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Blood Supply–Susceptible Formation of Melanin Pigment in Hair Bulb Melanocytes of Mice

Shogo Maeda, MD*†
Koichi Ueda, MD, PhD†
Hidenori Yamana, MD*
Junko Tashiro-Yamaji, PhD*
Minenori Ibata, MD, PhD†
Ayako Mikura, MD, PhD†
Masashi Okada, MD, PhD†
Emi Yasuda, MD, PhD‡
Yuro Shibayama, MD, PhD‡
Miya Yoshino, DDS, PhD§
Takahiro Kubota, MD, PhD*
Ryotaro Yoshida, MD, PhD*

Background: Allogeneic skin grafts onto C57BL/6 mice are rejected, and the rejected skin is replaced by surrounding skin with black hair. In contrast, syngeneic skin grafts are tolerated, and gray hair grows on the grafts.
Methods: To explore the mechanism of gray hair growing on the tolerated skin grafts, we prepared full-thickness skin (2-cm square) autografts, 2 (2 cm + 2 cm) horizontal or vertical parallel incisions, and U-shaped (2 cm × 2 cm × 2 cm) flaps with or without pedicle vessels. The grafts, incisions, and flaps were fixed by suturing with string and protected by a transparent bandage. On day 14 after the operation, the bandages were removed to observe the color of the hair growing on the skin.
Results: Skin autografts from wild-type or *hepatocyte growth factor*-transgenic (Tg) C57BL/6 mice survived with gray hair, whereas those from *steel factor* (*Kitl*)-Tg C57BL/6 mice survived with black hair. In addition, U-shaped flaps lacking both of the 2 main feeding vessels of wild-type mice had gray hair at the tip of the flaps. Light microscopy after staining with hematoxylin and eosin or dihydroxyphenylalanine showed that the formation of melanin pigment in the follicles, but not in the interadnexal skin, was susceptible to the blood supply.
Conclusions: Melanin pigment formation in the hair bulb melanocytes appeared to be susceptible to the blood supply, and melanocytosis was promoted in the follicles and in the epidermis of *Kitl*-Tg C57BL/6 mice. *(Plast Reconstr Surg Glob Open 2015;3:e328; doi: 10.1097/GOX.0000000000000284; Published online 18 March 2015.)*

Skin grafts from syngeneic strains of mice onto C57BL/6 mice (ie, isografts) are tolerated, whereas those from allogeneic (eg, BALB/c) strains of mice (ie, allografts) are rejected.[1] The alloreactive immune response is predominantly directed at the major histocompatibility complex (H-2 in mice) class I molecules,[2–11] and the rejected skin is replaced by surrounding skin with black hair.[5,12] Unexpectedly, however, mostly gray-colored hair grows on the syngeneic skin grafts to cover the original size of the grafts.[12]

Mature melanocytes are melanin-synthesizing dendritic cells located within the basal layer of the epidermis, hair bulb, and outer root sheath of hair follicles.[13] Hair melanin is formed by melanocytes situated in the hair bulb epithelium around the

*From the *Department of Physiology, Osaka Medical College, Takatsuki, Japan; †Department of Plastic and Reconstructive Surgery, Osaka Medical College, Takatsuki, Japan; ‡Department of Pathology, Osaka Medical College, Takatsuki, Japan; and §Division of Immunology, Department of Molecular and Cellular Biology, School of Life Science, Faculty of Medicine, Tottori University, Yonago, Japan.*
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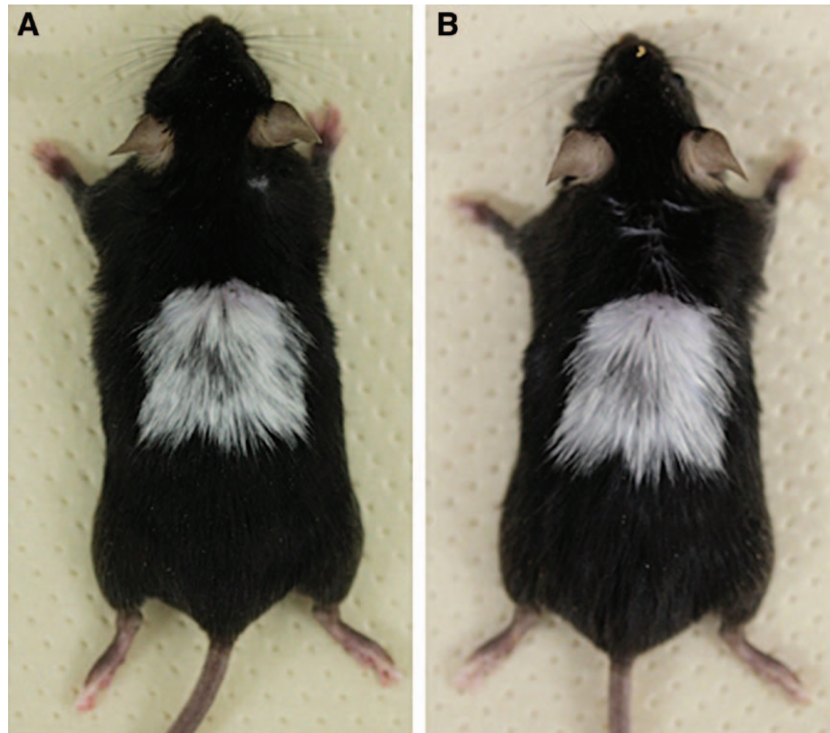

