



# Protective Effect of Hydroxygenkwanin against Hair Graying Induced by X-Ray Irradiation and Repetitive Plucking

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Hair graying in mice is caused by various injuries such as X-ray radiation and repeated plucking that ultimately damage melanocytes and their stem cells (melanocyte stem cells). In X-ray-induced hair graying, injuries first manifest as a loss-of-niche function of hair follicular keratinocyte stem cells to maintain melanocyte stem cells. Thus, we hypothesized that hair follicular keratinocyte stem cells could be a practical target to prevent hair graying. In this study, we investigated the in vivo effect of the flavonoid hydroxygenkwanin, which has been shown to exert the best protection on human epidermal keratinocytes against in vitro X-ray-induced cytological effects, using X-ray-induced and repeated hair plucking-induced hair graying mice models. We found that hydroxygenkwanin exerted a remarkable effect in preventing hair graying; however, when receptor Y kinase *Kit*-mutant mice were used, no prevention effect was observed. Therefore, we propose that Kit signaling might be involved in the hydroxygenkwanin-induced protective effect against hair graying.

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## INTRODUCTION

Injuries such as X-ray radiation and repeated plucking cause graying of hair in mice (Jo et al., 2018; Li and Hou, 2018). X-ray irradiation triggers premature differentiation of melanocytes from their stem cells (melanocyte stem cells [McSCs]) in the hair bulge, and the resultant DNA damage leads to hair graying (Inomata et al., 2009). Hair follicular keratinocyte (KC) stem cells (HFKSCs) provide a niche microenvironment for McSCs in the bulge region (Tanimura et al., 2011). X-ray-induced hair graying in mice does not directly affect the McSCs but causes a loss-of-the-niche function of HFKSCs (Aoki et al., 2013), therefore indicating that HFKSCs are also associated with hair graying. Therefore, we have focused on the involvement of HFKSCs, not that of McSCs, in hair graying and their potential as a practical target for its prevention.

Prevention of age-related beard graying in humans by the topical application of *Eriodictyon angustifolium* extract, containing a high concentration of flavonoids such as hydroxygenkwanin (HGK), sterubin, and luteolin, has been

reported previously (Taguchi et al., 2020a). Interestingly, *E. californicum*, which contains a small amount of HGK, sterubin, and luteolin, did not exert antihuman beard hair-graying effects or DNA damage-reducing effects (Taguchi et al., 2020a). In addition, dietary *E. angustifolium* tea can prevent X-ray-induced DNA damage and hair graying in mice (Taguchi et al., 2020b). These results indicate that the active ingredients in *E. angustifolium* that prevent hair graying are flavonoids, such as HGK.

One-time hair plucking induces melanogenesis in the regenerating hair bulb. The consequent hair hyperpigmentation and repeated hair plucking but not shaving cause inflammation in the hair follicles and injure the McSCs, leading to hair graying (Endou et al., 2014; Li and Hou, 2018; Iida et al., 2020). After five hair cycles (approximately five times of hair plucking), hair graying was observed in C57BL/6 mice (Endou et al., 2014); this repeated hair plucking-induced mouse model is the closest to age-related human hair graying.

In humans, hair graying appears at the average age of 35 years (Panhard et al., 2012; Pandhi and Khanna, 2013). Because hair cycle on the scalp lasts for approximately 7 years, hair begins to gray after five complete hair cycles (Damodaran and Gupta, 2011; Hartwig et al., 2003). However, drug screening for antihair graying agents using the repeated hair plucking mouse model has not been reported.

The Kit receptor Y kinase, which binds to its ligand Kitl, transmits signals that stimulate the proliferation, survival, and migration of cells, including melanocytes (Kunisada et al., 1998). Its functions manifest as white spotting or hair graying in *Kit* and *Kitl* mutant mice (Tosaki et al., 2006). Heterozygous loss-of-function mutants (*Kit*<sup>W/+</sup> and *Kit*<sup>V620A tg1/+</sup>) have white spots in their coats and are more susceptible to hair graying through X-ray irradiation or repeated hair plucking than the wild type (WT) (Aoki et al., 2011; Endou

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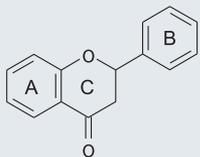
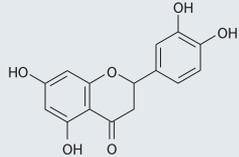
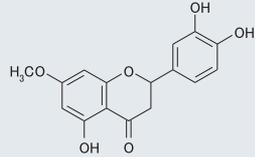
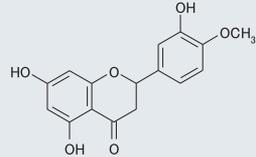
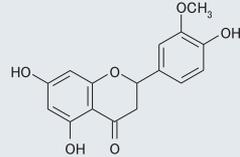
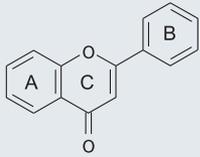
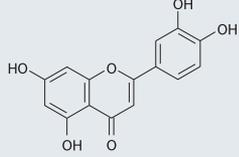
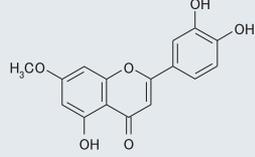
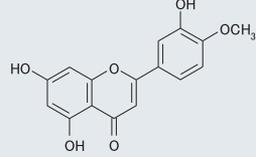
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Abbreviations: DCF, dichlorofluorescein; HFKSC, hair follicular keratinocyte stem cell; HGK, hydroxygenkwanin; KC, keratinocyte; McSC, melanocyte stem cell; NHEK, normal human epidermal keratinocyte; WT, wild type

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**Table 1. Flavonoids and Structures**

Type	Two OH Groups in the B-Ring			One OH Group in the B-Ring	
	Without –OCH <sub>3</sub>			With –OCH <sub>3</sub>	
					
Flavanone	Eriodictyol	Sterubin	Hesperetin	Homoeriodictyol	
					
Flavone	Luteolin	HGK	Diosmetin		

Abbreviations: HGK, hydroxygenkwanin; OCH<sub>3</sub>, methoxy; OH, hydroxyl.  
The table shows the skeletal structures of flavanones and flavones evaluated in this study.

et al., 2014). In contrast, exogenous expression of *Kitl* in the skin prevented X-ray-induced hair graying (Aoki et al., 2011).

