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Distinct stages of melanocyte differentiation revealed by analysis of nonuniform pigmentation patterns

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SUMMARY

The injection of an antagonistic anti-murine *c*-kit monoclonal antibody ACK2 during mouse embryonic development produced three distinctive pigmentation patterns on the coat of the offspring. Pattern 1 consisted of pigmentation in craniofacial and caudal regions and was induced by an ACK2 injection between 9.5 and 11.5 days post coitum (dpc). In pattern 2, the entire coat was unpigmented and was induced by the injection at around 13.0 dpc. Pattern 3 consisted of pigmented patches spreading ventrolaterally from the dorsoanterior trunk regions towards the anterior and posterior directions and it was induced by ACK2 administered at 14.5-15.0 dpc. We investigated the embryological basis of these nonuniform pigmentation patterns to elucidate the process of melanoblast differentiation between lineage commitment and colonization into developing hair follicles.

The results showed the following. (1) Melanocyte differentiation at the embryonic stage from 10.5 to 12.5 dpc progresses in a spatially nonuniform fashion, being faster in the craniofacial and caudal regions than in the trunk; pattern 1 reflects this. (2) Melanoblasts are activated to proliferate synchronously upon entering into the epidermis; pattern 2 correlates with this process. (3) *c*-kit functions as a survival signal for proliferating melanoblasts in the epidermis. (4) The melanoblasts that enter developing hair follicles can survive without a *c*-kit signal; pattern 3 essentially represents the hair follicles colonized by these cells. Analysis of the melanoblast distribution of *ls/ls* embryos that bear a loss-of-function mutation in the endothelin 3 gene suggested that endothelin 3 is required for early melanoblast differentiation before entering into the epidermis, whereas proliferation in the epidermis takes place without this molecule.

Based on these data, we propose 4 distinct steps of embryonic melanocyte differentiation: (1) migration in the dermis, which requires both *c*-kit and endothelin 3; (2) a stage before epidermal entry that is resistant to anti-*c*-kit mAb; (3) cell proliferation after entering the epidermal layer, which requires *c*-kit and endothelin receptor B but not endothelin 3 and (4) integration into developing hair follicles, which renders melanoblasts resistant to anti-*c*-kit mAb. Thus, melanoblast differentiation proceeds by alternately repeating *c*-kit-dependent and *c*-kit-independent stages and *c*-kit functions as a survival factor for the proliferating melanoblasts.

Key words: *c*-kit, TRP2, endothelin, pigmentation pattern, melanoblast, *lethal spotting*, mouse

INTRODUCTION

Mice with nonuniform pigmentation patterns have provided important model systems for investigating the development of melanocytes. Studies of mouse genetics have identified a number of loci, mutations of which cause nonuniform pigmentation patterns. According to the list presented in 'The Coat Colors of Mice' of Silvers (1979), those include *Piebald*, *Lethal spotting*, *Belted*, *Dominant spotting*, *Patch*, *Rump-white*, *Steel*, *Flexed tailed*, *Splotch*, *Varitint-waddler*, *Mottled*, *Microphthalmia*, *Fleck*, and *Belly spot and tail*. The underlined loci are those that have been characterized to some extent at the molecular level (Hosoda et al., 1994; Baynash et al., 1994; Chabot et al., 1988; Geissler et al., 1988; Stephenson et al., 1991; Nagle et al., 1994; Copeland et al., 1990; Huang et al., 1990; Zsebo et al., 1990; Epstein et al., 1991; Levinson et al., 1994; Hodgkinson et al., 1993). However, the present level of understanding of the molecular nature of each mutation is insufficient to explain the dynamic processes whereby mutations of a given gene lead to a particular set of pigmentation patterns.

Nonuniform pigmentation patterns can also be created by embryonic manipulations. Chimeric mice have been raised that consist of embryonic cells from mice that differ in coat color, either by making aggregation chimera (Mintz, 1967) or by an intraembryonal injection of cultured neural crest cells (Jaenisch, 1985). Huszar et al. (1991) have reported that a large area of the coat can be colonized by a very few, perhaps indi-

vidual melanocyte progenitors, an example of the remarkable proliferative capacity of the earliest melanocyte progenitors.

In addition, we described the manipulation of murine pigmentation patterns using the anti-*c*-kit monoclonal antibody (mAb) ACK2 (Nishikawa et al., 1991; Yoshida et al., 1993). Phenotypic analyses of the mutant mice of *c*-kit gene have revealed that *c*-kit and its ligand are required for the development of melanocytes in the embryo (see review in Besmer et al., 1993). Hence, inhibition of *c*-kit function by ACK2 should affect melanocyte development. In contrast to mutations in the *c*-kit gene, however, the ACK2-mediated inhibition of *c*-kit function can occur at any stage of embryogenesis. If a regional difference in the progression of the *c*-kit-dependent process of melanocyte differentiation is present, an ACK2 injection at a specific time should produce nonuniform pigmentation patterns that represent where this process has been completed or is still underway. We demonstrated that ACK2 injections at various embryonic stages produced a set of distinct pigmentation patterns, some of which were entirely different from those displayed in the mice bearing mutations in the coding region of the *c*-kit gene. Though extensive variation was present, three distinct patterns that might represent different process of melanocyte differentiation were discerned. The first was pattern 1, which consisted of pigmented areas in the craniofacial and caudal regions. This pattern was induced by an intraaminiotic injection of ACK2 in embryos younger than 12.5 dpc and resembled the pigmentation patterns displayed by *ls/ls*, *Ph/+* and W^{sh}/+ mice. While the pigmented area of pattern 1 was inclined to spread towards the trunk region as the ACK2 was injected later, this tendency was interrupted by pattern 2, induced by an injection at around 13 dpc, in which the entire coat was unpigmented. Pattern 3 consisted of complex pigmented spots spreading ventrolaterally from the dorsoanterior trunk towards the anterior and posterior directions. This pattern was produced by an ACK2 injection at 14.5-15.0 dpc. After this stage, ACK2 failed to alter the pigmentation pattern. While it is likely that these three patterns are the outcome of *c*-kit inhibition during distinct processes of melanocyte differentiation, our studies failed to specify which process in melanocyte differentiation corresponded to each pigmentation pattern.