Fig. 1. Autologous skin of a C57BL/6 mouse was engrafted. A, On day 21 after autologous skin grafting on the dorsum of a C57BL/6 mouse, gray hair had grown on the graft. When the gray hair was shaved off on the same day, 37 days later the gray hair had regrown on the graft (B).

upper half of the dermal papilla among the cells destined to form the hair cortex.[14] Melanogenic activity in the hair follicle is closely linked to the hair cycle. In the telogen follicle, nonmelanogenic melanocytes are found in the basal layer of the outer root sheath and in the secondary germ region.[15,16] These cells are assumed to be the precursors of active melanocytes during the next anagen phase, and they either migrate or are carried into the hair bulb in early anagen development. Melanogenic (ie, tyrosinase) activity becomes apparent in Anagen 3, pigment transfer to the cortical epithelium begins in the Anagen 4 stage of development, and melanogenesis ceases with the onset of catagen.[17–19] The fate of hair bulb melanocytes during catagen is uncertain.[20,21]

The wide range of genetically determined variations in coat color of the laboratory mouse provides an excellent model for the study of gene action within many biological processes.[22] More than 150 distinct mutations that affect pigmentation either directly or indirectly have been identified in murine models[23]: for example, *Steel*, *c-Kit*, *Splotch*, *Microphthalmia*, and *Dom* for melanocyte embryogenesis; *Silver*, *Beige*, and *OA-1* for melanosome structure and function; *Ashen* and *Dilute* for transport; *Albino/platinum*, *Brown*, and *Slaty* for melanogenic enzyme; and *Extension* and *Agouti* for regulation of melanogenesis. Many of these gene products have been shown to be involved in various clinical pigmentary diseases in man. In particular, the tyrosine kinase receptor Kit and its ligand (Steel factor, mast cell/stem cell growth factor, or Kitl) play a critical role in melanocyte physiology (eg, up-regulation of melanoblast proliferation and action as a survival factor for active migrating and proliferating melanoblasts).[24–26] Piebaldism, a genetic disorder, results from Kit receptor defects secondary to mutation of the *c-Kit* proto-oncogene, and patients with piebaldism present at birth with white patches of skin and hair that lack melanocytes.[27] Transgene expression of *steel factor* (*Kitl*) in the basal layer of the epidermis in a mouse model used for the study of melanocyte development promotes survival, proliferation, differentiation, and migration of melanocyte precursors,[28] and keratinocyte expression of transgenic (Tg) *hepatocyte growth factor* (*HGF*) affects melanocyte development, leading to dermal melanocytosis.[29]

Establishment of cultures of human melanocytes has led to an explosion of knowledge concerning the biology of melanocytes: in gray and white hair, the melanocytes in the basal layer of the hair matrix are greatly reduced in number or are absent.[30] However, cellular and molecular events that are responsible for

Fig. 2. A U-shaped incision was made in the dorsal wall of a C57BL/6 mouse. Arrows, 2 main feeding vessels.

the loss of melanogenesis in skin or hair after birth are nearly unknown. In the present study, we investigated the mechanisms of gray hair growth in autologous skin grafted onto the backs of C57BL/6 mice. The hair color did not change after making 2 horizontal or vertical parallel incisions or U-shaped flaps with pedicle vessels. However, U-shaped flaps lacking both of the 2 main feeding vessels had gray hair at the tip of the flaps, and skin autografts from wild-type C57BL/6 mice survived in the original size of the grafts with gray hair, implying blood supply-dependent melanin pigment formation. To explore which step(s) in the melanin pigment formation or which type(s) of cells was susceptible to the blood supply, we prepared skin autografts from *HGF*-Tg or *Kitl*-Tg C57BL/6 mice. Hematoxylin and eosin (H&E) and dihydroxyphenylalanine (DOPA) stainings showed that dermal (ie, interadnexal skin) melanocytosis was mainly stimulated in *HGF*-Tg C57BL/6 mice and that epidermal and follicular melanocytosis was induced in the *Kitl*-Tg C57BL/6 mice. Furthermore, there was mixture of black and gray hair growing on the tolerated skin grafts from *HGF*-Tg C57BL/6 mice, whereas there was unchanged accumulation of melanin granules in the upper half of dermal papilla of the grafts from *Kitl*-Tg C57BL/6 mice. These results showed that melanin pigment formation in the follicular melanocytes appeared to be susceptible to blood supply and that follicular melanocytosis was also stimulated in the *Kitl*-Tg C57BL/6 mice.

