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Original Article

Gremlin 2 suppresses differentiation of stem/progenitor cells in the human skin

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ABSTRACT

Introduction: The skin is comprised of various kinds of cells and has three layers, the epidermis, dermis and subcutaneous adipose tissue. Stem cells in each tissue duplicate themselves and differentiate to supply new cells that function in the tissue, and thereby maintain the tissue homeostasis. In contrast, senescent cells accumulate with age and secrete senescence-associated secretory phenotype (SASP) factors that impair surrounding cells and tissues, which lowers the capacity to maintain homeostasis in each tissue. Previously, we found Gremlin 2 (GREM2) as a novel SASP factor in the skin and reported that GREM2 suppressed the differentiation of adipose-derived stromal/stem cells. In the present study, we investigated the effects of GREM2 on stem cells in the epidermis and dermis.

Methods: To examine whether GREM2 expression and the differentiation levels in the epidermis and dermis are correlated, the expressions of GREM2, stem cell markers, an epidermal differentiation marker Keratin 10 (KRT10) and a dermal differentiation marker type 3 procollagen were examined in the skin samples (n = 14) randomly chosen from the elderly where GREM2 expression level is high and the individual differences of its expression are prominent. Next, to test whether GREM2 affects the differentiation of skin stem cells, cells from two established lines (an epidermal and a dermal stem/progenitor cell model) were cultured and induced to differentiate, and recombinant GREM2 protein was added.

Results: In the human skin, the expression levels of GREM2 varied among individuals both in the epidermis and dermis. The expression level of GREM2 was not correlated with the number of stem cells, but negatively correlated with those of both an epidermal and a dermal differentiation markers. The expression levels of epidermal differentiation markers were significantly suppressed by the addition of GREM2 in the three-dimensional (3D) epidermis generated with an epidermal stem/progenitor cell model. In addition, by differentiation induction, the expressions of dermal differentiation markers were induced in cells from a dermal stem/progenitor cell model, and the addition of GREM2 significantly suppressed the expressions of the dermal differentiation markers.

Conclusions: GREM2 expression level did not affect the numbers of stem cells in the epidermis and dermis but affects the differentiation and maturation levels of the tissues, and GREM2 suppressed the differentiation of stem/progenitor cells *in vitro*. These findings suggest that GREM2 may contribute to the age-related reduction in the capacity to maintain skin homeostasis by suppressing the differentiation of epidermal and dermal stem/progenitor cells.

Abbreviations: IL, Interleukin; TGF-β, Transforming growth factor beta; PFA, Paraformaldehyde phosphate buffer solution; KRT10, Keratin 10; KRT14, Keratin 14; ITGB1, Integrin beta 1; DAPI, 4′,6-diamidino-2-phenylindole.

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1. Introduction

The skin is a complicated organ comprised of various kinds of cells and has three layers, the epidermis, dermis and subcutaneous adipose tissue. The skin covers the whole body as a barrier and plays important roles in protection from environmental factors including physical and chemical stimuli as well as ultraviolet rays. Recent studies have revealed that the capacity of maintaining skin homeostasis is greatly attributed to stem cells. There are stem cells in the epidermis, dermis, subcutaneous adipose tissue and the bulge region of hair follicles, and stem cells in each tissue duplicate and differentiate to supply new cells that function to maintain or repair damaged or injured tissues [1–3].

In contrast, as age increases, the skin changes; for example, reduced function of each tissue, increased susceptibility to external stimuli and decreased capacity to maintain homeostasis are observed with age [4,5]. These changes may be attributed to senescent cells accumulated in aged skin [6–8]. Senescent cells secrete senescence-associated secretory phenotype (SASP) factors, such as proinflammatory cytokines, chemokines and matrix metalloproteinases (MMPs), and damage surrounding tissues, leading to a reduced function of the whole tissue [9–17]. Taken together, when the damaging effect of SASP factors on tissues surpasses the capacity of tissue regeneration driven by stem cells, aging features and functional reductions of tissues may appear. In addition, SASP factors may also directly affect stem cells, but the direct effect of SASP factors on stem cells remains unknown.