We therefore compared the melanoblast distribution in mice at a given embryonic stage with the pigmentation patterns that were induced by an ACK2 injection at the same stage. To identify melanocyte precursors, we used a mAb to *c*-kit and a polyclonal antibody to tyrosinase-related protein 2 (TRP2) (Jackson et al., 1992). We visualized the pigmentation pattern by means of whole-mount immunohistochemistry. This study showed that pattern 1 is the result of *c*-kit-inhibition during the migration of melanoblasts in the dermis, pattern 2 of the proliferation of melanoblasts in the epidermis and pattern 3 of melanoblasts colonizing into developing hair follicles.

MATERIALS AND METHODS

Mice

BDF1 and C3H/HeN mice were purchased from Japan SLC Inc.(Shizuoka, Japan). Ls/Le *ls/ls* mutant mice were donated by Dr

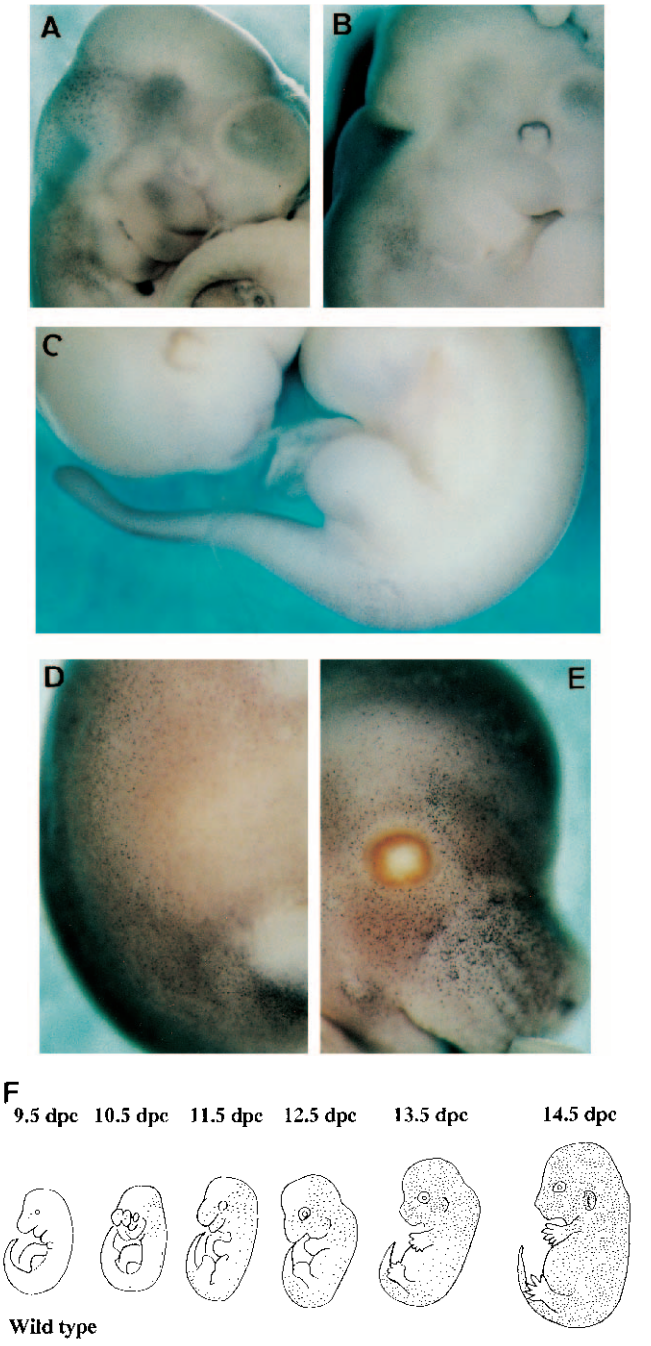

Fig. 1. Distribution of *c*-kit$^+$ and TRP2$^+$ cells in normal mouse embryos. (A) In the 10.5 dpc cranial region, *c*-kit$^+$ dendritic cells are distributed from the surface area over the midbrain to the nasal regions. (B) TRP-2$^+$ cells are visible in the ventrolateral, but not in dorsal region. (C) In the 11.5-12.0 dpc embryo, there is a cluster of *c*-kit$^+$ dendritic cells in the tail root region. (D,E) In the 13.5 dpc embryo, *c*-kit$^+$ dendritic cells are evenly distributed in the trunk region (D) and also in the facial region (E). (F) An illustrative summary of the melanoblast distribution pattern of wild type embryos at various developmental stages. These illustrations are based on a series of whole-mount staining of the embryos with ACK4 and anti-TRP2 antibody. The *c*-kit$^+$ spheric cells are not counted in this figure.