MATERIALS AND METHODS

Animals

Specific pathogen-free male C57BL/6 mice (7–12 weeks of age) were purchased from Japan SLC (Hamamatsu, Japan). *KRT14-Kitl*-Tg (*Kitl*-Tg) mice and *KRT14-HGF*-Tg (*HGF*-Tg) mice expressing mouse *Kitl* and human *HGF* driven by *KRT14* show hyperaccumulation of melanin granules in the epidermis and the dermis, respectively.[28,29] All experiments were carried out in accordance with The Guidelines on Animal Experiments of Osaka Medical College and the Japanese Government Notification on Feeding and Safekeeping of Animals (Notification No. 6 of the Prime Minister's Office), and the experimental protocol was approved by The Review Committee for Animal Experiments of Osaka Medical College.

Skin Grafts

C57BL/6 mice were anesthetized with inhaled isoflurane. We shaved off the dorsal hair with a razor and made four 2-cm incisions in the shape of a square into the dorsal wall. Full-thickness dorsal skin (2-cm square) was removed and then grafted back onto the original location by suturing it at intervals of 5 mm with a 5-0 nylon string, as described previously.[12] On day 14 after grafting, the grafts were scored as having survived when gray hair had grown to cover the original size of the grafts.

Two Horizontal or Vertical Parallel Incisions

We anesthetized C57BL/6 mice, shaved off the dorsal hair with a razor, and made 2 (2 cm + 2 cm) horizontal or vertical parallel incisions in the dorsal walls. The skin (2-cm square) was raised and then sutured back.

Skin Flaps

We prepared 3 types of flaps in the dorsal skin of C57BL/6 mice. (1) Axial pattern flap with 2 main feeding vessels: we made full-thickness incisions to prepare U-shaped flaps by making 3 incisions in different directions in the dorsal wall. The flap (2 cm × 2 cm × 2 cm) was raised and then sutured back.

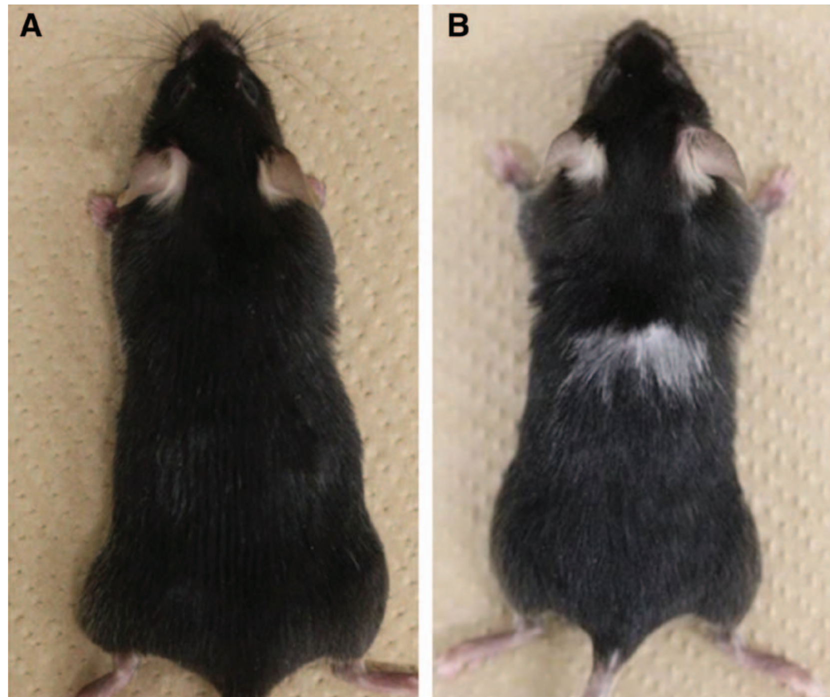

Fig. 3. Effect of cutting of 1 or both main feeding vessels of the U-shaped skin flap made in the dorsal walls of C57BL/6 mice. A, One of the 2 main feeding vessels was cut. B, Both vessels were cut. On day 21, the color of hair growing on the flap was determined.

(2) Flap with 1 of 2 main feeding vessels: we raised the U-shaped flap and cut 1 of the 2 main feeding vessels with a needle knife. (3) Random pattern flap lacking the 2 main feeding vessels: we raised the U-shaped flap and cut both main feeding vessels. Blood flow in this type of flap depends on capillaries in the skin.