We have investigated SASP factors and stem cells in the skin. Previously, we observed an increase of Gremlin 2 (GREM2) expression in senescence-induced keratinocytes and fibroblasts, in addition to an increase of major SASP factors in the skin, IL1B, IL6, MMP1 and MMP2. Therefore, we reported GREM2 as a novel SASP factor in the skin [18]. GREM2 is a DAN family antagonist of bone morphogenetic protein (BMP) of the TGF-β family, and the activity of BMP-responsive promoter is blocked by the binding of GREM2 to BMP-2 or BMP-4 [19–21]. In the skin, the involvement of BMP signaling in differentiation has been reported. BMP-2 and BMP-6 inhibit the proliferation of primary human interfollicular epidermal keratinocytes and induce their terminal differentiation [22]. The activation of BMP signaling during epidermal differentiation was also reported in human epidermal tissues [23]. BMP-4 promotes myocyte differentiation from human fetal fibroblasts [24]. These findings suggest that the blockage of BMP-responsive promoter affects the differentiation of keratinocytes and fibroblasts. In addition, GREM2 promotes the proliferation of myocardial progenitor cells and the differentiation of stem cells into myocytes. GREM2 is also known to suppress the differentiation of bone marrow-derived mesenchymal stem cells into osteoblasts [25–28]. These findings indicate that GREM2 has a great influence on the proliferation and differentiation of stem cells. In our previous study, we focused on GREM2 as a SASP factor in the skin and found that GREM2 suppressed the differentiation of stem cells in the subcutaneous adipose tissue [29]. However, whether GREM2 is expressed in the epidermis and dermis as well as its effect on epidermal and dermal stem cells were unclear. Here, we investigated the effect of GREM2 on these stem cells and tissues in the epidermis and dermis.

2. Materials and methods

2.1. Collection of human skin tissue

This study was conducted after approved by the ethics committee of Fujita Health University School of Medicine (approval number: HM17-223). Human skin tissues were obtained from patients who had skin surgery. Prior to surgery, informed consent was obtained from each patient. Normal skin tissues were trimmed out from surgically excised tissues, fixed with 4% PFA and embedded in paraffin. The skin samples used in this study were randomly chosen from skin tissues from the chest or abdomen of the elderly, and the information of the samples is shown in Table 1 (n = 14; 65–89 years of age, average 80.9 years). Our previous studies showed that GREM2 expression increased with age. Especially in subjects aged over 60 years, GREM2 expression level was higher and the individual differences of GREM2 expression level were significantly larger than in the young [18,24]. Therefore, to examine the effect of GREM2, the skin tissues from the elderly where GREM2 expression levels largely differ among individuals were used.

2.2. Immunohistochemistry and image analysis

Paraffin-embedded skin tissues were sectioned at 4 μm. Sectioned samples were deparaffinized and treated for antigen activation. After blocking with 3% BSA, 1% Triton-X in PBS at room temperature for 1 h, immunohistochemistry was performed in a conventional way against GREM2 (GeneTex, CA, USA), Integrin beta 1 (Abcam, Cambridge, UK), p75 NGF receptor/CD271 (ORIGENE, MD, USA), Endoglin/CD105 (Santa Cruz Biotechnology, TX, USA), Keratin 10 (Neomaekers, CA, USA), Keratin 14 (BioLegend, CA, USA) and Pro-COL3A1 (Santa Cruz Biotechnology) in the conditions shown in Table S1. Immunohistological images of three randomly chosen areas for each observation were captured with a fluorescence microscope (DMI 6000B-AFC, LAS X ver.1. x; Leica Microsystems, Wetzlar, DEU) at 200-times magnification. Conditions including exposure time and “gain” were kept consistent. Fluorescence intensities of the signals were analyzed using an image analysis software MetaMorph (Molecular Devices, CA, USA). For the expression levels of GREM2, KRT10 and KRT14, the average fluorescence intensities of all epidermal layers were measured. For the expression analyses in the dermis, the papillary layer (150 μm depth from the basal layer) was observed. The average fluorescence intensities of GREM2 in the areas surrounding DAPI signals where cell nuclei existed and Pro-COL3 in the entire target area were determined. For the analysis of stem cells in the epidermis and dermis, the basal cells and the papillary dermis were observed, respectively. The total cell numbers and the numbers of ITGB1, CD271 or CD105-positive cells in the observation areas were counted to determine the percentages of ITGB1, CD271 or CD105-positive cells (positive cells/total number of cells). For each

Table 1
List of human skin samples.

No.	Age	Sex	Body part
1	65	Male	Chest
2	67	Female	Abdomen
3	73	Male	Chest
4	75	Male	Chest
5	79	Male	Chest
6	83	Female	Abdomen
7	84	Male	Abdomen
8	85	Female	Abdomen
9	86	Male	Abdomen
10	86	Male	Chest
11	86	Female	Abdomen
12	87	Male	Abdomen
13	88	Male	Abdomen
14	89	Male	Abdomen