L. D. Shultz of The Jackson Laboratory and maintained in our animal colony for a few generations. Females were paired with males overnight and vaginal plugs were checked the following morning. Noon of the day when a vaginal plug was detected was designated day 0.5 of gestation (0.5 days postcoitum, 0.5 dpc). The developmental stage of the embryos was finally judged by their morphological appearance according to the staging procedure described in 'Methods in Experimental Embryology of the Mouse' (K. A. Rafferty, Jr., 1969.) and 'The Atlas of Mouse Development' (Kaufman, 1992).

Immunohistochemistry

Tissue fixation procedures were basically same as previously described (Yoshida et al., 1993) but microwave irradiation for prefixation proceded for 30 seconds twice at 600 W in a microwave cooking oven (Toshiba, ER-365S). Fixed specimens were embedded into polyester wax and sectioned at 5 μm as described (Kusakabe et al., 1984). Immunostaining procedures were as described (Yoshida et al., 1993) except that metal-enhanced diaminobenzidine (DAB) substrate kit (Pierce) was used without DAB/gold/silver intensification. Monoclonal rat anti-mouse-*c*-kit monoclonal antibodies, ACK2 and ACK4 were purified from the ascites fluid of nude mice inoculated with hybridoma cell lines (Nishikawa et al., 1991; Ogawa et al., 1991). The polyclonal rabbit anti-TRP2 antibody, α-PEP8 (Tsukamoto et al., 1992) was a gift from V. J. Hearing.

Whole-mount immunohistochemistry

Whole-mount immunohistostaining was performed according to either Davis (1993) or Wheatley et al. (1993) with some modifications. Embryos were fixed by 600 W of microwave irradiation for 30 seconds in 0.1 M sodium phosphate buffer (pH 7.4), then postfixed for 2-12 hours at 4°C in Dent's fixative, 80% methanol, 20% dimethyl sulfoxide (DMSO). Specimens may then be stored in 100% methanol at −20°C until required. To block endogenous peroxidase, the fixed specimens were soaked in methanol-DMSO-30% H_2O_2 (4:1:1) for 1.5-2 hours at room temperature. For staining, the rehydrated specimens were first blocked by two incubations in PBSMT (2% skim milk powder and 0.1% v/v Triton X-100 in PBS) containing 1% normal goat serum and 0.2% BSA for 1 hour at room temperature, then with PBSMT containing the monoclonal (0.1 μg/ml) or the polyclonal antibody (1:5000 dilution) overnight at 4°C. The specimens were washed 5 times in PBSMT each for 1 hour, at 4°C for the initial three washes and at room temperature for final two. The primary antibody was labeled by incubating the specimens with 1 μg/ml HRP-conjugated anti-rat or -rabbit-Ig antibodies overnight at 4°C. After more than 5 exchanges of PBSMT including a final 20 minutes wash in PBST (0.1% Triton X-100 in PBS) at room temperature, the specimens were soaked in PBST containing 0.05% $NiCl_2$ and 250 μg/ml diaminobenzidine (Dojin Chem.) for 10-30 minutes, then hydrogen peroxide was added to 0.01%. The enzymatic reaction proceeded until the desired color-intensity was reached, then the specimens were rinsed 3-4 times in PBST. Finally, the specimens were dehydrated by 100% methanol and stored at −20°C until they were photographed.

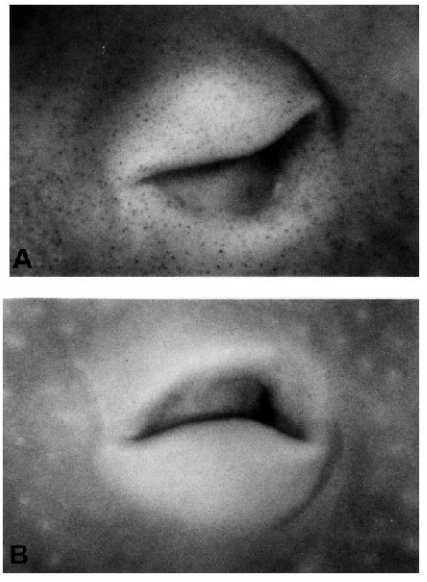

Fig. 2. (A) Depletion of TRP2$^+$ cells around the ear region of the 14.5 dpc embryo by a maternal injection of 2 mg ACK2 at 13.5 dpc. Many TRP2$^+$ cells are distributed in the same region of the control embryo (B). Note, melanoblasts were depleted from other areas in the same mouse, though the data are not shown.