DOPA and H&E Staining

Specimens of skin grafts were embedded in optimal cutting temperature compound and quick frozen in liquid nitrogen. Frozen serial sections (10-µm thickness) were cut, placed on poly-L-Lysine-coated glass slides, and air-dried. One section was stained with H&E, and the adjacent one was used for DOPA staining, as described previously.[31] These sections were fixed in 10% formalin solution for several hours and thereafter dehydrated in alcohol and xylol.

RESULTS

We removed full-thickness dorsal skin (2-cm square) from C57BL/6 mice and then grafted it back onto the original location (autografts). The autografts in C57BL/6 mice survived as the original size of the grafts with gray hair on day 21 after transplantation, and the gray hair was maintained for more than 5 months (data not shown). Similarly, 21 days after autologous skin transplantation, gray hair grew on the tolerated grafts (Fig. 1A) and was shaved off with a razor, and by day 37 after shaving, gray hair had regrown on the grafts (Fig. 1B).

To explore the mechanism of the change in the color of the hair growing on the tolerated skin grafts, we made 2 (2 cm + 2 cm) vertical or horizontal parallel incisions in the dorsal wall of C57BL/6 mice, raised the skin (2-cm square), and then sutured it back. As expected, the hair color did not change significantly (data not shown). Next, we made U-shaped skin flaps (2 cm × 2 cm × 2 cm) in the dorsal wall, raised the skin (2-cm square), and then sutured it back. Again, there was no change in the color of hair after making these U-shaped skin flaps (data not shown), demonstrating that 4 incisions (2-cm square) were essential for the growth of gray hair on the skin autografts.

Since the U-shaped skin flaps had their blood supply mainly from 2 feeding vessels coming from the underlying muscles (Fig. 2), we next examined the effects of cutting (with a needle knife) one or both of these main feeding vessels on the change in the hair color of the skin flap. When we made U-shaped skin flaps possessing 1 of these 2 main feeding vessels, the mice had black hair on the flaps (Fig. 3A). In contrast, U-shaped skin flaps lacking both vessels showed the growth of gray hair at the tip of the flaps (Fig. 3B), indicating that an adequate blood supply to the flaps might be essential for the growth of pigmented hair at the tip of the flaps.