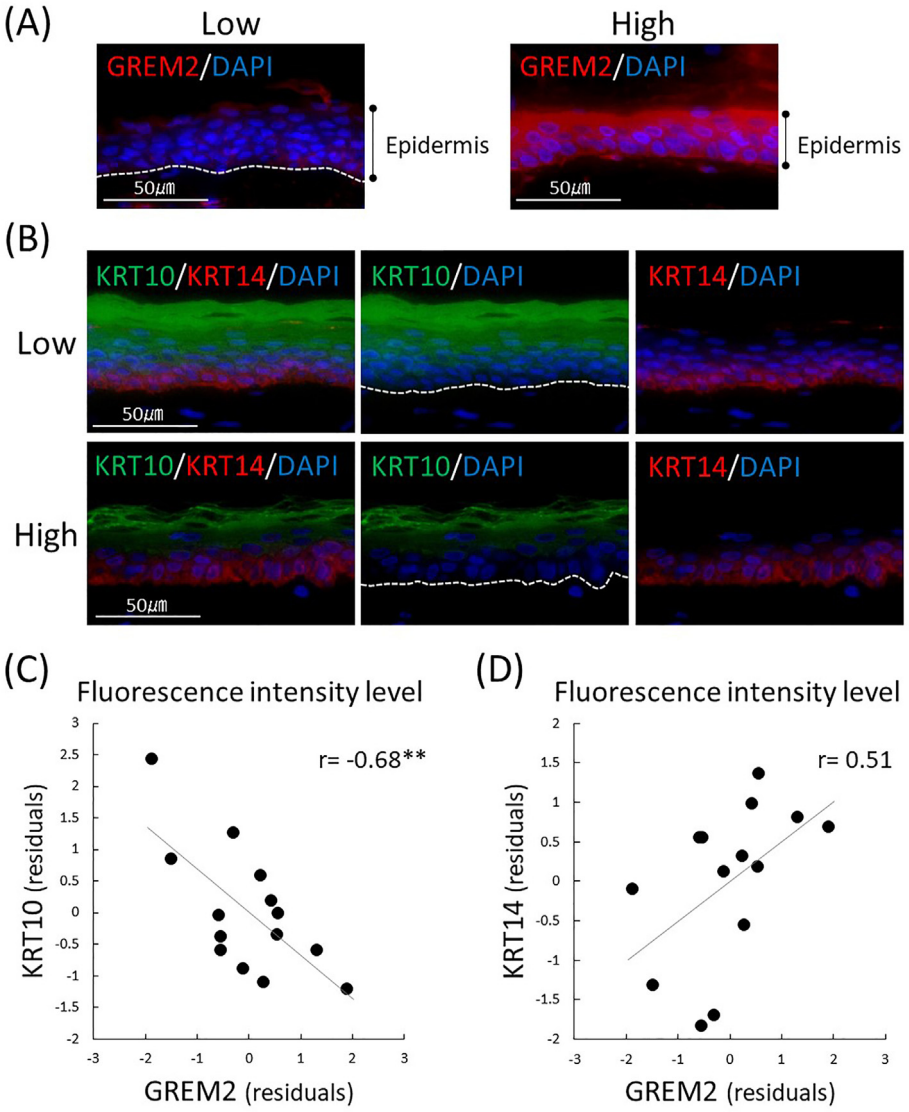

Fig. 1. The expressions of GREM2 and differentiation markers in the epidermis. To examine GREM2 expression in the epidermis and epidermal differentiation, immunohistochemistry against GREM2 and epidermal differentiation markers, Keratin 10 (KRT10) and Keratin 14 (KRT14), was performed (n = 14; 65–89 years of age, average 80.9 years of age). (A) Representative images of a low and a high GREM2 expression in the epidermis. The skin samples were from the donors No. 6 (Low, 83 years of age) and No. 4 (High, 75 years of age) in Table 1. Red, GREM2; blue, nuclei. (B) Double staining with KRT10 and KRT14 in the epidermis of the same samples as (A). Red, KRT14; green, KRT10; blue nuclei. (C, D) Fluorescence intensities of GREM2, KRT10 and KRT14 per unit area in the epidermis were measured, and the average value of each of the three proteins for each donor was calculated. The average values for the donor No. 1 (Table 1, 65 years of age) were set as 1, and the relative values of (other) donors were calculated. Then the residuals of the relative values against age were plotted. (C) Partial correlation analysis revealed that the expression level of KRT10 was negatively correlated with that of GREM2 (*$p < 0.05$). (D) Partial correlation analysis did not reveal any significant correlation between the expression levels of GREM2 and KRT14.

sample, the average of three areas observed was calculated, and the reproducibility was examined with other sections of the sample.

2.3. Cell culture

Human keratinocytes (HDK1), a cell line established by Egawa et al. [30], were used as an epidermal stem/progenitor cell model. HDK1 cells have been reported to be epidermal stem cells with a potential to form three-dimensional (3D) reconstructed epidermis in culture and positive for Integrin beta-1 (ITGB1) and Integrin alpha-6 (ITGA6) that are both known as epidermal stem cell markers [31,32]. SF8428 line established by Ban S et al. was used as a dermal stem/progenitor cell model [33,34]. SF8428 has been reported to be mesenchymal stem cell-like fibroblasts with multiple differentiation potentials [35]. We showed that SF8428 expressed mesenchymal stem cell markers, CD105 and CD44 (Fig. S1). HDK1 and SF8428 were cultured in Keratinocyte-SFM (KSFM; Invitrogen, CA, USA) and in DMEM supplemented with 10% fetal bovine serum (FBS; Sigma–Aldrich, MO, USA), respectively.