In this study, we compared the topical application of HGK between WT and *Kit*-mutant mice using X-ray-induced and repeated hair plucking-induced hair graying mice models. As a result, HGK prevented hair graying in WT mice, but not in the *Kit* loss-of-function mutant mice. Kit signaling-dependent action of HGK flavonoid species against mechanical injury-induced hair graying provides the biological basis for the prevention and control of age-related hair graying.

## RESULTS

### HGK significantly suppresses X-ray-induced cell death in normal human epidermal KCs

HFKSCs, which provide a niche for melanocytes, have been suggested to play indispensable roles in age-associated (Tanimura et al., 2011) and X-ray-induced hair graying (Aoki et al., 2013). Because the niche function of HFKSCs is fulfilled by KCs expressing *Kitl* (Kunisada et al., 1998), we used normal human epidermal KCs (NHEKs), known to constantly express *Kitl*, as HFKSC equivalents. The flavonoid sterubin was recently studied for its ability to protect cultured NHEKs from X-ray irradiation (Taguchi et al., 2018).

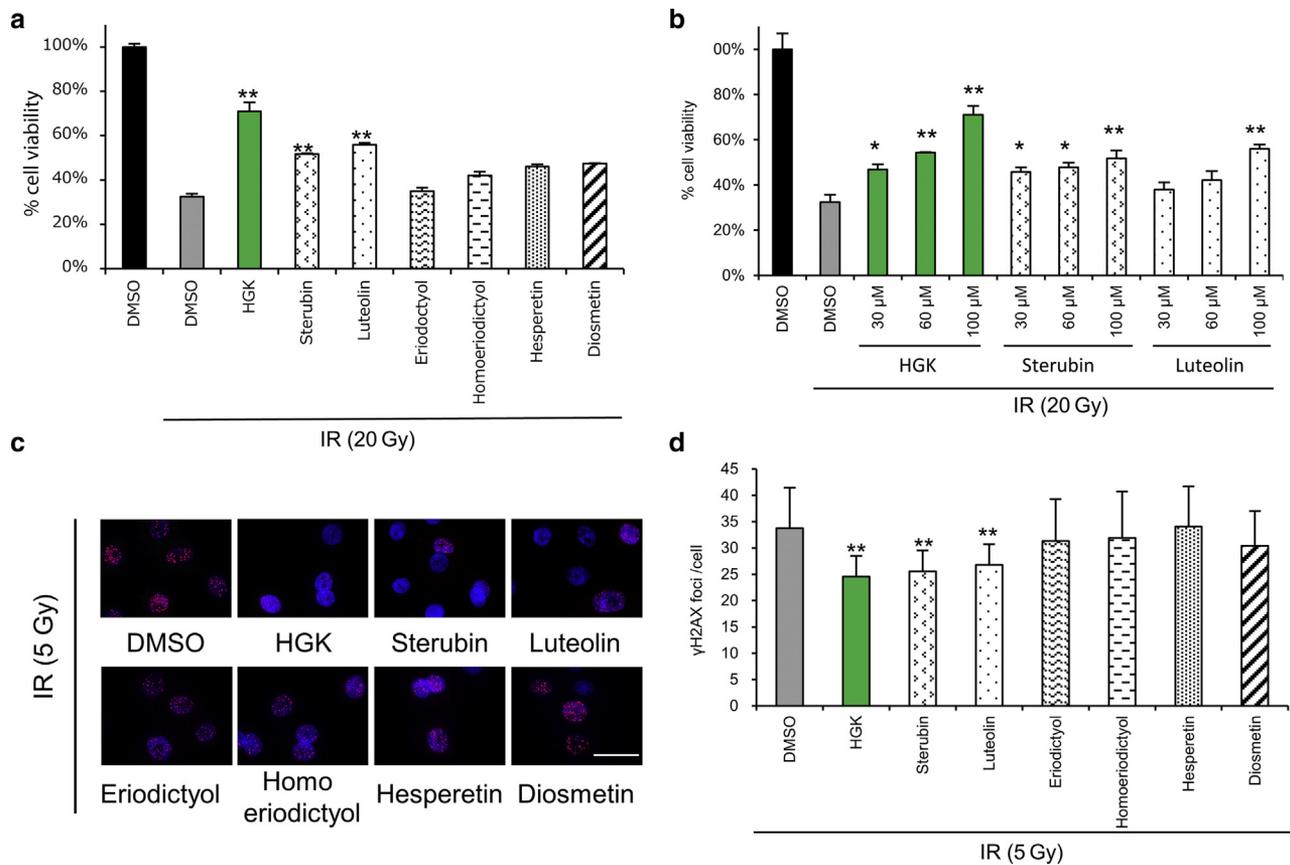
We investigated whether flavonoids could rescue NHEKs from X-ray-induced cell death (Table 1). Pre-exposure to HGK, sterubin, and luteolin rescued NHEKs from X-ray-induced cell death in a concentration-dependent manner (Figure 1a and b). In addition, HGK, sterubin, and luteolin significantly decreased the number of phosphorylated H2AX foci per cell after X-ray irradiation, which is indicative of reduced DNA damage (Figure 1c and d). In this study, we compared the protective effects of seven flavonoids (Table 1) and found that the three most effective flavonoids (HGK, sterubin, and luteolin) contain two hydroxyl groups in the B-

ring of their framework, suggesting that only certain types of flavonoid exhibit protective effects against X-rays. Hereafter, we used HGK as a representative, which has been shown to be most effective, for subsequent experiments.

We next investigated whether HGK could scavenge X-ray-induced intracellular ROS using the dichlorofluorescein (DCF) diacetate assay. After exposure to 20 Gy of X-ray, increased fluorescence intensity was observed in NHEKs. As shown in Figure 2a and b, HGK significantly suppressed X-ray-induced generation of ROS. ROS destroys mitochondrial DNA and membrane proteins, resulting in the impairment of mitochondrial function and hair graying (Kujoth et al., 2005). Mito-Tracker staining results revealed that pretreatment with HGK prevents the severe deformation of the mitochondrial mass in NHEKs (Figure 2c and d).

