig. 4B). After extensive observation of eight different litters, no melanoblast was seen in any sections of embryos containing the oral region. Therefore, we concluded that melanocyte precursors never reach the mouth epithelium of normal mice. The number of melanoblasts in the epidermal skin at this stage was comparable in transgenic and wild-type mice as shown in Fig. 4C and D, respectively. In the newborn transgenic animals, the number of melanoblasts in the oral epithelium and epidermal skin was increased significantly (Fig. 4E,F) when compared to those of 16.5 dpc. Still, no TRP2-positive cells were observed in normal littermates (Fig. 4G). As expected from the increasing pigmentation of transgenic mice after birth, melanoblasts of the newborn transgenic skin outnumbered those of wild-type litters (compare Fig. 4F and H). In most cases, TRP2-positive melanoblasts were found inside of the primordial molar teeth as indicated by the arrows in Fig. 4E. In pawpads, TRP2-positive melanoblasts were located in transgenic 16.5 dpc embryos but no TRP2-positive melanoblasts were detected in wild-type littermates (data not shown). Collectively, these data suggest that it is highly likely that exogenous SLF stimulated the migration of epidermal melanoblasts of as early as 16.5 dpc embryos to reach the entire oral epithelium and pads of paws, and then these precursor cells continued to differentiate into mature melanocytes in situ.

As mentioned in the previous section, mature melanocytes were observed in the ears of normal mice. By histological analysis, mature melanocytes were found mostly in the dermis of the ears of normal mice as shown by an arrowhead in Fig. 4I. Interestingly, the majority of melanocytes in SLF transgenic mice were confined to the epidermis as shown in Fig. 4J. As melanocytes of the ears of normal mice might be maintained by endogenous SLF expressed in the dermis of the ears (Yoshida et al., 1996a), movement of the melanocytes of the transgenic mice to the ear epidermis might be caused by the relatively higher...
concentration of local SLF derived from the transgene. Also, in this case, SLF might play a role in the migration of melanoblasts.

**Extended coat pigmentation of Sl and W mutant mice in the presence of hk14-SLF transgene**

Melanoblasts originated from the neural crest migrate through a dorsolateral pathway and cover most of the haired skin region (Le Douarin, 1982; Hirobe, 1992; Bronner-Frazer, 1993; Erickson and Perris, 1993; Bennett, 1993; Anderson, 1997). During this migration phase, melanoblasts are located exclusively in the dermis and then invade the epidermis around 12 to 13 dpc (Kunisada et al., 1996). Because c-Kit signaling through SLF is indispensable at least for the survival of melanoblasts, mutations in the W and Sl loci exhibit reduced or complete loss of coat color pigmentation. Among these mutations, W<sup>57</sup> showed reduced expression of the c-kit gene of trunk melanoblasts, giving rise to an irregular white band in the trunk region as indicated by W<sup>57</sup>/W<sup>57</sup>; +/+ in Fig. 5A. When this mutation is expressed as a compound mutation with W in which a nonfunctional c-Kit molecule without the transmembrane region is produced (Hayashi et al., 1991), the amount of c-Kit is further reduced and results in an almost complete loss of pigmentation of the hair of the entire body (W/W<sup>57</sup>; +/+ of Fig. 5A). In the presence of the SLF transgene, the white band of W<sup>57</sup>/W<sup>57</sup> was greatly reduced in size (W<sup>57</sup>/W<sup>57</sup>; tg/+ of Fig. 5A) and colored skin regions appeared in the head and rump of W/W<sup>57</sup> mice (W/W<sup>57</sup>; tg/+ of Fig. 5A). In addition, a clear unpigmented band in the mid-trunk region characteristic of b/bt mutant mice (b/bt; +/+ of Fig. 5B; its responsible gene is not identified at present, see Mayer and Maltby, 1964) was reduced in the presence of the transgene (b/bt; tg/+ of Fig. 5B).