2.4. The effect of GREM2 on skin stem cell differentiation

The effects of GREM2 on the differentiation of an epidermal stem/progenitor cell model HDK1 and a dermal stem/progenitor cell model SF8428 were examined. HDK1 cells were induced to differentiate and generate 3D epidermis as described in Inoue et al. [31]. To examine the effect of GREM2 on the differentiation of HDK1 cells, human recombinant GREM2 Protein (NOVUS Biologicals, CO,

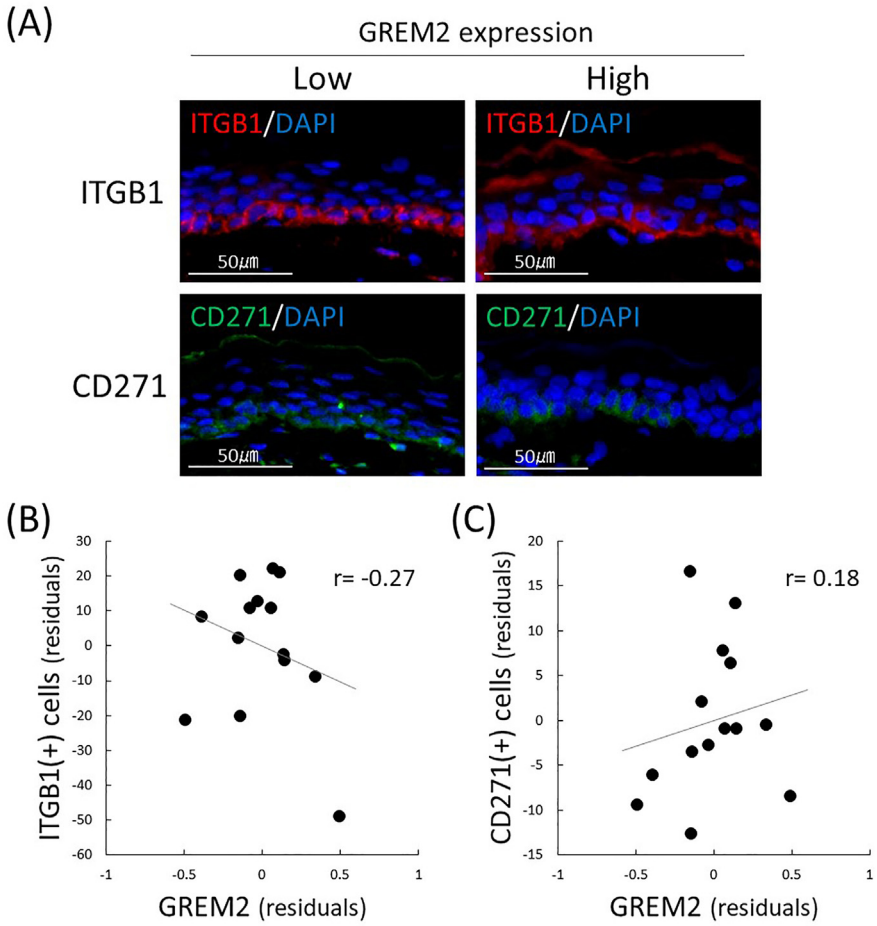

Fig. 2. The expressions of stem cell markers in the epidermis with different levels of GREM2. Immunostaining against epidermal stem cell markers, ITGB1 and CD271, was performed. (A) Immunohistological images against ITGB1 (red in the upper panels) and CD271 (green in the lower panels) of representative samples with a low and a high GREM2 expression (Low, No. 6, 83 years of age; High, No. 4, 75 years of age), which were from the same samples shown in Fig. 1. Blue, nuclei. (B) The percentage of ITGB1-positive cells per basal cells was calculated. The residuals of GREM2 expression level and the ratio of ITGB1-positive cells (%) against age were plotted. Partial correlation analysis revealed no significant correlation. (C) The percentage of CD271-positive cells per basal cells was calculated. The residuals of GREM2 expression level and the ratio of CD271-positive cells (%) against age were plotted. Partial correlation analysis revealed no significant correlation.

USA) was added to the epidermal differentiation medium at final concentrations of 12.5 and 25 ng/mL, and cells were cultured for seven days. Then the gene expressions were analyzed. Sections of 3D reconstructed epidermis were prepared, and HE staining and immunohistochemistry against KRT14 and KRT10 were performed in a conventional way to observe histological changes and examine the levels of gene expressions.

For the differentiation induction of SF8428 cells, TGF-β1 (PeproTech, NJ, USA) was added to the medium at 5 ng/mL as described in Bettinger et al. [36]. To examine the effect of GREM2 on the differentiation of SF8428, human recombinant GREM2 Protein (NOVUS Biologicals) was added at final concentrations of 12.5 and 25 ng/mL to the medium, and cells were cultured for 24 h. Then the gene expressions were analyzed. In addition, cells were fixed in 4% PFA four days after the differentiation induction, and immunohistochemistry against COL1A1 (LB-1190; LSL, Tokyo, JPN) or COL3A1 (LB-1300, LSL) was performed in a conventional way. The expression levels of these proteins were analyzed to evaluate the differentiation levels.