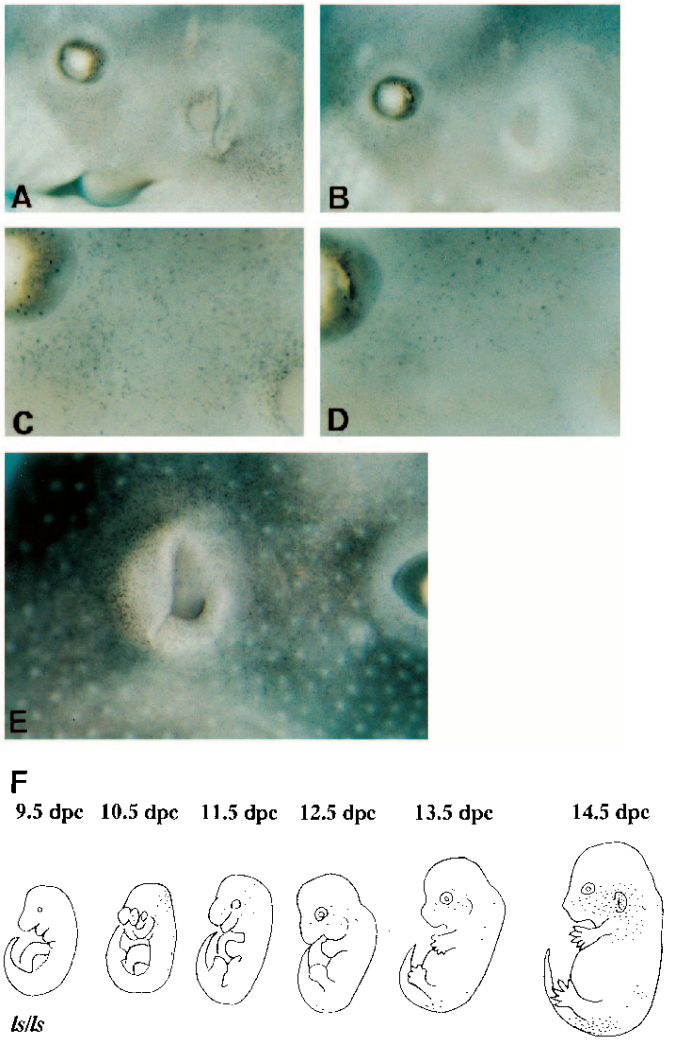

Fig. 3. Distribution of *c*-kit$^+$ cells in the facial area of the 13.0 dpc +/*ls* and *ls*/*ls* embryos. While both *c*-kit$^+$ dendritic and spheric cells are present in the surface of +/*ls* embryo (A,C), none of the former are found in the same region of the *ls*/*ls* embryo (B,D). (D) Note that *c*-kit$^+$ spheric cells are present in the *ls*/*ls* embryo. (E) Distribution of the *c*-kit$^+$ dendritic cells around the ear region of the *ls*/*ls* embryo at 14.5 dpc. (F) Schematic illustration of the distribution of melanoblasts during the development of *ls*/*ls* mice.

RESULTS

Pigmentation patterns originating from spatial differences during the initial stage of melanoblast differentiation

The initial stage of melanocyte differentiation consists of the commitment of neural crest cells into melanoblasts, the migration of melanoblasts in the dorsolateral pathway and the entry of melanoblasts into the epidermis, which occurs between 9.0 and 12.5 dpc (Mayer, 1973; Erickson and Weston, 1983; Kunisada et al., 1996). Since pattern 1 with craniofacial and caudal pigmented areas were induced by an ACK2 injection at this stage (Yoshida et al., 1993), it is likely that melanoblast differentiation at this stage progresses in a spatially nonuniform fashion. Thus, the prototype of the pattern 1 should be presented as a progression map of melanoblast differentiation in the embryo. To confirm this notion, we studied the localization of melanoblasts in the embryo.

Whereas our studies and those of others using *c*-kit and TRP2 as melanocyte markers demonstrated where and when in embryogenesis the cells expressing these markers are present (Nishikawa et al., 1991; Manova and Bachvarova, 1991; Yoshida et al., 1993; Jackson et al., 1992; Steel et al., 1992; Pavan and Tilghman, 1994), we reevaluated these results by means of whole-mount immunohistochemistry, which provides a clearer view of melanocyte distribution. Whole-mount immunohistochemistry with ACK4 or ACK2 mAb distinguishes two types of *c*-kit$^+$ cells, spherical cells of hematopoietic lineage and cells with a dendritic morphology. About 70 to 80% of *c*-kit$^+$ dendritic cells were also positive in TRP2 staining, indicating that *c*-kit$^+$ dendritic cells essentially represent melanoblasts (Kunisada et al., 1996). We stained the BDF1 embryos from 9.5 to 14.5 dpc with either the anti-*c*-kit mAb ACK4 or a polyclonal anti-TRP2 antibody (Fig. 1).

The site where we could first detect *c*-kit$^+$ dendritic cells was the region between olfactory placode and optic eminence in 9.5 dpc embryos (data not shown), though they were so few that they could be detected only occasionally. From 10.0 dpc, they became detectable in all embryos. They were most dense in the region overlying the caudal part of the midbrain and distributed laterally, reaching the nasal region anteriorly and the second branchial arch posteriorly (Fig. 1A). At this stage, most *c*-kit$^+$ dendritic cells, particularly those in the midbrain region, did not express TRP2 (Fig. 1B). A few *c*-kit$^+$ dendritic cells were also visible in more posterior parts, but they were distributed only in the most dorsal part of somites. No *c*-kit$^+$ dendritic cells were found distal to the hindlimb bud or in the most anterior region. At 11.5 dpc, a cluster of *c*-kit$^+$ dendritic cells appeared between the hindlimb bud and tail bud and its density increased during the following day (Fig. 1C). In the trunk, the area containing *c*-kit$^+$ dendritic cells extended ventrally down to the lateral ridge of the body wall. However, the trunk was less densely populated than the craniofacial and caudal regions. In the 12.5 dpc embryo, the pattern of the 11.5 dpc embryo was essentially preserved, while the overall density increased. Histological examination demonstrated that some of the TRP2$^+$ cells were already located in the epidermal layer in the craniofacial and caudal regions (Kunisada et al., 1996). Within the next 2 days, the entry of melanoblasts into the epidermis seemed to be complete and they were distributed evenly over the entire body surface of the 14.5 dpc embryo except for the umbilical, digital and tail tip regions (Fig. 1D,E).