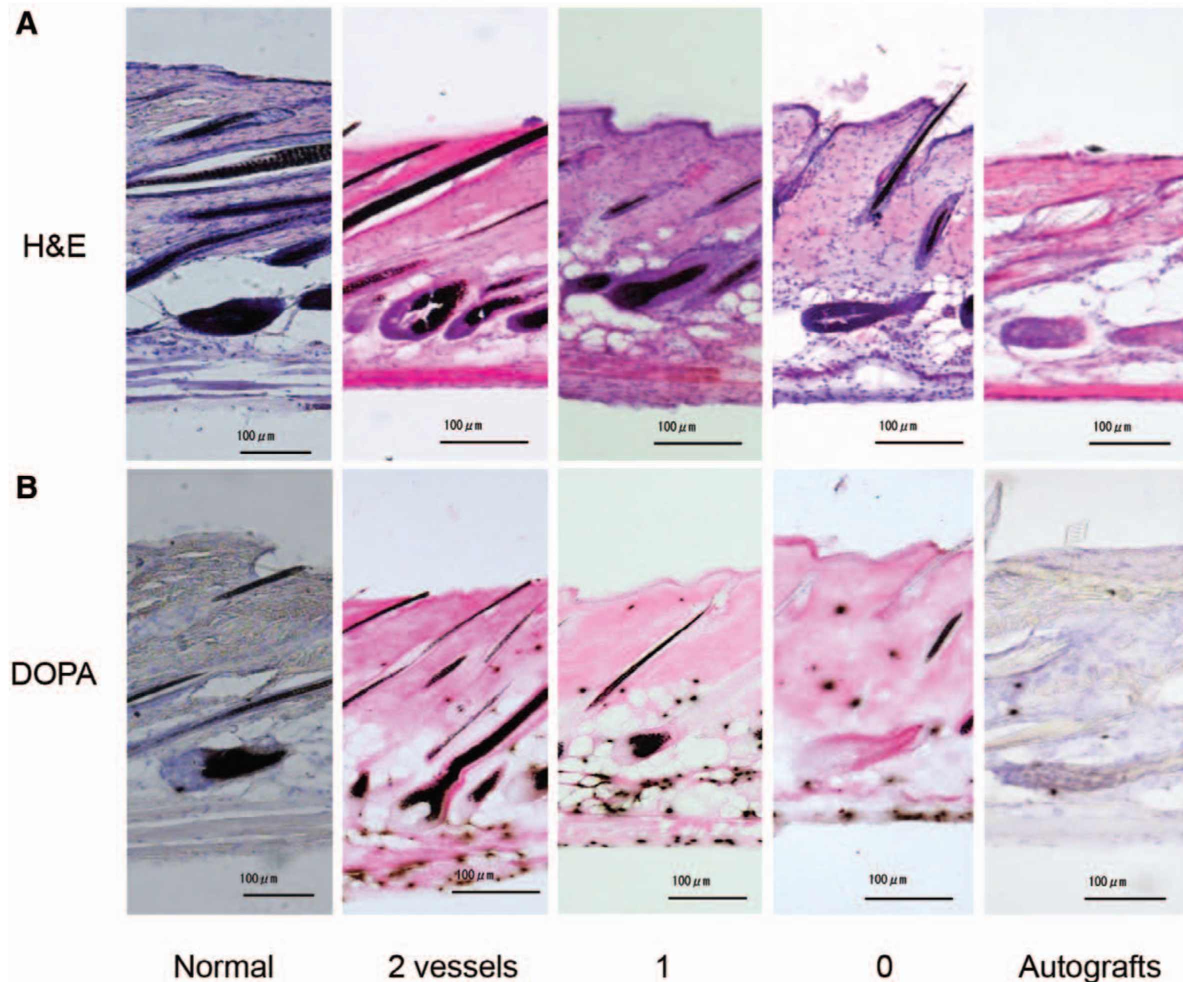

Fig. 4. H&E (A)- and DOPA (B)-stained sections of normal dorsal skin, U-shaped skin flaps possessing 2, 1, or no feeding vessels, or autografts from C57BL/6 mice. Scale bars = 100 μm. The difference in the numbers of DOPA⁺ follicles between "2 feeding vessels" and "no feeding vessels" and between "1 feeding vessel" and "no feeding vessels" is significant (P = 0.002105 and 0.003756, respectively), whereas the difference between "2 feeding vessels" and "1 feeding vessel" is not significant (P = 0.7978) according to Student's t test.

To examine histologically the mechanism of gray hair growth on the tolerated skin autografts, we made serial frozen sections of dorsal skin from C57BL/6 mice and stained them in alternate fashion, that is, with H&E for one section and with DOPA for the next one (Fig. 4). On light microscopy, the H&E-stained sections (Fig. 4A) showed almost constant numbers of follicles in the dermis (10.4±1.5 follicles/[×100] field [mean ± SD, n = 5], 7.8±1.3, 7.5±2.4, 7.8±0.8, and 6.2±1.1 for untreated skin, the tip of the U-shaped skin flaps possessing both, 1, or neither of the 2 main feeding vessels, and tolerated autografts, respectively). It also showed more clear deposition of melanin in the dermal (ie, interadnexal skin) layer in the tip of U-shaped skin flaps possessing both (107.0±17.6 melanin⁺ granules/[×100] field [mean ± SD, n = 5]), 1 (159.8±15.3), or neither (130.4±20.6) of the 2 main feeding vessels or in the tolerated autografts (29.8±9.1) than in the untreated skin (10.6±1.1). By contrast, a few (1.8±1.3) or very few (0.6±0.9) hair shafts had melanin pigments in the tip of U-shaped skin flaps lacking the 2 main feeding vessels or in the tolerated autografts, respectively.

To explore whether the melanocytes from U-shaped skin flaps lacking both main feeding vessels or tolerated autografts exhibited normal melanogenic activity, we used other adjacent sections for DOPA staining. Light microscopy after DOPA staining (Fig. 4B) confirmed melanin pigment production (10.4±1.5 DOPA⁺ follicles [n = 5]) in skin sections from untreated mice and in most of the hair follicles at the tip of the U-shaped skin flaps possessing both (7.0±0.7 DOPA⁺ follicles [n = 5]) or 1 (6.8±1.7 DOPA⁺ follicles [n = 4]) of the 2 main feeding vessels. In contrast, melanin pigment was not produced