2.5. Gene expression analysis

To examine the effects of GREM2 on the differentiation of epidermal and dermal stem cells, the expression levels of differentiation marker genes were analyzed. Total RNA was purified from cells with RNAiso Plus (Takara Bio, Shiga, JPN) in a conventional way. One μg of RNA was reverse transcribed into cDNA with PrimeScript TM RT Master Mix (Takara Bio). Quantitative real-time PCR was performed using TB Green® Premix Ex Taq™ II (Takara Bio) with a StepOnePlus (Life Technologies Japan, Tokyo, JPN). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control for normalization, and the relative differences in values among samples were analyzed with a $\Delta\Delta Ct$ method. The primer sequences (*IVL*, *KRT10*, *COL1A1, COL3A1* and *GAPDH*) were listed in Table S2.

2.6. Statistical analysis

All statistical analyses were performed by R program (version 3.1.1, R Development Core Team 2012). Partial correlation analysis was performed using donor age as a control variable to examine relationships between fluorescence intensities of immunohistological images. Bar graphs represent the mean values, and error bars represent standard deviation. Correlation analysis was performed by Pearson's product moment analysis for normally distributed data. Statistical significances were tested employing Student's *t*-test or Tukey's multiple comparison test. A p value of <0.05 was considered statistically significant.