### Prevention of X-ray-induced hair graying after HGK application in WT mice

In this study, an X-ray-induced hair graying mouse model was used (Figure 3a) to investigate in vivo the effect of topical application of HGK and other flavonoids on hair graying. Topical application of HGK, sterubin, and luteolin but not that of eriodictyol, homoeriodictyol, hesperetin, and diosmetin prevented X-ray-induced hair graying (Figure 3b–g). To avoid entry of the mice in anagen stage, during which hair follicles are resistant to X-ray-induced hair graying, all mice were confirmed to be in the telogen stage of hair cycle by inspecting their skin color. In addition, treatment with minoxidil, a strong inducer of the anagen hair cycle, instead of with flavonoids did not prevent X-ray-induced hair graying (Figure 3h). Quantitation of hair darkness using the CIE-LAB (CIE L\*a\*b\*) color system (Figure 3i) confirmed the antihair graying effect of HGK, sterubin, and luteolin. Furthermore, DNA damage, as evidenced by  $\gamma$ H2AX foci, was significantly reduced by HGK in CD34-positive hair follicle bulge-derived KCs, including HFKSCs (Tremplus et al.,



**Figure 1. HGK, sterubin, and luteolin inhibit radiation-induced cytotoxicity in NHEKs in a concentration-dependent manner.** (a, b) NHEKs were pretreated with flavonoids for 1 h before irradiation with 20 Gy X-ray and were incubated for 72 h. Cell viability was determined using the MTT assay. Data represent the mean  $\pm$  SD (n = 3). (c) Immunofluorescence activity of  $\gamma$ H2AX foci (red) in NHEKs pretreated with 100  $\mu$ M flavonoids for 1 h, irradiated with 5 Gy X-ray, and incubated for 5 h. Nuclei were stained with DAPI (blue). Bar = 5  $\mu$ m. (d) The average number of  $\gamma$ H2AX foci per cell was obtained from the fluorescent images in c. At least 50 cells were counted from 10 randomly chosen fields. Data represent the mean  $\pm$  SD. \* $P$  < 0.05 and \*\* $P$  < 0.01 analyzed using one-way ANOVA with Bonferroni posthoc multiple comparison test. h, hour; HGK, hydroxygenkwanin; IR, irradiation; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NHEK, normal human epidermal keratinocyte.

2003) (Figure 3j and k). This observation confirms that HFKSCs provide niches for follicular McSCs.

Interestingly, after the induction of hair graying through X-ray irradiation, the topical application of HGK for 2 months did not reverse the formation of gray hair (Figure 3l–n). These results indicated that at least in this test system, the effects of HGK are limited to the prevention of hair graying.

#### Prevention of plucking-induced hair graying after HGK application in WT mice

We employed the repeated hair plucking–induced hair graying mouse model to confirm the antihair graying effect of HGK, sterubin, and luteolin. The number of gray hair in C57BL/6 mice increased after a minimum of five rounds of hair plucking, which was significantly reduced after daily treatment with HGK, sterubin, or luteolin (Figure 4), indicating that these agents possess antihair graying effects.

#### Prevention of X-ray–induced hair graying after HGK application is canceled in *Kit*-mutant mice

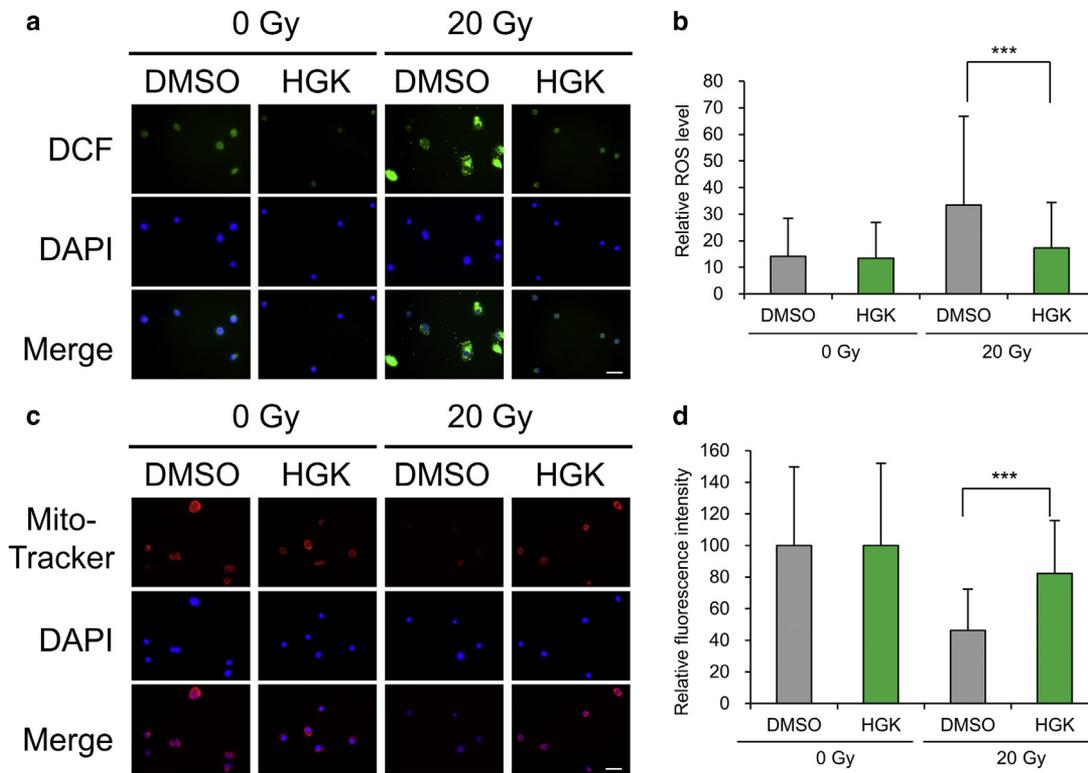
The stimulation of Kit signaling in follicular melanocytes has been reported to protect hair graying induced by X-ray irradiation; in contrast, the reduction in Kit signaling facilitated X-ray–induced hair graying (Aoki et al., 2011). To investigate

the mechanisms underlying the preventive effect of HGK, we subjected *Kit* loss-of-function mutant mice (*Kit*<sup>W/+</sup> and *Kit*<sup>V620A tg1/+</sup>) to HGK treatment. The *Kit* loss-of-function mutant mice (*Kit*<sup>W/+</sup> and *Kit*<sup>V620A tg1/+</sup>) treated with HGK, compared with those treated with ethanol, exhibited no significant antihair graying effect (Figure 5a–d). In addition, no significant reduction in DNA damage was observed in HFKSCs (Figure 5e–g).