This histological analysis showed that the distribution of *c*-kit$^+$ dendritic cells in the normal embryo from 10.5 to 12.5 dpc was nonuniform. Namely, melanoblast distribution proceeded faster in the craniofacial and caudal regions than in the trunk region. This distribution resembled that of pigmentation pattern 1.

Synchronous activation of melanoblast proliferation after entering the epidermis

After completing migration, melanoblasts enter the epidermis during 12.5 to 13.5 dpc (Kunisada et al., 1996). The density of melanoblasts, particularly of those in the trunk region, increased rapidly to produce a homogeneous distribution over the next 24 hours (Fig. 1F). This narrow time window was the stage when ACK2 induced pattern 2, in which the entire coat was unpigmented. The fact that all melanoblasts at this stage of the embryo were affected by ACK2 indicates that their differentiation, which has hitherto progressed in a spatially nonuniform fashion, is now synchronized with respect to *c*-kit-dependence.

To investigate the function of *c*-kit at this stage, we injected ACK2 into pregnant mice at 13.5 dpc and analyzed the distribution of TRP2$^+$ cells of the embryo on the next day. Fig. 2 shows that all TRP2$^+$ cells were depleted from the embryo. Considering that this procedure produces offspring without melanocytes (Nishikawa et al., 1991), the rapid disappearance of melanoblasts induced by ACK2 suggests that *c*-kit is an absolute requirement for their survival at this stage.

The notion that melanoblasts actively proliferate upon entry into the epidermis was based upon the melanoblast distribution pattern of the *ls/ls* mouse, which has a mutation in the endothelin 3 gene (Baynash et al., 1994). As summarized in Fig. 3A-F, the early phase of melanoblast distribution was severely inhibited in this mutant embryo as only few, if any, melanoblasts were found around the ear and tail roots of the 13.5 dpc embryo. Consistent with the studies of Baynash et al. (1994), this indicates that endothelin 3 is required for melanoblast development at the migratory phase. While the melanoblast distribution pattern at 13.5 dpc was similar among 20 *ls/ls* embryos, the size of the regions containing melanoblasts varied substantially at 14.5 dpc. Moreover, the variation was much more extensive in the eventual pigmentation patterns of adult *ls/ls* mice (Fig. 6A; Silvers, 1979). These results indicated that melanoblast proliferation, which is suppressed in the dermis of *ls/ls* embryo, is activated upon entry into the epidermis. Because of the extensive increase of melanoblasts in the epidermis of *ls/ls* mouse, any reduction in the number of melanoblasts that have completed the dermal stage of differentiation can be corrected at the subsequent stage. This indicates that endothelin 3 is not required for the proliferation of melanoblasts in the epidermis.

Colonization of developing hair follicles by melanoblasts renders them *c*-kit-independent

An ACK2 injection at 14.5 dpc produces pattern 3, which consists of complex pigmentation areas of various sizes (Nishikawa et al., 1991). In this pattern, the pigmentation extended ventrolaterally from the patches in the dorsoanterior trunk region towards the anterior and posterior directions (Fig. 4; see detail in Nishikawa et al., 1991).

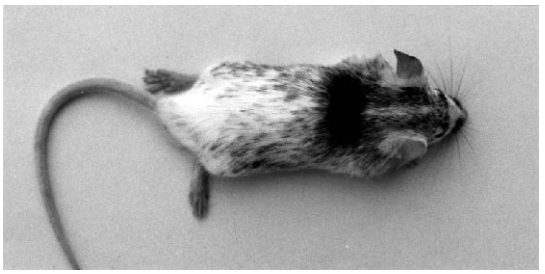

Fig. 4. Typical pigmentation of the offspring of a pregnant mouse that received a subcutaneous injection of 2 mg ACK2 at 14.5 dpc. Other patterns were published in our previous manuscript (Nishikawa et al., 1991).

Given that the entry of the melanoblasts is completed before 14.5 dpc and that an ACK2 injection inhibits the survival of the melanoblasts in the epidermal layer, the pigmented areas in pattern 3 might correspond to the region where the melanoblasts have completed the stage during which *c*-kit is required for their survival.

What is the event that renders the melanoblasts in the epidermal layer *c*-kit-independent? Byrne et al. (1994) have shown expression of keratin 5 and the transcription factor AP2 in developing pelage hair germs. The distribution of keratin 5 or AP2 in the body surface of the 13.5 dpc embryo presented in their study resembles the pigmentation pattern of the 14.5 dpc ACK2-injected mice (Fig. 4). Thus, we speculated that melanocyte precursors become resistant to ACK2 as they start to be incorporated in developing hair follicles. If so, we predicted that an ACK2 injection at 14.5 dpc depletes the melanoblasts from the epidermal layer, while leaving the melanoblasts that have colonized the developing follicles unaffected.