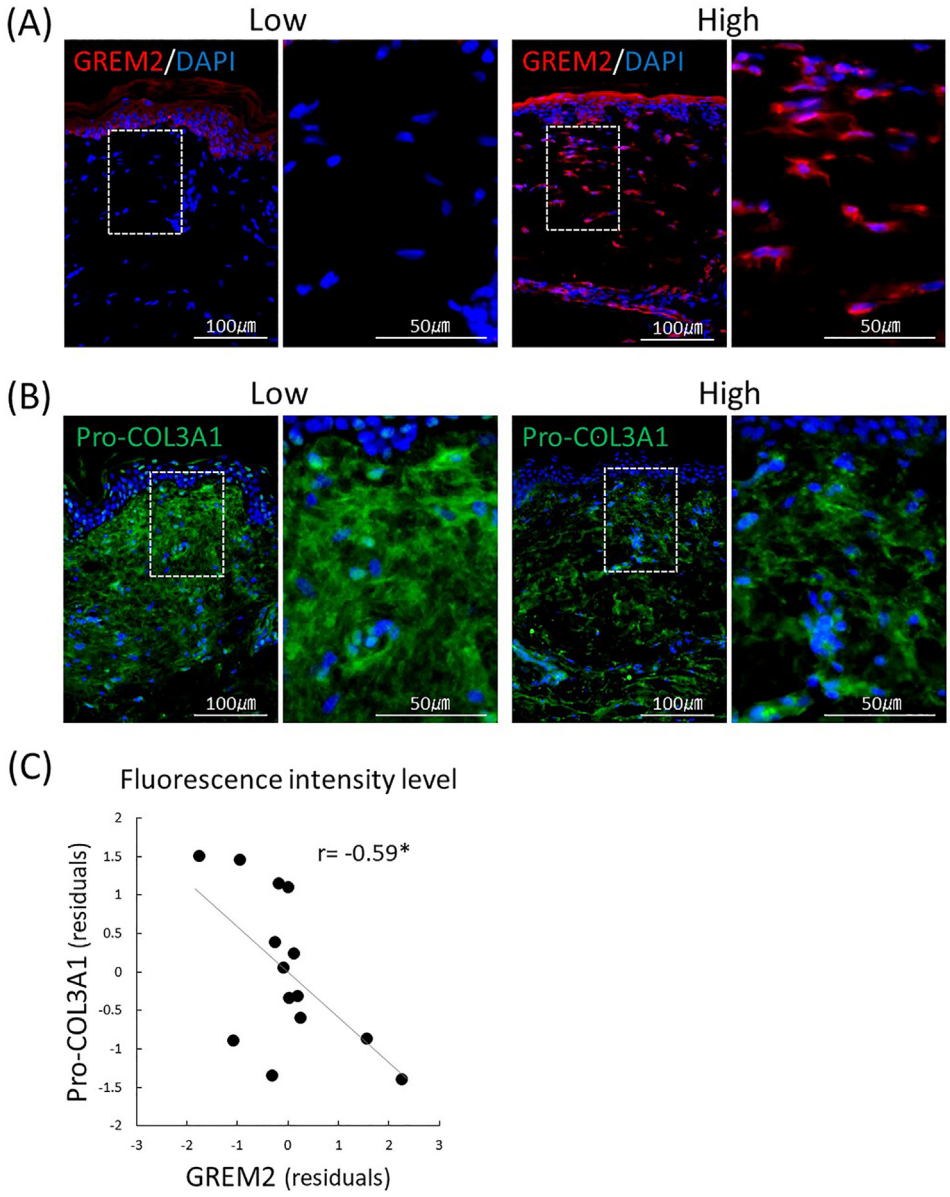

Fig. 3. The expressions of GREM2 and type 3 procollagen in the dermis. To examine GREM2 expression and dermal differentiation, immunohistochemistry against GREM2 and a dermal differentiation marker, type 3 procollagen (Pro-COL3A1), was performed in the dermis (n = 14; 65–89 years of age, average 80.9 years of age). (A) Representative images of a low and a high GREM2 expression in the dermis. The boxed areas in the left panels are magnified and shown in the right. The samples were from the donors No. 9 (Low, 86 years of age) and No. 11 (High, 86 years of age) in Table 1. Red, GREM2; blue, nuclei. (B) Immunohistochemistry against type 3 procollagen (green) in the dermis of the same samples as (A). Blue, nuclei. (C) Fluorescence intensities of GREM2 and type 3 procollagen per unit area in the dermal papillary layer were measured, and the average value of each of the two proteins for each donor was calculated. The average values for the donor No. 1 (Table 1 and 65 years of age) were set as 1, and the relative values of (other) donors were calculated. Then the residuals of the relative values against age were plotted. Partial correlation analysis revealed that the expression level of type 3 procollagen was negatively correlated with that of GREM2 (*$p < 0.05$).

3. Results

3.1. GREM2 suppressed Keratin10 (KRT10) expression in the epidermis

In the epidermis, epidermal stem cells in the basal layer provide new cells. Newly produced cells gradually differentiate and mature into keratinocytes, move toward upper layers and comprise the epidermal tissue [37]. To examine whether GREM2 expression and the differentiation level in the epidermis are correlated, immunohistochemistry was performed against GREM2, an epidermal undifferentiation marker KRT14 and an epidermal differentiation marker KRT10 [38,39] on human skin sections (n = 14), and expression levels were analyzed. Since our previous study showed that GREM2 expression levels prominently varied among elderly individuals [18], we examined skin samples from the elderly (65–89 years of age). GREM2 expression levels in the epidermis were found to vary among individuals (Fig. 1A). In skin samples showing high GREM2 expression, the epidermis was thinner and KRT10 expression was obviously lower than in samples with low GREM2 expression (Fig. 1B). To examine the correlation between the expression levels of GREM2 and KRT10 or KRT14, partial correlation analysis controlled for age was performed. We found that the expression level of KRT10 was negatively correlated with that of