Although complex interactions between follicular melanocytes and KCs have been identified, Kit signaling is necessary to maintain the niche function for X-ray–induced hair graying. The reduced Kit signaling in *Kit* loss-of-function mutants may have prevented the sufficient recovery of hair graying induced by the protective function of HGK through the Kit signaling pathway.

#### Prevention of plucking-induced hair graying after HGK application in WT mice but not in *Kit*-mutant mice

The protective effect of HGK against repetitive hair plucking using *Kit*-mutant mice was also evaluated. HGK application showed no effect on the extent of hair graying in both *Kit*<sup>W/+</sup> and *Kit*<sup>V620Atg1/+</sup> mouse models (Figure 5h–k). Thus, Kit signaling is suggested to be involved in the protective effect



**Figure 2. HGK reduces radiation-induced ROS production and deformation of the mitochondrial mass.** (a) NHEKs pretreated with HGK were irradiated with 20 Gy X-ray, and ROS production was measured on the basis of the intensity of the DCF-DA probe. Bar = 20  $\mu$ m. (b) Quantitation of relative ROS levels. At least 50 cells were counted from 10 randomly chosen fields of view. (c) NHEKs pretreated with HGK were irradiated with 20 Gy X-ray, and the mitochondrial membrane integrity of irradiated NHEKs pretreated with HGK was determined using Mito-Tracker. Bar = 20  $\mu$ m. (d) The relative fluorescence intensity of the Mito-Tracker was quantitated. At least 50 cells were counted from 10 randomly chosen fields of view. Data are presented as the mean  $\pm$  SD. \*\*\* $P < 0.001$  analyzed by *t*-test. DCF, dichlorofluorescein; DCF-DA, dichlorofluorescein-diacetate; HGK, hydroxygenkwanin; NHEK, normal human epidermal keratinocyte.

of HGK against repetitive hair plucking-induced hair graying.

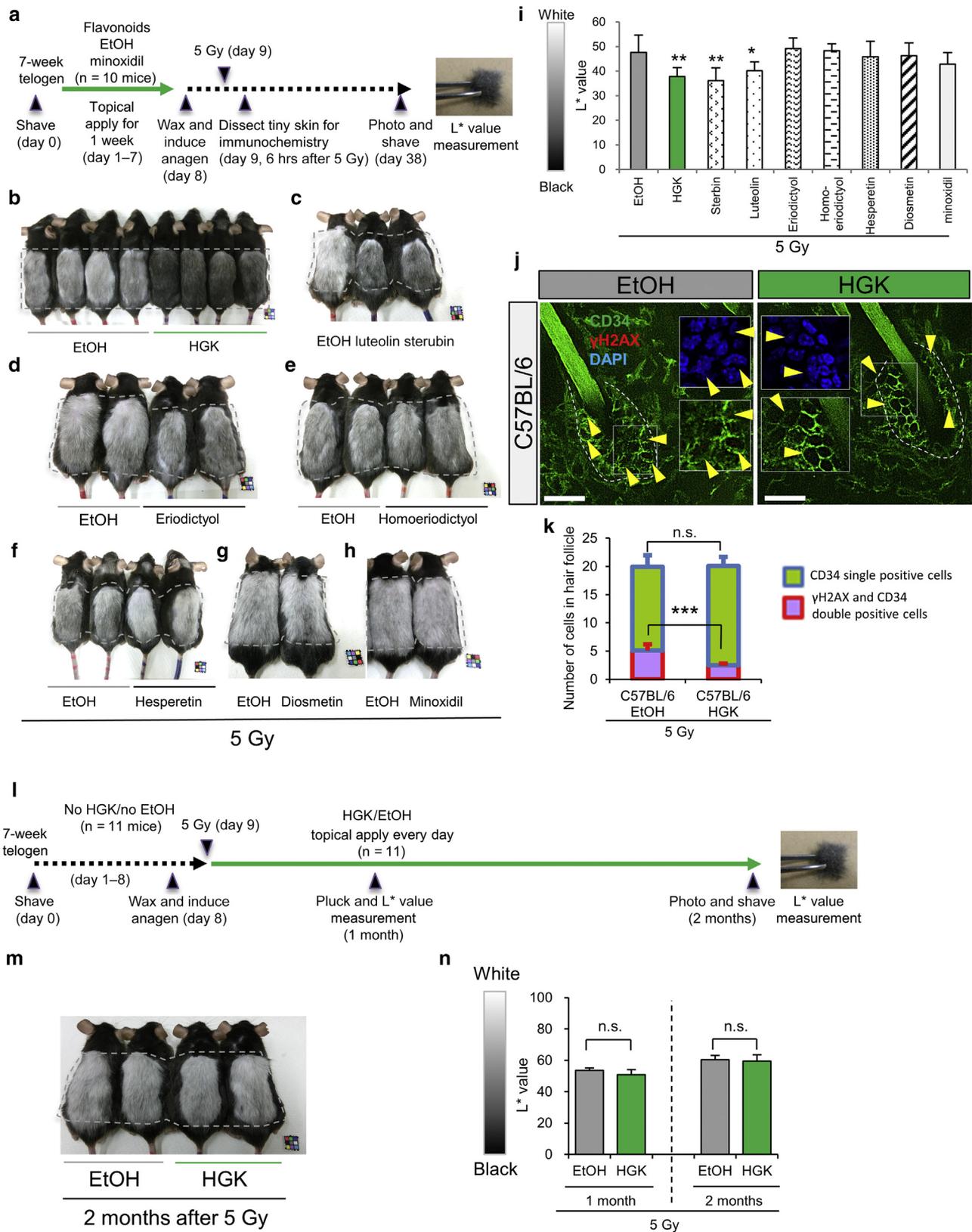
## DISCUSSION

Active reagents for improving hair graying can be easily screened in vitro using cultured melanocytes or melanoma cells, and such tested natural extracts and chemical compounds that activate melanin synthesis are commonly available. In particular, most of these agents have been reported to enhance melanin production in differentiated melanocytes (Jeon et al., 2009; Liu-Smith and Meyskens, 2016; Taguchi et al., 2018; Takekoshi et al., 2014) and are expected to stimulate melanogenesis of differentiated melanocytes in hair bulbs. Although there are some clinical reports of hair repigmentation (Yale et al., 2020), only a few drugs have been proven to prevent hair graying. For example, in our previous study, the topical application of *E. angustifolium* extract, which contains a high concentration of HGK, sterubin, and luteolin, prevented age-related human beard hair graying (Taguchi et al., 2020a). The *E. angustifolium* extract also showed a protective effect against DNA damage induced by X-ray irradiation in NHEKs. *E. californicum*, which contains a small amount of HGK, sterubin, and luteolin, did not exert antihuman beard hair graying effect or DNA damage-reducing effect (Taguchi et al., 2020a). In addition, dietary *E. angustifolium* tea can prevent X-ray-induced DNA damage and hair graying in mice (Taguchi et al., 2020b). These results indicate that the active ingredients in *E. angustifolium*