These results could be explained by the stimulation of migration of melanoblasts as well as stimulation of growth and/or survival of melanoblasts by the exogenous SLF expressed in the epidermis. We think that compensation of reduced melanoblasts by the SLF transgene occurred 14.5 dpc or later because the extension of the migration area of melanoblasts in 16.5 dpc transgenic embryos was not observed in 14.5 dpc embryos. In this stage, most of the melanoblasts were located in the basal layer of the epidermis, where the SLF transgene was expressed. As observed in the embryos of lethal spotting (ls/ls) mutant mice (Yoshida et al., 1996), melanoblasts are able to migrate through the epidermis of 14.5 dpc or later embryos.

**Maintenance of c-Kit-independent stem cells of melanocytes and their differentiation in the postnatal skin of hk14-SLF transgenic mice**

When SLF transgenic mice treated with anti-c-Kit function-blocking antibody ACK2 described in the previous section were raised for a month (Fig. 6A, unshaved ventral view of the mice), dorsal to lateral skin was uniformly repigmented (Fig. 6B) and black pigmented spots appeared in ventral skin (Fig. 6C), unhaired skin of ears, mouth, foot (Fig. 6A) and tail (Fig. 6D). Considering that there was previously complete depigmentation (Fig. 2) and no detectable TRP-2-expressing melanocyte precursors (data not shown) in the skin of day 10 transgenic mice treated with ACK2, the observed repigmentation seems to be caused by the regeneration of melanocytes differentiated from stem cells that were maintained independent of c-Kit signaling. Each spot of Fig. 6C thus represents a single stem cell clone. The pigmented spots of ventral skin continued to expand and, 6 months after ACK2 administration, all spots were completely fused (Fig. 6E). Histological analysis showed uniformly distributed melanocytes in the epidermis of repigmented skin (data not shown). This would further support a role of SLF in the skin to promote migration of melanocytes originated from small numbers of stem cells. The expansion of pigmented spots is c-Kit dependent because the size of each spot remained unchanged and the color of the spots faded after the ACK2 treatment of 1-month-old SLF transgenic mice that had developed clear pigmented spots due to the neonatal ACK2 treatment (compare Fig. 6G and H).

**DISCUSSION**

Multiple distinct developmental stages of melanocytes are known to be affected by c-Kit and its cognate ligand SLF (Yoshida et al., 1996a; Kluppel et al., 1997). By the continuous expression of SLF in the epidermis, we have clearly found evidence for the precise control of survival, growth, differentiation and migration of melanocytes attained by SLF-c-Kit signaling.

In normal mice, DOPA reaction-positive cells (melanoblasts and melanocytes) found in the epidermis at birth decrease in number from postnatal day 4 and then virtually disappear after 1 month except for those in hair follicles (Hirobe, 1984). The number of these interfolllicular melanocyte lineage cells was increased in the skin of SLF transgenic mice after birth, suggesting that the survival of melanocyte precursors was extended by the locally produced SLF. Immediate loss of TRP2-positive melanoblasts from the neonatal skin by the c-kit function-blocking antibody (ACK2) injection and lack of significant recovery of melanoblasts until day 10 after ceasing injection of ACK2-treated mice (Fig. 2) also support the conclusion that SLF promotes the survival of melanocyte lineages in vivo. The fully pigmented melanocytes of SLF transgenic mice were resistant to ACK2 treatment (H. Y. et al., unpublished data) suggesting that SLF affects the survival of early stages of melanocyte lineage cells.

Unexpectedly, the ACK2-induced complete loss of melanoblasts from the skin of SLF transgenic mice was followed by the nascent regeneration of melanocytes a month after ceasing the ACK2 treatment. This strongly suggests the maintenance of c-Kit-independent stem cells for melanocytes in the SLF transgenic skin. The presence of such a kind of c-Kit-independent melanoblasts was previously reported in the hair follicles of normal mice (Nishikawa et al., 1991; Okura et al., 1995). It was also shown that melanoblasts pass through several alternating c-Kit-dependent and c-Kit-independent stages during their normal development (Yoshida et al., 1993). Thus, in the presence of ectopic SLF, c-Kit-independent melanoblasts are likely to be constantly generated from the more immature c-Kit-dependent melanoblasts. These c-Kit-independent cells are derived from c-Kit-dependent melanoblasts because ACK2 treatment of SLF transgenic embryos produced mice that were completely depigmented even after a year (T. K. et al., unpublished data).