To test this theory, pregnant mice were given an intradermal injection of 2 mg ACK2 at 14 dpc. When the embryos were recovered at 16.5 dpc, $TRP2^+$ cells were found only in the developing hair follicles of the ACK2-injected mouse, but they were present both in the epidermal layer and hair follicles of the control mice (Fig. 5A,B).

Time window for melanoblast entry into the hair follicle

We found that an ACK2 injection given after 15 dpc failed to affect the coat color of the offspring (Nishikawa et al, 1991). Given that the resistance of the melanoblasts to the ACK2 is endowed by the integration of melanoblasts into the developing hair follicles, our results implied that hair follicle development extends rapidly and completes in the entire coat at around 15 dpc. If so, an ACK2 injection into *ls/ls* mice at 16 dpc, when epidermal expansion of melanoblasts may still be underway, whereas hair follicle development would have completed, should induce an entirely different pigmentation pattern that represents how far melanoblasts have expanded in the epidermis at 16 dpc.

We injected 2 mg of ACK2 into pregnant *ls/ls* mice at 16.0 dpc and examined the pigmentation pattern of the offspring. None of the offspring displayed pattern 3 and the pigmented areas were distributed in the craniofacial, and sometimes in the caudal area (Fig. 6). This result indicated that the expansion of the melanoblasts in the epidermis continued in the 16.0 dpc *ls/ls* embryo. Moreover, this result indicated that melanoblasts can colonize developing hair follicles at later embryonic stages.

DISCUSSION

While it is established that melanocytes differentiate from neural crest cells and migrate through a dorsolateral pathway to eventually colonize the developing hair follicle (Mayer, 1973; Silvers, 1979; Le Douarin, 1982; Erickson and Weston, 1983), little is known about the processes between these events. Wehrle-Haller and Weston (1995) have demonstrated that the soluble ligand for *c*-kit induces dispersion of the earliest melanoblasts into the dorsolateral pathway, while the membrane anchored ligand is required for supporting migration within this pathway. Consistent with this report, our whole-mount immunohistochemical studies with an anti-*c*-kit mAb revealed melanoblasts in the dorsocranial region of 10.5dpc Sl^d/Sl^d embryo (Kunisada et al., 1996). Moreover, in agreement with the results of Steel et al. (1992), we found that those melanoblasts disappeared completely over the next 24 hours (Kunisada et al., 1996). This rapid disappearance suggested that the membrane-anchored form of the *c*-kit ligand is requisite for supporting the survival of the migrating melanoblasts (Murphy et al., 1992; Morrison-Graham and Weston, 1993). In fact, we showed that an ACK2 injection at this stage inhibited the *c*-kit-dependent survival of the migrating melanoblasts, thereby resulting in pattern 1, in which the craniofacial and caudal regions are pigmented but not those of the trunk regions. Given the role of *c*-kit in the survival of migrating melanoblasts, the pigmented areas in pattern 1 imply that a proportion of melanoblasts in the craniofacial and caudal regions have already acquired *c*-kit-independence for their survival, while most melanoblasts in the trunk region still remain at the *c*-kit-dependent stage (summarized in Fig. 7). In fact, the melanoblast distribution pattern of the embryos (Fig. 1F) showed that the melanoblast differentiation starts and reaches to the *c*-kit-independent stage earlier in the craniofacial and caudal regions than in the trunk. Thus, pattern 1 basically reflects this regional difference in the time schedule of melanoblast differentiation.

The melanoblast differentiation that has proceeded in a spatially non-uniform fashion is synchronized upon entry into the epidermal layer. A comparison of the melanoblast distribution in *ls/ls* embryos before and after epidermal entry demonstrated that melanoblast proliferation is active in the epidermis. This rapid proliferation seems to be the most important factor in changing the melanoblast distribution from non-uniform to uniform, as the melanoblast density of the trunk region, that is, below the craniofacial region in the 13.5 dpc embryo, became comparable to that of other areas in the 14.5 dpc embryo.

Here, we provided evidence that *c*-kit is again required for the survival of the proliferating melanoblasts in the epidermis. The effects of ACK2 on melanocytes at various stages indicate that *c*-kit functions as a cell survival factor for melanoblasts during proliferation, whereas it is not required at the dormant stage. Moreover, our analysis of the *ls/ls* mouse indicated that endothelin 3, which is required for the early process of the melanoblast development is not involved in melanoblast pro-

liferation in the epidermis. Comparing the pigmentation of *ls/ls* and s^l/s^l mice, however, the pigmented area in s^l/s^l mice remains restricted to narrow areas in the craniofacial and caudal regions, suggesting that the melanoblast proliferation in the epidermis of s^l/s^l embryo is severely suppressed. As s^l/s^l mice bear a loss-of-function mutation in the endothelin receptor B gene, the pigmentation patterns suggest that endothelin receptor B is still required for melanoblast proliferation in the epidermis, while endothelin 3 might be compensated by other endothelins that can bind to the endothelin receptor B (Imokawa et al., 1992; Yanagisawa, 1994).

Finally, we demonstrated that the survival of melanoblasts colonizing the developing hair follicles was not affected by ACK2. Thus the melanoblasts entered a stage during which inhibition of *c*-kit has no effect on their survival until they are reactivated upon initiation of the first wave of the hair cycle after birth. Since the follicular epithelium differentiates from the epidermal epithelium to form a independent unit (Hardy, 1992), it would be of interest to understand how this epithelial differentiation creates the microenvironement where melanoblasts can survive without the *c*-kit signal. In addition, we showed, by a series of pigmentation pattern 3, how hair follicle development spreads in the embryonic coat and that an ACK2 injection into the 16.0 dpc *ls/ls* embryo inhibited the expansion of the pigmented area as compared with untreated offspring. This indicated that the period during which the developing hair follicle can tolerate melanoblast colonization might be long, although this process is usually completed before 15.5 dpc in the normal embryo.