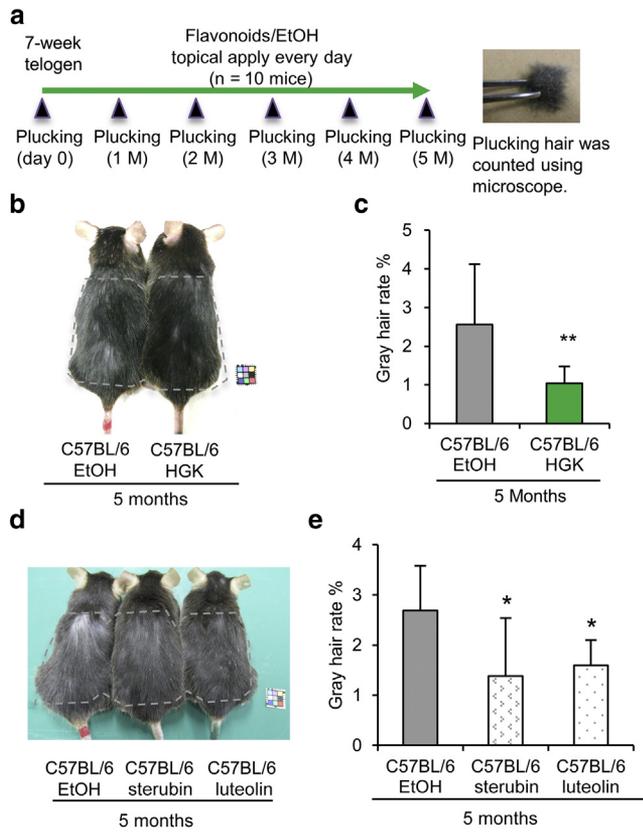
exerting hair-graying preventive effects could be flavonoids, such as HGK.

X-ray-irradiated mice have been used as a convenient animal model for age-related hair graying (Inomata et al., 2009; Taguchi et al., 2020b); however, damage induced by X-ray-induced ionized oxygen species to various biomolecules, including DNA double-strand breaks, is not the same as the age-related cellular damage induced by factors such as stresses, which are considered the main cause of hair graying (Jo et al., 2018; Li and Hou, 2018). A previous study showed that in the repeated hair plucking-induced hair graying C57BL/6 mouse model, hair graying increased after five hair cycles (Endou et al., 2014). This suggests that this model is more similar to age-related human hair graying than the X-ray-induced acute hair graying model. In this study, three flavonoid species, namely HGK, sterubin, and luteolin, which were shown to be effective in vitro, were found to have preventive effects against hair graying induced by X-ray irradiation and repeated hair plucking.

To gain insights into the molecular mechanisms of flavonoids in the prevention of hair graying, we investigated the effect of HGK on *Kit* loss-of-function mutant mice, which have been reported to be susceptible to X-ray-induced hair graying (Aoki et al., 2011). If HGK is mostly acting independently from *Kit* signaling, hair graying in these mutant mice induced by X-ray is sure to be protected by HGK treatment, as in the WT controls. However, we did not observe the protective effects of HGK in two hair-graying



**Figure 3. Prevention of X-ray–induced hair graying after HGK, sterubin, and luteolin application.** (a) Experimental scheme: X-ray–induced hair graying. (b–h) The coat color of C57BL/6 mice pretreated with 0.1% w/v flavonoids, minoxidil, or 50% EtOH after 5 Gy irradiation. (i) Quantitative analysis of hair pigmentation. L\* values were analyzed from all shaved hairs (n = 10 each). Data represent the mean ± SD. \**P* < 0.05 and \*\**P* < 0.01 analyzed by one-way ANOVA with Bonferroni posthoc multiple comparisons test. (j) Immunohistochemical analysis of γH2AX foci (red) in CD34<sup>+</sup> cells (green) or DAPI (blue) 6 h after irradiation. Bar = 25 μm. (k) Immunofluorescence detection. \*\*\**P* < 0.001 analyzed by *t*-test. (l) Experimental scheme. (m) Coat color of C57BL/6 mice treated with 0.1% w/v HGK and 50% EtOH for 1 or 2 months after 5 Gy irradiation. (n) Quantitative analysis of hair pigmentation. L\* values were analyzed from all shaved hairs (n = 11 each). *t*-Test revealed no significant differences (n.s.). EtOH, ethanol; h, hour; HGK, hydroxygenkwanin; n.s., not significant.



**Figure 4. Prevention of plucking-induced hair graying after HGK, sterubin, and luteolin application.** (a) Experimental scheme: plucking-induced hair graying. (b) The coat color of C57BL/6 mice treated with 0.1% w/v HGK or 50% EtOH. (c) Quantitative analysis of hair pigmentation. L\* values were analyzed from plucked hairs ( $\geq 300$  hairs) of each mouse ( $n = 10$  each). \*\* $P < 0.01$  analyzed by Mann–Whitney *U* test. (d) The coat color of C57BL/6 mice pretreated with 0.1% w/v sterubin, luteolin, or 50% EtOH. (e) Quantitative analysis of hair pigmentation. L\* values were analyzed from plucked hairs ( $\geq 300$  hairs) of each mouse ( $n = 10$  each). \* $P < 0.05$  analyzed by one-way ANOVA with Bonferroni posthoc multiple comparisons test. EtOH, ethanol; HGK, hydroxygenkwanin; M, months.

mouse models with *Kit* loss-of-function mutation. These results suggest that HGK acts on signaling pathways involving the Kit receptor Y kinase. Anderson et al. (2021) have recently reported that the topical application of RT1640, a pigment-promoting drug and immunophilin ligand consisting of cyclosporin A, minoxidil, and RT175, effectively reversed the formation of gray hair in an X-ray-irradiated mouse model. Although their experimental method was slightly different from ours in terms of shaving and the number of daily applications after X-ray irradiation, HGK did not show such an effect (Figure 3m and n). Therefore, the mechanism underlying the hair-graying effects of HGK may be different from that of RT1640.