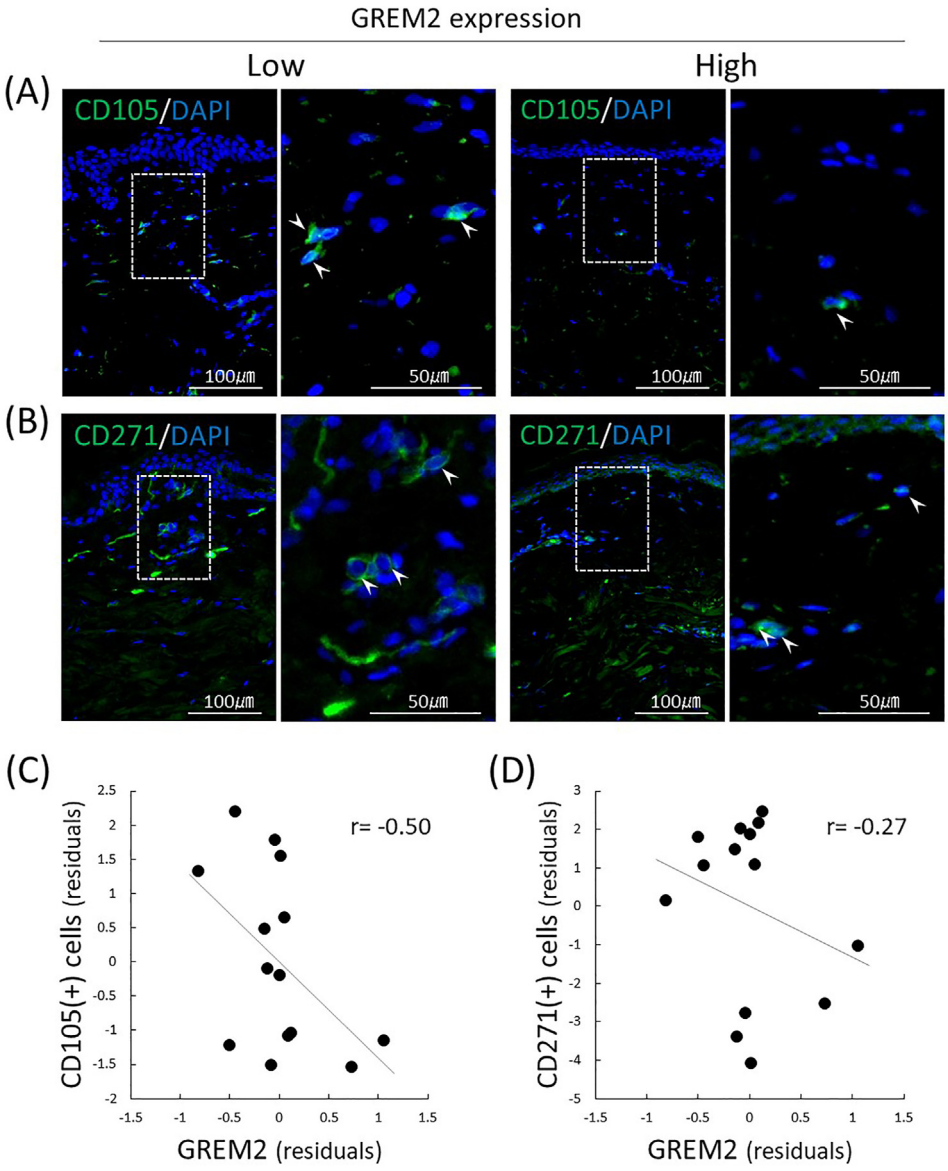

Fig. 4. The expressions of stem cell markers in the dermis with different levels of GREM2. Immunostaining against dermal stem cell markers, CD105 and CD271, was performed. (A and B) Immunohistological images against CD105 (green in A) and CD271 (green in B) of representative samples with a low and a high GREM2 expression (Low, No. 9, 86 years of age; High, No. 11, 86 years of age), which were from the same samples shown in Fig. 3. Blue, nuclei. The arrowheads indicate positive cells for CD105 in A or CD271 in B. (C) The percentage of CD105-positive cells per total number of cells in the dermal papillary layer was calculated. The residuals of GREM2 expression level and the ratio of CD105-positive cells (%) against age were plotted. Partial correlation analysis revealed no significant correlation. (D) The percentage of CD271-positive cells per total number of cells in the dermal papillary layer was calculated. The residuals of GREM2 expression level and the ratio of CD271-positive cells (%) against age were plotted. Partial correlation analysis revealed no significant correlation.

GREM2 (Fig. 1C), but no significant correlation was observed between GREM2 and KRT14 expression levels (Fig. 1D). These results suggest that GREM2 negatively affects the differentiation and maturation of the epidermis.

Next, we examined whether GREM2 expression in the epidermis affected the number of stem/progenitor cells. The tissue sections from the same samples used for Fig. 1 were immunostained with epidermal stem/progenitor cell markers ITGB1 [40,41] and CD271 [42–45], and the fluorescent signals were analyzed (Fig. 2A). No significant correlation was observed between GREM2 expression level and the number of either ITGB1- or CD271-positive stem/progenitor cells (Fig. 2B and C), suggesting that GREM2 in the epidermis does not affect the proliferation of stem/progenitor cells in the basal layer.

3.2. GREM2 suppressed type 3 procollagen expression in the dermis

In the dermis, dermal stem cells provide new cells and differentiate into fibroblasts, and during the differentiation and maturation process, fibroblasts produce distinct types of collagen, which maintains the homeostasis of the dermis [2,46–48]. Type 3 procollagen is expressed at an early differentiation stage of fibroblasts [49–51] and used as a marker to indicate the differentiation level in the present study. To examine GREM2 expression and the differentiation level in the dermis, we performed immunohistochemistry against GREM2 and type 3 procollagen on human skin samples ($n = 14$) and observed the dermal papillary layer, where dermal stem cells are localized [45,52]. Similar to in the epidermis, GREM2 expression levels in the dermis also varied among individuals