The existence of numerous melanoblasts in the skin of SLF transgenic mice clearly suggests the promotion of proliferation by the exogenous SLF. The blockage of further expansion of the pigmented spots observed in the second ACK2 treatment
of SLF transgenic mice (Fig. 6H) indicates that the proliferation of the melanocyte lineage cells in the developed interfollicular transgenic skin is promoted directly by the exogenous SLF.

Differentiation of melanoblasts was also affected by SLF. In SLFTg2-1, which showed extensive pigmentation of the entire skin accompanied by massive mast cell accumulation (Kunisada et al., 1998), pigmentation was visible in all regions of newborn skin. In the case of one extraordinary transgenic mouse, the whole epidermal region was severely pigmented at birth with apparent epidermal hyperplasia possibly caused by a massive number of mature melanocytes (data not shown). Since melanoblasts/melanocytes found in the newborn skin of normal mice are rarely pigmented, this may imply that SLF promotes differentiation of melanocyte lineages. Interestingly, an unpigmented ventral region is a characteristic of SLFTg2-1 (shown by an arrow in Fig. 1H). The relatively abundant transgenic SLF may allow migrating melanocytes to differentiate into mature melanocytes before reaching abdominal skin. This observation thus provides additional support that SLF may act as a stimulus for melanocyte differentiation in vivo. While it is well established that in vitro culture of neural crest cells in the presence of SLF promotes mainly the survival of melanoblasts (Murphy et al., 1992; Steel et al., 1992; Morrison-Graham and Weston, 1993), SLF or other extracellular signals have been reported to activate the transcription factor MITF (Hemesath et al., 1998; Bertolotto et al., 1998) in melanocyte cell line. As MITF is known to have the capability to differentiate fibroblasts into melanocyte-like cells (Tachibana et al., 1996), SLF may promote the differentiation of melanocyte lineages via MITF in the skin of the transgenic mice.