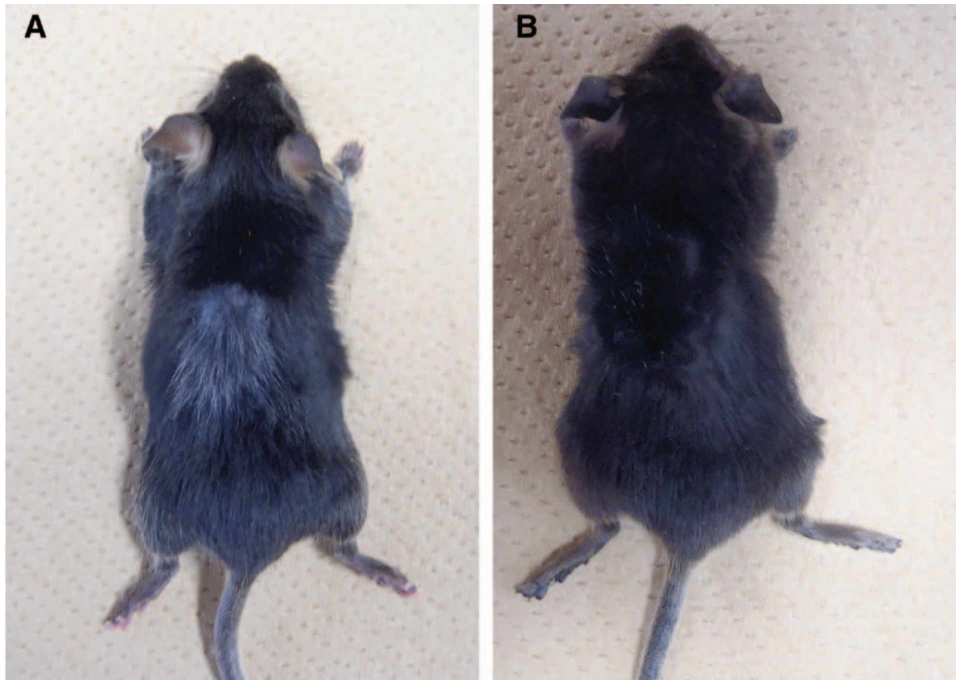

Fig. 5. *HGF*-Tg (A) and *Kitl*-Tg (B) C57BL/6 mice after skin autografting.

in some hair follicles (1.8±1.3 DOPA$^+$ follicles [n = 5]) at the tip of the U-shaped skin flaps lacking both vessels. In the case of tolerated autografts, there was almost no melanin pigment production (0.4±0.5 DOPA$^+$ follicles [n = 5]) in the hair follicles, revealing that melanin pigment formation in the follicular melanocytes was blood supply susceptible.

To examine which step(s) in the melanin pigment formation or which type(s) of cells was susceptible to blood supply, we performed skin autografting in *Kitl*-Tg and *HGF*-Tg C57BL/6 mice. An *HGF*-Tg C57BL/6 mouse with dermal melanocytosis had black hair before transplantation, and dermal melanocytosis was observed after shaving (data not shown). However, the mouse produced gray hair covering the original size of the grafts (Fig. 5A), suggesting specific impairment in the follicular melanocytosis after skin autografting. In the case of the *Kitl*-Tg C57BL/6 mice with epidermal melanocytosis, these mice without transplantation had black hair; and marked epidermal melanocytosis was observed after shaving (data not shown). In addition, there was no gray hair on the grafts after autologous skin transplantation (Fig. 5B), suggesting that melanocytosis in the epidermis and the follicles of the *Kitl*-Tg C57BL/6 mice might have become resistant to insufficient blood supply after skin autografting.

Light microscopy after staining with H&E (Fig. 6A) or DOPA (Fig. 6B) clearly showed that dermal (ie, interadnexal skin) melanocytosis was stimulated in *HGF*-Tg C57BL/6 mice before (1388.8±239.3 melanin$^+$ granules [n = 5]) and after (1414.4±214.8 melanin$^+$ granules [n = 5]) skin autografting, whereas follicular melanocytosis on the tolerated grafts was reduced after it: before (7.0±0.7 DOPA$^+$ follicles [n = 5]) and after (2.6±0.9 DOPA$^+$ follicles [n = 5]). In contrast, there was enhanced epidermal melanocytosis in the *Kitl*-Tg mice before transplantation, and there was unchanged accumulation of melanin granules in the epidermis and in the hair follicles: before (7.8±1.3 DOPA$^+$ follicles [n = 5]) and after (4.6±1.7 DOPA$^+$ follicles [n = 5]).

DISCUSSION

As observed with skin isografts,[12] skin autografts in C57BL/6 mice survived in the original size of the grafts and developed gray hair (Fig. 1). U-shaped flaps with 1 or both of the 2 main feeding vessels had black hair, whereas U-shaped flaps lacking these 2 main feeding vessels had gray hair at the tip of the flaps (Figs. 2, 3), implying that an adequate blood supply to melanocytes or their progenitor melanoblasts might have been essential to the growth of pigmented hair. In fact, the numbers of DOPA$^+$ follicles increased in a blood supply-dependent manner (Fig. 4): tolerated autografts (0.4±0.5); tip (1.8±1.3), middle (7.2±0.8), or bottom (7.4±0.9) part of U-shaped flaps lacking the 2 main feeding vessels; U-shaped flaps with 1 (6.8±1.7) or both (7.0±0.7) of 2 main feeding vessels; and untreated skin (10.4±1.5).