Notably, in two hair-graying mouse models, the stimulation of Kit signaling by the forced expression of *Kitl* in the skin showed a protective effect against graying (Aoki et al., 2011; Endou et al., 2014). In the X-ray-irradiated model, HFKSCs have been suggested to be mainly responsible for the protection of McSCs (Aoki et al., 2011); therefore, the primary target of HGK is likely to be HFKSCs. Kit signaling starts from the binding of the ligand (*Kitl*) to the receptor (*Kit*) and it is

modulated in various steps downstream of the signal (Lennartsson and Rönstrand, 2012).

As a next step, human clinical test with HGK or the gene expression in HFKSCs treated with HGK should be investigated to determine the molecular mechanism underlying the protective effect against hair graying.

In summary, the preventive effect of the flavonoid HGK against hair graying was confirmed in two mouse models, suggesting it as a potential drug against hair graying. Because HGK did not prevent hair graying in the *Kit* loss-of-function mutant mice, it is proposed that Kit signaling might be involved in the HGK-induced protective effect against hair graying.

## MATERIALS AND METHODS

### Materials

Sterubin was synthesized by Kanto Chemical (Tokyo, Japan). Luteolin was purchased from LKT Laboratories (St. Paul, MN). HGK was purchased from Phytolab (Vestenbergsgreuth, Germany). Eriodictyol, homoeriodictyol, hesperetin, and diosmetin were purchased from Extrasynthese (Lyon, France).

### Measurement of $\gamma$ H2AX activation in NHEKs after irradiation

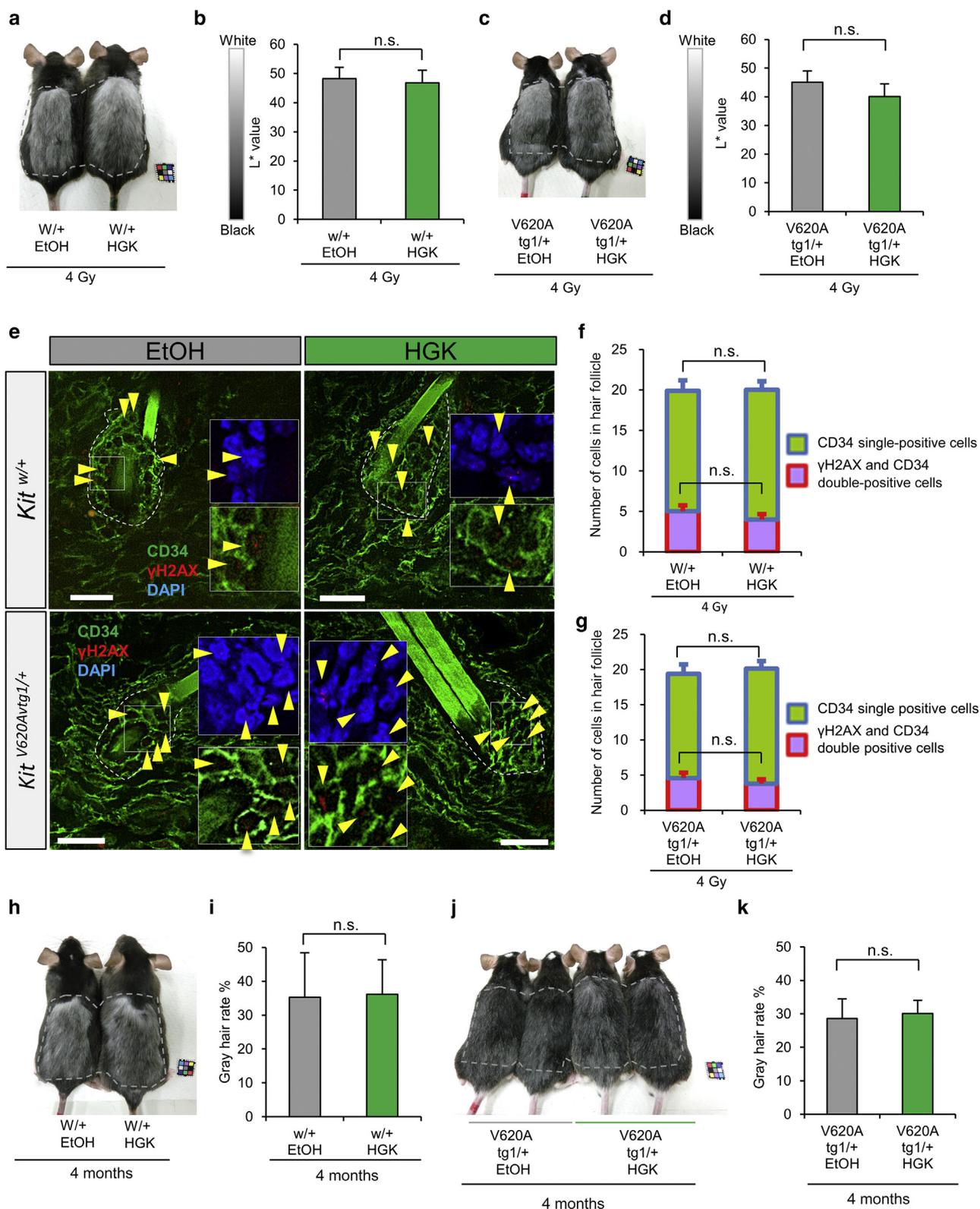
NHEKs (Kurabo, Osaka, Japan) were seeded on coverslips in 12-well plates for 12 hours and preincubated with 30, 60, or 100  $\mu$ M flavonoids for 1 hour before exposure to 5 Gy X-rays using a Hitachi MBR-1520 (Hitachi Medical, Tokyo, Japan). Cells were washed with PBS, fixed with freshly prepared 4% paraformaldehyde, and permeabilized with Triton X-100. The anti- $\gamma$ H2AX antibody (Cell Signaling Technology, Beverly, MA) was diluted to 1:500 in PBS containing 1% BSA, added to the cells, and incubated for 12 hours at 4 °C, followed by incubation with a secondary antibody (1:500; anti-mouse, Abcam, Cambridge, United Kingdom) for 2 hours at 25  $\pm$  1 °C. The cells were counterstained with DAPI to visualize the cell nuclei. The number of  $\gamma$ H2AX foci was counted under a fluorescence microscope. The irradiation dose was chosen because it has been previously shown to cause DNA damage in human KCs (Zhu et al., 2014). Cell viability was measured 72 hours after 20 Gy X-ray irradiation using the MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay (Nacalai Tesque, Kyoto, Japan).