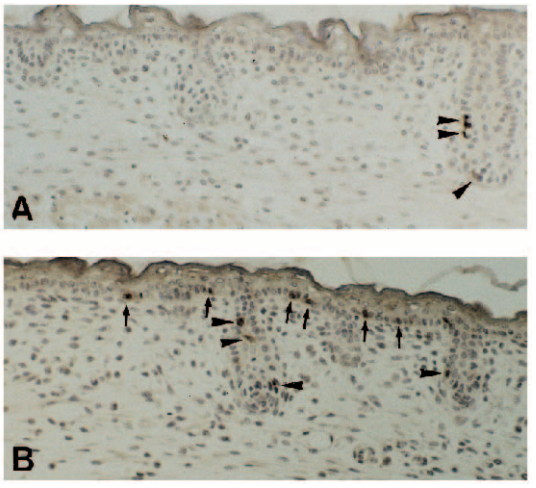

Fig. 5. Resistance of the melanoblasts in developing hair follicles to ACK2. Pregnant mice were given a subcutaneous injection of 2 mg ACK2 at 14.5 dpc and the embryos were recovered at 16.5 dpc for immunohistological analysis with anti-TRP2 antibody. A few TRP2-positive cells persist in the developing hair follicles of the ACK2-treated embryos (arrowheads) but none in the interfollicular epidermal layer, nor in the more immature hair follicle buds (A). (B) In the skin of the untreated embryo at the same stage, many $TRP2^+$ cells are present in both developing follicles (arrowheads) and the interfollicular epidermis (arrows).

In conclusion, melanocyte differentiation proceeds by the alternate repetition of *c*-kit-dependent and *c*-kit-independent stages in terms of their survival (Fig. 7). At present, we do not understand the molecular mechanism that renders the melanoblasts able to survive without the *c*-kit signal. However, since it can be functionally verified by ACK2-mediated manipulation, this feature provides useful hallmark with which to dissect the process of melanocyte differentiation. Based on the result of this study, we propose 4 distinctive stages of embryonic melanocyte differentiation after *c*-kit expression: (1) a stage of dorsolateral path migration when *c*-kit and endothelin 3/endothelin receptor B are functionally required; (2) the first *c*-kit-independent stage before entry into the epidermal layer; (3) a stage of proliferation in the epidermis activated in a *c*-kit- and endothelin receptor B-dependent but endothelin 3-independent manner and (4) the second *c*-kit-independent stage after being integrated into the developing hair follicles (Fig. 7). As we demonstrated that melanoblasts in the hair follicles proliferate and differentiate in a *c*-kit-dependent manner in coordination with the postnatal hair cycle (Nishikawa et al., 1991), this stage may constitute stage 5. As pigmented spots of the s^l/s^l mouse are maintained during postnatal life, it is likely that endothelin receptor B is no longer required in the final stage, whereas *c*-kit continues to function in the adult hair follicles.

After dissecting the embryonic process of melanocyte differentiation, the next issues to be determined are how *c*-kit and endothelin receptor B collaborate to regulate the proliferation and differentiation of melanoblasts at each distinctive stages, and the molecular mechanisms that allow the melanoblasts

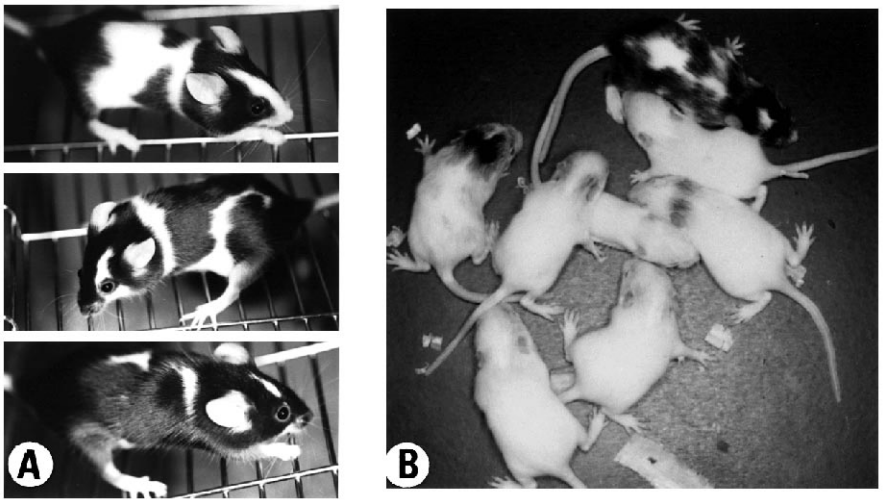

Fig. 6. (A) Some typical coat color pigmentation patterns of *ls/ls* mice maintained in our laboratory. All mutant mice have pigmentation around the ears or tail end, but the size of the pigmented areas are variable and occasionally cover most of the coat (bottom). (B) Coat color pigmentation patterns of an *ls/ls* mouse littermates treated with a maternal injection of ACK2 at 16.0 dpc. Different pigmentation patterns among littermates indicate the difference in the extent of melanoblast expansion in the epidermis of the *ls/ls* mouse.