**Fig. 4.** Distribution of TRP2-positive melanoblasts in SLF transgenic mice. Coronal sections through head were stained by anti-TRP2 antibody. In 14.5 dpc embryo, TRP2-positive melanoblasts were detected in the dermoepidermal junction of oral epithelium cells (A). In this stage, TRP2-positive cells (indicated by an arrowhead) were restricted to the epithelium of alveolar ridges. While no TRP2-positive cells were detected in the corresponding regions of littermates (B), the basal cell layer of body wall skin contained approximately equal densities of TRP2-positive cells in SLF transgenic (C) and littermate (D) mice. In newborn transgenic mice, the entire oral epithelium was filled with TRP2-positive cells (E). In this stage, TRP2-positive cells were detected in the primodium of teeth, as indicated by an arrow in E. Body wall skin in this stage was also filled with TRP2-positive cells in the transgenic mouse (F). Note that the density of TRP2-positive cells was higher in transgenic skin than in that of normal littermates (H). No TRP2-positive cells were found in the oral epithelium of normal littermates (G). In the ears of 2-month-old normal mice, pigmented melanocytes (arrowhead) were mostly distributed in the dermal area (J) whereas melanocytes (arrowhead) were restricted to the epidermis (I). Asterisks in I and J indicate hair follicles. Abbreviations: oc, oral cavity; tg, tongue; hf developing hair follicle; pmt, primodium of molar tooth; nc, nasal cavity. Scale bar represents 100 μm in A-H and 50 μm in I and J.
Promotion of the migrative/chemotactic activity by SLF was suggested previously in melanocyte, mast cell and germ cell lineages during embryonic development (Galli et al., 1993). One direct experimental approach taken has been in vitro measurement of chemotactic activity of c-Kit-expressing endothelial cells (Blume-Jensen et al., 1991) or mast cells (Meininger et al., 1992) in response to recombinant SLF. Human melanocytes cultured in vitro did not show the induction of chemotactic activity by SLF (Horikawa et al., 1995). Although subcutaneous injection of SLF resulted in the appearance of a large number of mast cells at the injection site in the mouse (Zsebo et al., 1990; Tsai et al., 1991) and melanocytes in humans (Harrist et al., 1995; Costa et al., 1996), the circulating nature of mast cell precursors and the presence of melanocytes before SLF injection in the human skin also raise the possibility that SLF might only have promoted growth and differentiation, not migration of these cells. By using a cytokeratin promoter, we have induced targeted expression of SLF in the whole epidermal layer including the regions where no pigmentation is observed in the normal mouse. In these regions, especially in the oral epithelium and footpads, no melanocyte precursors are detected in any developmental stages of normal mice. Accordingly, melanoblasts found in these regions of SLF transgenic mice are likely to have had their limit of migration extended by the exogenous SLF. Simple overgrowth might not explain the observation that the density of melanoblasts in transgenic embryos remained comparable to that of normal littermates when precursors had reached the oral epithelium at 16.5 dpc (Fig. 4C,D). Likewise, in Sl<sup>con</sup> mutant mice, melanocytes are known to accumulate in the reproductive tract and oviduct and in these tissues ectopic expression of SLF mRNA was observed (Bedell et al., 1995). The pigmentation defects, i.e. the absence of melanocytes in the mid-trunk region of W/W<sup>57</sup>, W<sup>57</sup>/W<sup>57</sup> or bt/bt mutations were significantly rescued by crossing with SLF transgenic mice. Under the transgenic background, as shown in Fig. 5, pigmented areas of the skin were enlarged (W/W<sup>57</sup>; tg/+ and bt/bt; tg/+ or newly formed on the originally white coat (W/W<sup>57</sup>; tg/+). Apparently, even in a white coat phenotype such as W/W<sup>57</sup>, melanoblasts generated as early as 9.5 dpc survive until 12.5 to 13.5 dpc, when they enter the basal layer of epidermis and utilize transgenic SLF expressed in situ. Provided that there exist other factor(s) independently conferring migratory activity on melanoblasts, these surviving melanoblasts at least migrate normally and distribute over the whole coat skin. Subsequently, more or less randomly

**Fig. 5.** Coat phenotypes of W<sup>57</sup>/W<sup>57</sup> and W/W<sup>57</sup> mutant mice (A) or bt/bt mutant mouse (B) crossed with SLF transgenic mice. Phenotypes were consistent in the several independent crosses of both mutants.