### Measurement of ROS

ROS levels of NHEKs were determined using the ROS-sensitive dye 2',7'-DCF diacetate (Cell Biolabs, San Diego, CA), which is converted by ROS into the highly fluorescent DCF. NHEKs were pretreated with 100  $\mu$ M HGK for 1 hour before irradiation, washed with PBS, and incubated with DCF diacetate for 30 minutes. DCF fluorescence was measured using a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan). The relative fluorescence intensity was calculated using ImageJ software (National Institutes of Health, Bethesda, MD).

### Assessment of mitochondrial mass

Mito-Tracker was used to measure the mitochondrial membrane potential. Cells were pretreated with 100  $\mu$ M HGK for 1 hour, subjected to 20 Gy X-ray irradiation, and incubated for 30 minutes in the dark with Mito-Tracker red fluorescent stain (Molecular Probes, Eugene, OR) dissolved in serum-free medium at 37 °C. DAPI was used to stain the nucleus. Relative fluorescence intensity was calculated using ImageJ software.



**Figure 5. HGK application does not prevent X-ray-induced hair graying in *Kit*<sup>W/+</sup> and *Kit*<sup>V620A tg1/+</sup> mice.** (a, c) Coat color of (a) *Kit*<sup>W/+</sup> and (c) *Kit*<sup>V620A tg1/+</sup> mice pretreated with 0.1% w/v HGK or 50% EtOH after 4 Gy irradiation. (b, d) Quantitative analysis of hair pigmentation in (b) *Kit*<sup>W/+</sup> and (d) *Kit*<sup>V620A tg1/+</sup> mice. L\* values were analyzed from all shaved hairs (n = 10 each). (e) Immunohistochemical analysis of γH2AX foci (red) in CD34+ cells (green) or DAPI (blue) 6 h after irradiation. Bar = 25 μm. (f, g) Immunofluorescence detection. (h, j) Coat color of (h) *Kit*<sup>W/+</sup> and (j) *Kit*<sup>V620A tg1/+</sup> mice treated with 0.1% w/v HGK or 50% EtOH. (i, k) Quantitative analysis of hair pigmentation. L\* values were analyzed from plucked hairs (≥300 hairs) of each mouse (n = 10 each). (b, d, f, g, i, k) t-test revealed no significant differences (n.s.). EtOH, ethanol; h, hour; HGK, hydroxygenkwanin; n.s., not significant.

## Mice

All animal experiments were approved by the Animal Research Committee of the Graduate School of Medicine, Gifu University (Gifu, Japan). All mice were housed in standard animal rooms with food ad libitum under controlled humidity and temperature ( $22 \pm 2$  °C).

**X-ray–induced hair graying method.** The experimental scheme is shown in Figure 3a. Mice aged 7 weeks were shaved, and flavonoids (0.1% w/v) or minoxidil (1.0% w/v) was dissolved in 50% ethanol. These solutions (200  $\mu$ l) were applied daily to the shaved area for 1 week. On day 8, all mice were confirmed to be in the telogen stage by inspecting their skin color, and the dorsal skins were waxed to induce the anagen hair cycle and irradiated with 5 Gy X-ray on day 9. Because *Kit*-mutant mice are susceptible to X-ray, the radiation was reduced to 4 Gy for *Kit*<sup>W/+</sup> and *Kit*<sup>V620A tg1/+</sup> mice. Because the application of 4 Gy radiation to *Kit*-mutant mice and 5 Gy radiation to WT mice resulted in almost equal levels of L\* after hair regeneration, the effect of HGK could be assessed. After 6 hours of exposure, tiny skin tissue portions were dissected and fixed by overnight immersion in 10% formalin. The incision was carefully stitched. The detailed methods for X-ray radiation, dissection, and immunohistochemical analysis have been described previously (Aoki et al., 2013, 2011; Taguchi et al., 2020b). Briefly, a Histofine Kit (Nichirei Biosciences, Tokyo, Japan) was used to detect the antiphosphorylated histone H2AX using rabbit antibodies (1:500; Cell Signaling Technology, Danvers, MA) and antimouse CD34, a marker for HFKSC (1:500; eBioscience, San Diego, CA). The secondary antibodies goat anti-rat IgG-FITC (Santa Cruz Biotechnology, Dallas, TX) and donkey anti-rabbit IgG-Alexa Fluor 594 (Invitrogen, Carlsbad, CA) were used at a 1:1,000 dilution for 60 minutes at room temperature. At least 10 hair follicles were inspected for 10 mice in each immunohistochemical analysis. Hair lightening was assessed on hair shaved 1 month after irradiation (day 38) and quantified using the L\*a\*b\* system. All shaved hairs were well mixed and measured 10 times through color spectroscopy (CM-700d, Konica Minolta, Tokyo, Japan), and the average L\* value is shown as the result (Taguchi et al., 2020b). The L\* value indicates hair brightness from 0 (black) to 100 (white).

**Repeated hair plucking–induced hair graying method.** The experimental scheme is shown in Figure 4a. Mice aged 7 weeks were plucked once per month, and the flavonoid solution (0.1% w/v, 200  $\mu$ l) was applied to the plucking area once daily. Photos were acquired before hair plucking. The plucked hairs ( $\geq 300$  hairs) from each mouse were arranged on a slide and randomly counted under a microscope (Endou et al., 2014).

## Statistical analysis

Statistics were calculated using GraphPad Prism 8 (GraphPad Software, La Jolla, CA). After confirming the normality of the data using Shapiro–Wilk test and variance equivalency using *F* test, group means were compared using Student's *t*-test, Welch's *t*-test, or Mann–Whitney *U* test. For multiple comparisons, one-way ANOVA with Bonferroni posthoc test was used. Data are shown as the mean  $\pm$  SD. \**P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001 were considered as statistically significant, and n.s. means not significant.

## Data availability statement

No datasets were generated or analyzed during this study.

