**Communication to the Editor**

Flavonoids with Two OH Groups in the B-Ring Promote Pigmented Hair Regeneration

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During the process of skin regeneration following a skin injury, de novo hair follicle regeneration is initiated after wounding; however, these regenerated hairs are mostly unpigmented. The activation of epidermal melanocyte stem cells and their differentiation into regenerating hair follicles have been shown to be necessary for the pigmented hair regeneration after wounding. To determine the role of flavonoids in the regeneration of pigmented hairs, we applied the candidate flavonoids to the regenerating hair follicles after wounding and identified the flavonoid species that maximally induced pigmented hair regeneration. Flavonoids with two OH groups in the B-ring, such as sterubin, luteolin, and hydroxygenkwanin, showed promising effects in regenerating black pigmented hairs, while those with one OH group in the B-ring showed no significant change. Thus, flavonoids with two OH groups in their B-ring could be studied further as potential wound healing agents with the ability to regenerate pigmented hair.

**Key words** flavonoid; wound healing; pigmentation; hair regeneration

### INTRODUCTION

During wound healing in the skin, hair follicles are regenerated under severe wounding conditions. In adult mice, regenerated hairs are mostly unpigmented because follicular melanocytes are not activated during the regeneration process. Recently, melanocyte stem cells have been reported to respond to wounding and migrate to the nearby epidermis from the existing hair follicles residing very close to the regenerating skin. These skin regeneration-associated melanocytes have the potential to differentiate into follicular melanocytes of regenerating hair follicles to form pigmented hairs. Wnt signaling, first produced in the follicular keratinocyte stem cells, is known to stimulate both hair follicular keratinocytes and follicular melanocyte stem cells to induce coordinated pigmented hair follicle regeneration after wounding. Furthermore, efficient regeneration of pigmented hairs was observed when mice were wounded at 5 weeks after birth. During this process, the follicles were in the anagen phase, and the expression of Wnt7a, a known inducer of wound healing associated with hair follicle regeneration, was highest in the epidermis. Pigmented hair regeneration markedly reduced after wounding during the telogen phase at 7 weeks after birth. However, when drugs activating Wnt signaling, such as LiCl, were applied to the wounded skin during this period, a higher proportion of pigmented hairs in the regenerated hair follicles was observed.

Flavonoids are plant-derived polyphenolic compounds claimed to help alleviate a variety of conditions owing to their antioxidant, anti-inflammatory effects, wound healing, and melanogenesis effects. Luteolin treatment has been reported to promote wound healing possibly via its epithelization promoting and free-radical scavenging activities. In our previous study, sterubin was found to promote melanin production through the Wnt signaling pathway in the human melanoma cell line HMVII and prevent the progression of hair graying. Luteolin, diosmetin, and hesperetin are known to promote

### Table 1. Flavonoids and Their Structures

| Type | Two OH groups in the B-ring | One OH group in the B-ring |
|------|----------------------------|---------------------------|
| Flavonone | Sterubin, Eriodictyol | Hesperetin, Homoeordictyol |
| Flavone | Luteolin, Hydroxygenkwanin | Diosmetin |

The skeleton structure of flavonone and flavone with the rings named. The chemical structure diagrams were drawn manually using ISIS/Draw (MDL Information Systems, Inc., CA, U.S.A.).

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Fig. 1. Regeneration of Pigmented Hairs after Wounding

(A) Representative photos of regenerated dorsal skin area at 80d after wounding. Scale bar = 1 mm. (B) The percentage of regenerated hairs and their pigmentation. The flavonoids (0.1% w/v, 200 µL/d) were applied to the wounded area for one week. Hair regeneration and hair color were assessed under a microscope at 80d after resection. Hairs only on re-epithelialized areas containing at least 10 regenerated hairs were counted, and only areas with more than one-third of regenerated hairs with pigmentation were designated as areas containing pigmented regenerated hairs. The number of mice used for the experiments is shown. The results were analyzed using chi-square test (hair regeneration: \( *p < 0.05 \), \( **p < 0.01 \), pigmented hair regeneration: \( \dagger\dagger p < 0.01 \)).
melanin production in cultured cells; therefore, their effects on the darkening of hair are being considered.7–9

In this study, flavonoids with two OH groups in the B-ring, such as sterubin, luteolin, hydroxygenkwanin (HGK), and eriodictyol, and one OH group in the B-ring, such as hesperetin, homoeriodictyol, and diosmetin, that possibly have the potential to induce regeneration of pigmented hairs during wound healing were evaluated, and their effects were compared with each other. We hypothesized that flavonoids applied onto the wounded skin of mice can induce appropriate signals to activate follicular keratinocytes and melanocytes necessary for regeneration of the pigmented hairs and/or to prevent inflammation during the regeneration process; therefore, a higher proportion of pigmented hairs could be expected in the treated regenerated hair follicles.