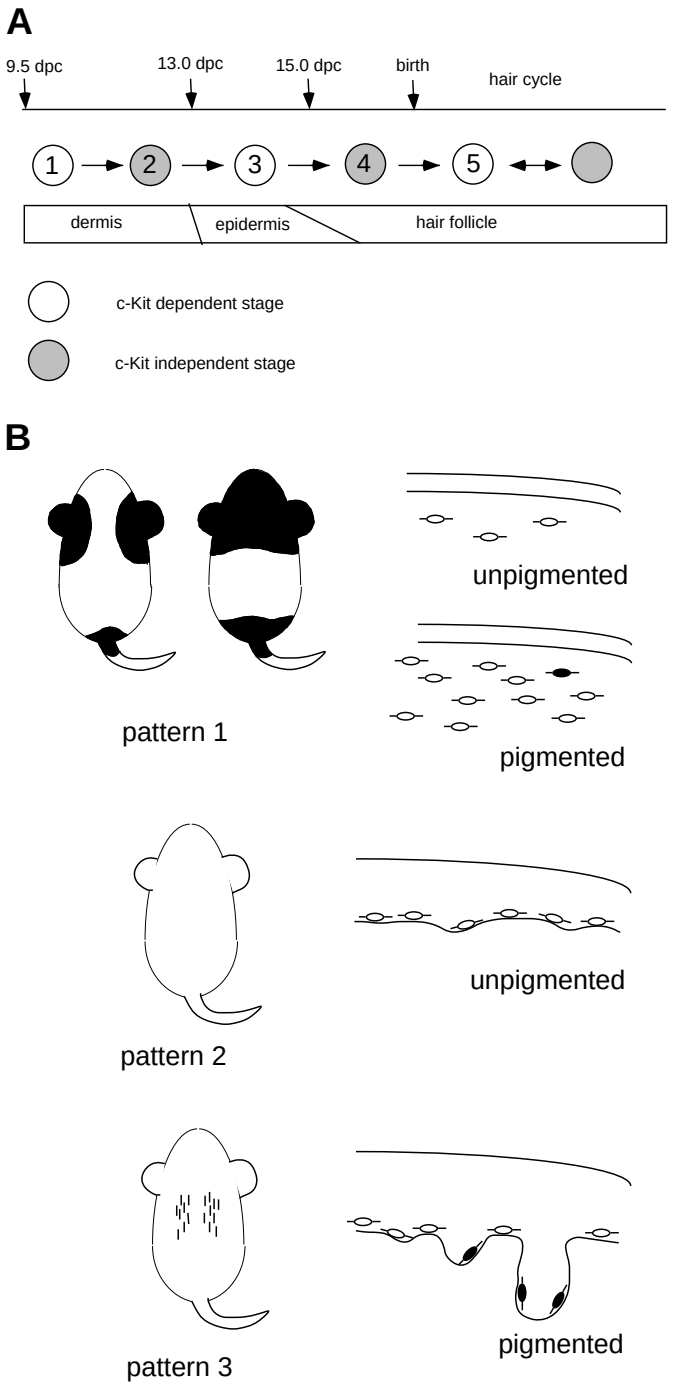

Fig. 7. A model of melanocyte differentiation. (A) Alternate repetition of *c*-kit-dependent and -independent stages during melanocyte differentiation. Two types of melanoblast are distinguished by ACK2 injection; one is resistant to this treatment and produce pigmented skin (indicated by filled circle), and the other is deleted by this treatment (indicated by open circle). This figure combines these stages as revealed by a series of ACK2 injection experiments (Nishikawa et al., 1991; Yoshida et al., 1993) with the localization of melanoblasts during the embryogenesis and postnatal development. The number in the circle corresponds to the stage described in the text (p 1212). (B) Interpretation of the three patterns induced by ACK2 injection. (Pattern 1) Pattern 1 is induced by ACK2 injection at the stage when melanoblasts are in the dermis. In the area where only ACK2-sensitive melanoblasts are present (stage 1), skin is basically unpigmented. In contrast, the area where some ACK2-resistant melanoblasts (stage 2) reside produces pigmented skin. (Pattern 2) Upon entry of melanoblasts into the epidermis, all of them become *c*-kit-dependent (stage 3). Therefore, ACK2 injection at this stage deletes basically all melanoblasts, thereby producing an unpigmented mouse (Pattern 2). (Pattern 3) Melanoblasts again become resistant to ACK2-treatment after migrating into developing hair follicles (stage 4). Thus, pattern 3 represents hair follicles that are colonized by melanoblasts. As we showed previously (Nishikawa et al. 1991), melanoblasts in hair follicles are activated to proliferate again with the postnatal hair cycles. This *c*-kit-dependent melanoblasts correspond to stage 5.

survive without *c*-kit before entering into the epidermis, in the developing hair follicle of the embryos and in the resting hair follicle of the adult mouse. To this end, it is important to separate and culture the subsets of melanoblasts from the developing embryo to analyze their phenotypes and functions. We have purified melanoblasts from the dermis and epidermis of the prenatal embryo using a fluorescence activated cell sorter (Kunisada et al., 1996). The molecules controlling the cell cycle progression and survival of those purified melanoblasts are currently underway.

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