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Conceptualization: NT; Data Curation: NT; Formal Analysis: NT, RK, TA, TN; Funding Acquisition: NT, HA, TK; Investigation: NT, RK, TA, TN; Methodology: HA; Project Administration: NT, HA; Resources: NT, HA, TK; Supervision: TK; Validation: NT, RK, TA, TN; Visualization: NT, RK, TA; Writing - Original Draft Preparation: NT, TK

## CONFLICT OF INTEREST

NT and TN are employees of Hoyu. The remaining authors state no conflict of interest.

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## REFERENCES

- Anderson ZT, Palmer JW, Idris MI, Villavicencio KM, Le G, Cowart J, et al. Topical RT1640 treatment effectively reverses gray hair and stem cell loss in a mouse model of radiation-induced canities [published correction appears in *Pigment Cell Melanoma Res* 2021;34:1141] *Pigment Cell Melanoma Res* 2021;34:89–100.
- Aoki H, Hara A, Motohashi T, Kunisada T. Protective effect of Kit signaling for melanocyte stem cells against radiation-induced genotoxic stress. *J Invest Dermatol* 2011;131:1906–15.
- Aoki H, Hara A, Motohashi T, Kunisada T. Keratinocyte stem cells but not melanocyte stem cells are the primary target for radiation-induced hair graying. *J Invest Dermatol* 2013;133:2143–51.
- Damodaran RG, Gupta R. Hair loss and the applied techniques for identification of novel hair growth promoters for hair re-growth. *Pharmacogn J* 2011;3:1–5.
- Endou M, Aoki H, Kobayashi T, Kunisada T. Prevention of hair graying by factors that promote the growth and differentiation of melanocytes. *J Dermatol* 2014;41:716–23.
- Hartwig S, Auwärter V, Pragst F. Fatty acid ethyl esters in scalp, pubic, axillary, beard and body hair as markers for alcohol misuse. *Alcohol Alcohol* 2003;38:163–7.
- Iida M, Tazaki A, Yajima I, Ohgami N, Taguchi N, Goto Y, et al. Hair graying with aging in mice carrying oncogenic RET. *Aging Cell* 2020;19:e13273.
- Inomata K, Aoto T, Binh NT, Okamoto N, Tanimura S, Wakayama T, et al. Genotoxic stress abrogates renewal of melanocyte stem cells by triggering their differentiation. *Cell* 2009;137:1088–99.
- Jeon S, Kim NH, Koo BS, Kim JY, Lee AY. Lotus (*Nelumbo nuficera*) flower essential oil increased melanogenesis in normal human melanocytes. *Exp Mol Med* 2009;41:517–25.
- Jo SK, Lee JY, Lee Y, Kim CD, Lee JH, Lee YH. Three streams for the mechanism of hair graying. *Ann Dermatol* 2018;30:397–401.
- Kujoth GC, Hiona A, Pugh TD, Someya S, Panzer K, Wohlgemuth SE, et al. Mitochondrial DNA mutations, oxidative stress, and apoptosis in mammalian aging. *Science* 2005;309:481–4.
- Kunisada T, Yoshida H, Yamazaki H, Miyamoto A, Hemmi H, Nishimura E, et al. Transgene expression of steel factor in the basal layer of epidermis promotes survival, proliferation, differentiation and migration of melanocyte precursors. *Development* 1998;125:2915–23.
- Lennartsson J, Rönstrand L. Stem cell factor receptor/c-Kit: from basic science to clinical implications. *Physiol Rev* 2012;92:1619–49.
- Li H, Hou L. Regulation of melanocyte stem cell behavior by the niche microenvironment. *Pigment Cell Melanoma Res* 2018;31:556–69.
- Liu-Smith F, Meyskens FL. Molecular mechanisms of flavonoids in melanin synthesis and the potential for the prevention and treatment of melanoma. *Mol Nutr Food Res* 2016;60:1264–74.
- Pandhi D, Khanna D. Premature graying of hair. *Indian J Dermatol Venereol Leprol* 2013;79:641–53.

- Panhard S, Lozano I, Loussouarn G. Greying of the human hair: a world-wide survey, revisiting the '50' rule of thumb. *Br J Dermatol* 2012;167:865–73.
- Taguchi N, Hata T, Kamiya E, Homma T, Kobayashi A, Aoki H, et al. *Eriodictyon angustifolium* extract, but not *Eriodictyon californicum* extract, reduces human hair greying. *Int J Cosmet Sci* 2020a;42:336–45.
- Taguchi N, Hata T, Kamiya E, Kobayashi A, Aoki H, Kunisada T. Reduction in human hair graying by sterubin, an active flavonoid of *Eriodictyon angustifolium*. *J Dermatol Sci* 2018;92:286–9.
- Taguchi N, Homma T, Aoki H, Kunisada T. Dietary *Eriodictyon angustifolium* tea supports prevention of hair graying by reducing DNA damage in CD34+ hair follicular keratinocyte stem cells. *Biol Pharm Bull* 2020b;43:1451–4.
- Takekoshi S, Nagata H, Kitatani K. Flavonoids enhance melanogenesis in human melanoma cells. *Tokai J Exp Clin Med* 2014;39:116–21.
- Tanimura S, Tadokoro Y, Inomata K, Binh NT, Nishie W, Yamazaki S, et al. Hair follicle stem cells provide a functional niche for melanocyte stem cells. *Cell Stem Cell* 2011;8:177–87.
- Tosaki H, Kunisada T, Motohashi T, Aoki H, Yoshida H, Kitajima Y. Mice transgenic for Kit(V620A): recapitulation of piebaldism but not progressive depigmentation seen in humans with this mutation. *J Invest Dermatol* 2006;126:1111–8.
- Tremplus CS, Morris RJ, Bortner CD, Cotsarelis G, Faircloth RS, Reece JM, et al. Enrichment for living murine keratinocytes from the hair follicle bulge with the cell surface marker CD34. *J Invest Dermatol* 2003;120:501–11.
- Yale K, Juhasz M, Atanaskova Mesinkovska N. Medication-induced repigmentation of gray hair: a systematic review. *Skin Appendage Disord* 2020;6:1–10.
- Zhu W, Xu J, Ge Y, Cao H, Ge X, Luo J, et al. Epigallocatechin-3-gallate (EGCG) protects skin cells from ionizing radiation via heme oxygenase-1 (HO-1) overexpression. *J Radiat Res* 2014;55:1056–65.



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