MATERIALS AND METHODS

Dorsal skin samples (2.25 cm²) were excised from 7-week-old C57BL/6 mice after trimming the surrounding hairs with electric clippers. All animal experiments were approved by the Animal Research Committee of the Graduate School of Medicine, Gifu University. The flavonoids (0.1% w/v) shown in Table 1 were dissolved in 50% ethanol, and 200 µL of the flavonoid solution was applied daily to the wounded area for one week. Sterubin used was synthesized in the laboratory,6 and the other flavonoids: luteolin (LKT Laboratories, MN, U.S.A.), hydroxygenkwanin (Phytolab, Vestenbergsgreuth, Germany), eriodictyol (Extrasythene, Lyon, France), hesperetin (Alexis Corporation, Nottingham, U.K.), homoeriodictyol (Extrasythene) and diosmetin (LKT Laboratories) were all commercially available products. Hair regeneration and hair color were assessed using a microscope (Moritex Corporation, Saitama, Japan) at 80 d after resection. Hairs only on re-epithelialized areas containing at least 10 regenerated hairs were counted, and only areas with more than one-third of pigmented regenerated hairs were designated as areas containing pigmented regenerated hairs.4

RESULTS AND DISCUSSION

To evaluate the pigmented hair regeneration during the wound healing process of the mouse treated with flavonoids, we shaved the hair around the reepithelialized areas and then photographed the regenerated hair. On day 80, the rate of hair regeneration including black pigmented and unpigmented hairs was as follows: no drug (20.5%), 50% ethanol (22.7%), sterubin (70.6%), luteolin (58.8%), HGK (42.9%), eriodictyol (42.1%), hesperetin (11.1%) homoeriodictyol (9.5%), and diosmetin (5.3%). Sterubin and luteolin significantly activated hair regeneration, but homoeriodictyol and diosmetin significantly inhibited hair regeneration in comparison with the other flavonoids and controls (Figs. 1A, 1B). Furthermore, the ratio of pigmented hair regeneration was as follows: no drug (0.0%), 50% ethanol (2.3%), sterubin (47.1%), luteolin (52.9%), HGK (42.9%), eriodictyol (21.1%), hesperetin (5.6%) homoeriodictyol (4.8%), and diosmetin (5.3%). Sterubin, luteolin, and HGK activated melanocyte stem cells to successfully regenerate pigmented hairs in comparison with the other flavonoids and controls (Figs. 1A, B). Horibe et al. reported that the one OH group-containing diosmetin induced melanogenesis to a greater extent than the two OH groups-containing luteolin in B16F10 cells by upregulating tyrosinase through cAMP response element binding protein (CREB).13 In our study, however, the one OH group-containing flavonoids did not significantly induce overall hair follicle regeneration. Regeneration of hair follicles after wounding requires the concerted activation of melanocyte stem cells, including their differentiation, and the stimulation of melanin synthesis.10 Therefore, the one OH group-containing flavonoids that had the least effect on hair regeneration are not considered suitable.

Flavonoids with two OH groups in the B-ring have been reported to have a greater antioxidant activity than those with one OH group in the B-ring.11 Sterubin (two OH groups) has been shown to have the highest antioxidant activity among flavonoids isolated from the resinous exudate of Heliotropium sinuatun and Erodioctyos angustifolium.6,12,13 Sterubin and eriodictyol (two OH groups) have greater neuroprotective effects than homoeriodictyol (one OH group). In addition, sterubin showed three times more anti-inflammatory activity than eriodictyol, and O-methylation on the A-ring increases the overall lipophilicity, bioavailability, and metabolic stability of sterubin.16 The two OH groups in the B-ring also inhibited the phosphorylation of protein kinase Cδ (PKCδ) than no or one OH group in the B-ring.11 The two OH groups in the B-ring could bind with C1B domain of PKCδ phorbol ester binding site better than the others and thus expected to effectively reduce PKCδ activity. Because proliferation of melanocytes is inversely related with PKCδ activity,15 two OH groups-containing flavonoids are expected to increase hair pigmentation.

Pigmented hair regeneration after wounding has recently been reported in a geriatric patient with a large wound on the scalp.60 To date, there have been no reports on pigmented hair regeneration in human skin after wounding.

In conclusion, flavonoids with two OH groups in the B-ring effectively regenerate pigmented hairs after skin wounding, whereas those with one OH group in the B-ring do not. Our finding may guide further exploration of novel agents that induce pigmented hair regeneration.

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Conflict of Interest Nobuhiko Taguchi is an employee of Hoyu Co., Ltd. The other authors declare no conflict of interest.

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