**Fig. 6.** Repigmentation of SLF transgenic mice treated with ACK2. SLFTg1-1 mice were injected with ACK2 on days 0, 2 and 4 after birth and raised for a month. Most of the hair is unpigmented in the belly skin (A) and a significant portion of the hair of the back skin is pigmented (B). The interfollicular region of the back skin is completely pigmented (shaved area of B) and pigmented spots are observed in the belly skin (C), tail (D), foot and external genitalia (A). When these ACK2-treated mice was raised for 2 additional months, the belly skin (E) and tail (F) were completely pigmented. This expansion of pigmented spots was prevented by a second administration of ACK2 antibody; the 1-month-old mouse shown in G was injected with ACK2 every other day for a total of 3 injections and photographed after a month (H).
distributed pigmented patches derived from these melanoblasts might be produced. However, in our observations, pigmented areas appear exclusively in the head and posterior trunk regions as shown in Fig. 5. This distribution pattern is consistent with the observation that TRP2-positive melanoblasts were far more abundant in these regions than in the mid-trunk region (Yoshida et al., 1996). Therefore, surviving melanoblasts in these W or Sl mutants do not seem to actively migrate during 9.5 to 12.5 dpc. However, the possibility that the main function of the SLF transgene in these genetic cross experiments is only to complement growth and/or survival of affected melanoblasts of the W or Sl mutant can not be ruled out. Melanoblasts derived from the most recently generated neural crest cells might be saved by transgenic SLF after the entry into epidermis. These saved melanoblasts might generate pigmented areas in the head and tail regions. Another line of evidence came from the pigmented spots formed after ACK2 treatment of SLF transgenic mice. They first appeared as small dots and then rapidly increased their size, and this process was blocked by the second ACK2 treatment. Taken together, these results strongly suggest a migratory role of SLF; however, the phenotype expressed as an alteration of coat pigmentation pattern could not be rigorously attributed to either proliferative or migratory roles of SLF. The additional observation that melanocytes in the ear of SLF transgenic mouse were distributed closely under the epidermal layer whereas they mostly reside in the dermal layer in the normal control might indicate the migratory or chemotaxic roles of SLF on the melanocyte lineage cells.

The various pigmentation phenotypes that appeared among SLF transgenic mice indicate that the expression level of SLF affects the distribution of melanocytes in the skin. Expression levels of transgenic SLF in the skin examined are SLFTg2-1>SLFTg1-1>SLFTg4-3 (Kunisada et al., 1998; T. K., unpublished data). In SLFTg4-1 to SLFTg4-3, tail, external genitalia or ears were pigmented (Fig. 11-K). In SLFTg1-1 and SLFTg1-2, unhaired skin as well as the entire haired skin were pigmented (Fig. 1D,E). As described in the previous paper, the entire skin of SLFTg2-1, SLFTg2-2, SLFTg2-3 and SLFTg4-2 mice was severely pigmented and varying sizes of completely unpigmented areas appeared in the belly, feet or tail. Although they are not found in haired skin, mature melanocytes reside in tail, external genitalia and ears of normal mice; therefore, a small amount of SLF may promote the survival of melanoblasts in these tissues of SLFTg4 mice. Along with the reduction of endogenous SLF in the haired skin region, surviving melanoblasts in the haired skin may disappear. For the continuous proliferation or differentiation of melanocytes in the haired skin, the expression of SLF at greater levels than that of SLFTg1 skin might be required.

In summary, analysis of the SLF transgenic mice described in this report has clearly demonstrated that SLF acts as a key factor to support the temporally and spatially coordinated survival, proliferation, differentiation and migration of melanocyte lineages. In addition, some of the transgenic lines established enabled us to study these developmental processes in the skin of postnatal mice. In particular, the repigmentation process after ACK2 treatment of SLF transgenic mice reported here is similar to the repigmentation phase of vitiligo patients.

The use of the transgenic mice may serve to search for an effective method to promote vitiligo repigmentation.

We thank Dr. T. Shibahara of the Laboratory Animal Research Center of Tottori University for maintenance of the transgenic mice and Ms. T. Shinohara for secretarial assistance and also thank Dr. E. Fuchs for his 4 promoter sequence, and Dr. V. Hearing for anti-TRP-2 antibody. This work was supported in part by grants from the Ministry of Education, Science, Sports and Culture of Japan, Special Coordination Funds for Promoting Science and Technology from the Science and Technology Agency of Japan, Uehara Memorial Foundation (Tokyo, Japan). We also thank for the support from the Cellular Technology Institute, Otsuka Pharmaceutical Co., Ltd (Tokushima, Japan).

REFERENCES


Bell, D. C., Bra, C. I., Evans, E. P., Coppen, N. G., Jenkins, N. A. and Donovan, P. J. (1995). DNA rearrangements located over 100 kb 5′ of the Steel (St) coding region in Steel-panda and Steel-contrasted mice deregulate Sl expression and cause female sterility by disrupting ovarian follicle development. Genes Dev. 9, 455-470.