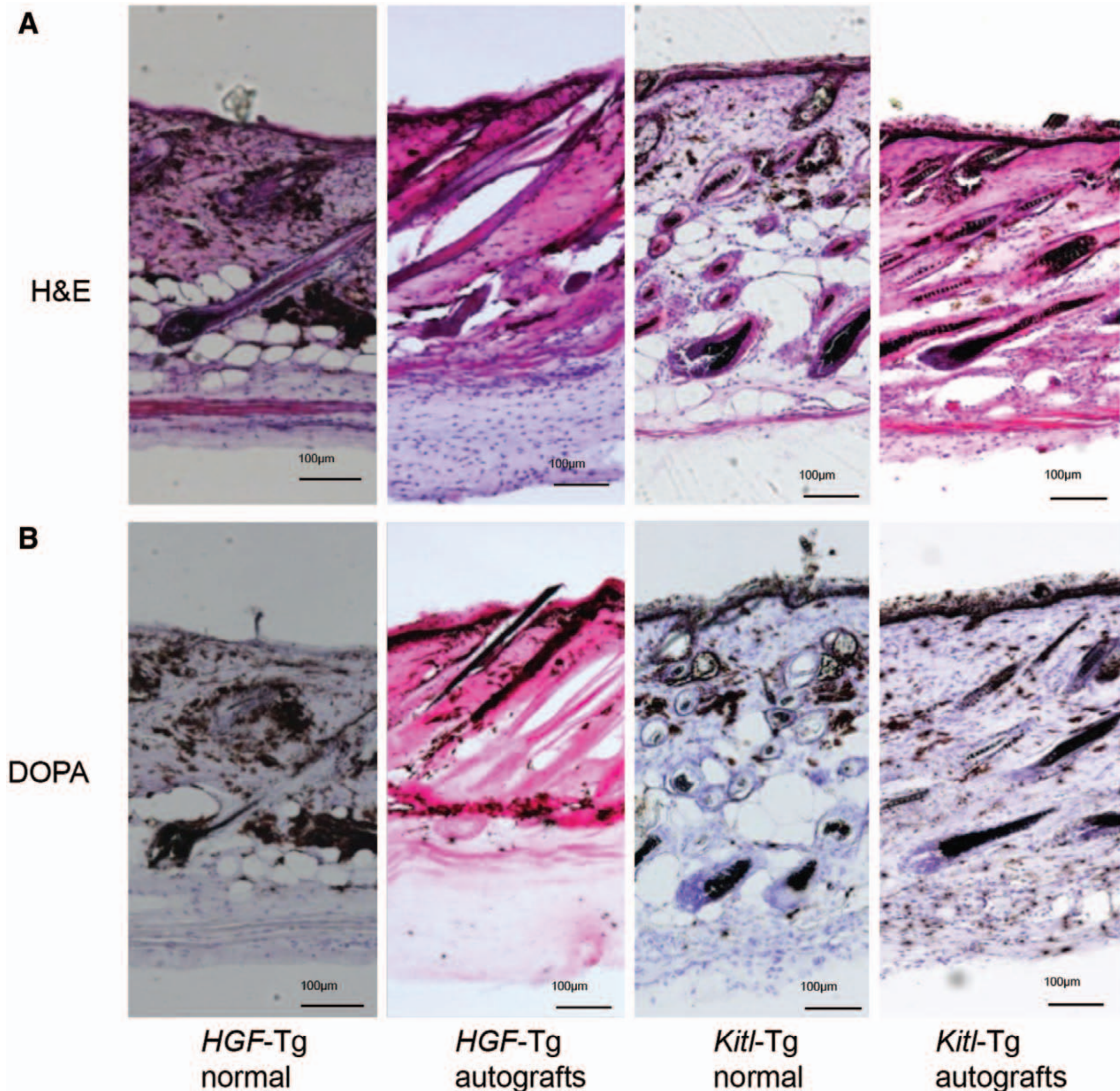

Fig. 6. H&E (A)- and DOPA (B)-stained sections of normal dorsal skin or skin autografts from *HGF*-Tg and *Kitl*-Tg C57BL/6 mice. Scale bars = 100 μm. The difference in the numbers of DOPA+ follicles between "*HGF*-Tg normal" and "*HGF*-Tg autografts" is significant ($P = 0.0001$), whereas the difference between "*Kitl*-Tg normal" and "*Kitl*-Tg autografts" is not significant ($P = 0.0723$) according to Student's *t* test.

Keratinocyte expression of Tg *HGF* and epidermal expression of Tg *Kitl* promote the survival and proliferation of melanoblasts in the dermis[29] and in the basal layer of the epidermis,[28] respectively. Therefore, we expected the growth of black and gray hair on the tolerated skin autografts of *HGF*-Tg and *Kitl*-Tg C57BL/6 mice, respectively. Unexpectedly, however, the hair color on tolerated skin grafts from *Kitl*-Tg mice was black and that from *HGF*-Tg mice, a mixture of black and gray (Fig. 5). Melanocytosis in the interadnexal skin was stimulated in *HGF*-Tg C57BL/6 mice before and after skin autografting, whereas follicular melanocytosis on the tolerated grafts was reduced after it (Fig. 6). In the case of *Kitl*-Tg mice, there was unchanged accumulation of melanin granules in the epidermis and the follicles of the tolerated grafts (Fig. 6), demonstrating that melanocytosis was promoted in the follicles and in the epidermis of the *Kitl*-Tg mice.

The primary distinguishing feature of follicular melanogenesis, compared with the continuous melanogenesis in the epidermis, is the tight coupling of hair follicle melanogenesis to the hair growth cycle[15–19]: the hair cycle involves melanocyte death

via apoptosis during early catagen.[32] In addition, follicular melanogenesis (ie, melanogenic activity of follicular melanocytes, transfer of melanin granules into cortical and medullary keratinocytes, and formation of pigmented hair shafts)[33] is more sensitive to aging influences than melanocytes in the epidermis, resulting in hair graying/canities.[31] The melanogenic activity appeared to be impaired at the tip of U-shaped flaps lacking the 2 main feeding vessels or in the tolerated autografts (Fig. 4), and the hair shafts without pigmentation elongated normally to be maintained for more than 5 months (Fig. 1). Therefore, the nonmelanogenic melanocytes, a progenitor of active melanocytes during the next anagen phase, in the telogen follicle[15,16] would appear to be susceptible to the poor blood supply.

Recently, Tanimura et al[34] reported that the transmembrane protein collagen XVII is highly expressed in hair follicle stem cells (HFSCs) and is required for the maintenance not only of HFSCs but also of melanocyte stem cells. They also showed that mice lacking collagen XVII show premature hair graying and hair loss and that forced expression of collagen XVII in basal keratinocytes, including HFSCs, in collagen XVII-null mice rescues melanocyte stem cells from premature differentiation and restores transforming growth factor-β signaling. In the present study, however, skin autografts from wild-type or *HGF*-Tg C57BL/6 mice survived with gray hair and without hair loss and the hair shafts without pigmentation elongated normally suggesting a collagen XVII/transforming growth factor-β-independent mechanism of hair graying (Figs. 1, 3, 5).

Patients with piebaldism, a genetic disorder of the Kit receptor, present at birth with white patches of skin and hair that lack melanocytes.[27] In contrast, vitiligo is an acquired idiopathic and, in the majority of cases, a progressive, unpredictable disorder of the skin; skin with such lesions is characterized by the lack of epidermal melanocytes.[35–42] Tobin et al[43] reported that melanocytes are never completely absent in the depigmented epidermis of vitiligo and that these melanocytes can recover their functionality in vivo and in vitro upon the removal of H_2O_2. During vitiligo repigmentation, hypertrophic pigmented melanocytes appear in the outer root sheath of the hair follicle and migrate from the infundibulum into the nearby epidermis.[44] The melanin pigment formation was stimulated mainly in the epidermis of *Kitl*-Tg C57BL/6 mice (Figs. 5, 6); the follicular melanocytes from vitiligo patients are to some extent intact at the first onset of the disease.[35] These results imply a possibility that oxidative stress by an insufficient blood supply and impairment in c-Kit and/or its ligand expression might induce melanocyte-specific lesions in the epidermal (at the first onset) and/or follicular (at the later stages) melanin unit.

U-shaped skin flaps possessing 1 of the 2 main feeding vessels had black-colored hair, whereas those lacking both vessels showed the growth of gray-colored hair at the tip of the flaps (Fig. 3). In addition, as inhibition of the c-Kit function, by injection of an anti-c-Kit monoclonal antibody, results in the growth of unpigmented hair[45] and as there was unchanged follicular melanocytosis on the tolerated skin grafts from *Kitl*-Tg mice (Fig. 6), the *c-Kit* gene might play a critical role in this process in mice. These results suggest that generation of at least 1 feeding vessel at a skin transplantation site might be useful for protection against gray hair growth in mice and that topical treatment with c-Kit and/or its ligand might not only achieve a normal dermal structure and pigmented hair but also induce epidermal melanocytosis for piebaldism, vitiligo, and so forth in humans.

Koichi Ueda, MD, PhD
Department of Plastic and Reconstructive Surgery
Osaka Medical College, 2–7 Daigaku-machi
Takatsuki 569–8686, Japan
E-mail: pla007@poh.osaka-med.ac.jp

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