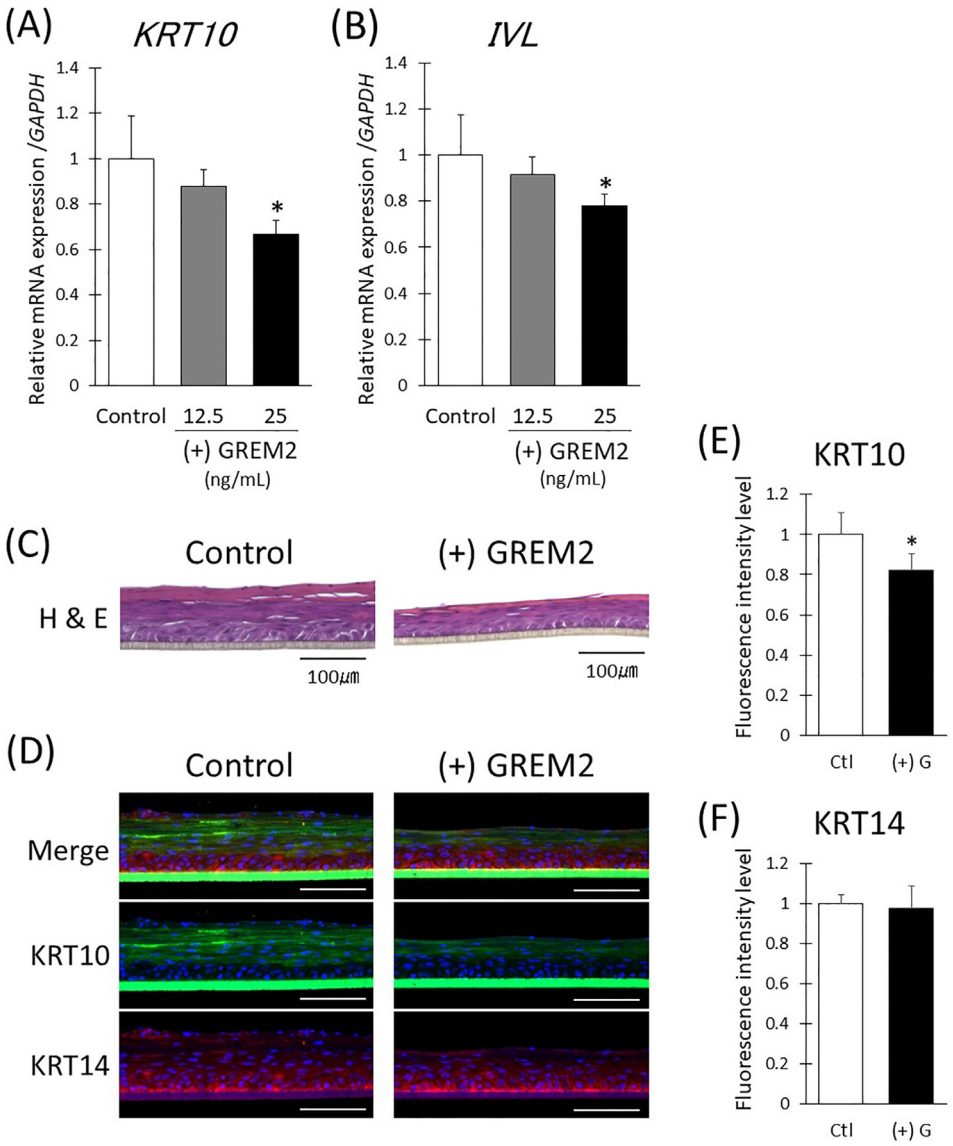

Fig. 5. The effect of GREM2 on the differentiation of HDK1cells. HDK1 cells were cultured for seven days to generate 3D epidermis in the differentiation medium with GREM2. (A, B) mRNA was collected from 3D reconstructed epidermis, and the gene expression levels of differentiation markers, *Keratin 10* (*KRT10* in A) and *Involucrin* (*IVL* in B), were examined. The expressions of both genes were suppressed by GREM2 in a dose-dependent manner and a significant suppression was observed when GREM2 was added at 25 ng/ml (n = 3, mean ± SD, *$p < 0.05$). (C–F) Sections of 3D reconstructed epidermis (cultured with GREM2 at 25 ng/mL or without GREM2) were prepared, and HE staining (C) and immunohistochemistry against KRT10 and KRT14 (D) were performed. The 3D reconstructed epidermis cultured with GREM2 was thinner than GREM2-negative control. Fluorescence intensities of KRT10 (E) and KRT14 (F) in immunohistochemical images were measured and compared between when cultured with and without GREM2. The expression of KRT10, a differentiation marker, was significantly lower in the 3D epidermis cultured with GREM2 (n = 3, mean ± SD, *$p < 0.05$). The addition of GREM2 did not change the expression level of KRT14, an undifferentiation marker, in the 3D reconstructed epidermis, especially in the lower layers.

(Fig. 3A). To examine the correlation between the expression levels of GREM2 and type 3 procollagen, partial correlation analysis controlled for age was performed, and we found that the expression level of type 3 procollagen was negatively correlated with that of GREM2 (Fig. 3B and C). These results suggest that GREM2 negatively affects the differentiation and maturation of the dermis.

We next examined whether GREM2 expression level in the dermis affect the number of stem/progenitor cells. Using the sections from the same samples used for Fig. 3, immunohistochemistry was performed against dermal stem/progenitor cell markers, CD105 [53–55] and CD271 [42,43,56,57] and the fluorescent signals were analyzed (Fig. 4A and B). No significant correlation was observed between GREM2 expression level and the number of either CD105-or CD271-positive stem/progenitor cells (Fig. 4C and D). This finding supports that GREM2 in the dermis does not affect the proliferation of stem/progenitor cells.

3.3. *GREM2 suppressed epidermal stem/progenitor cell differentiation*

GREM2 may negatively affect epidermal differentiation (Fig. 1), and therefore, we examined the effect of GREM2 on the differentiation of epidermal stem/progenitor cells *in vitro*. As an epidermal stem/progenitor cell model, HDK1 cells were cultured with GREM2 to generate 3D epidermis, and the gene expression levels of epidermal differentiation markers were analyzed. Human recombinant GREM2 protein was added at a concentration of 12.5 or

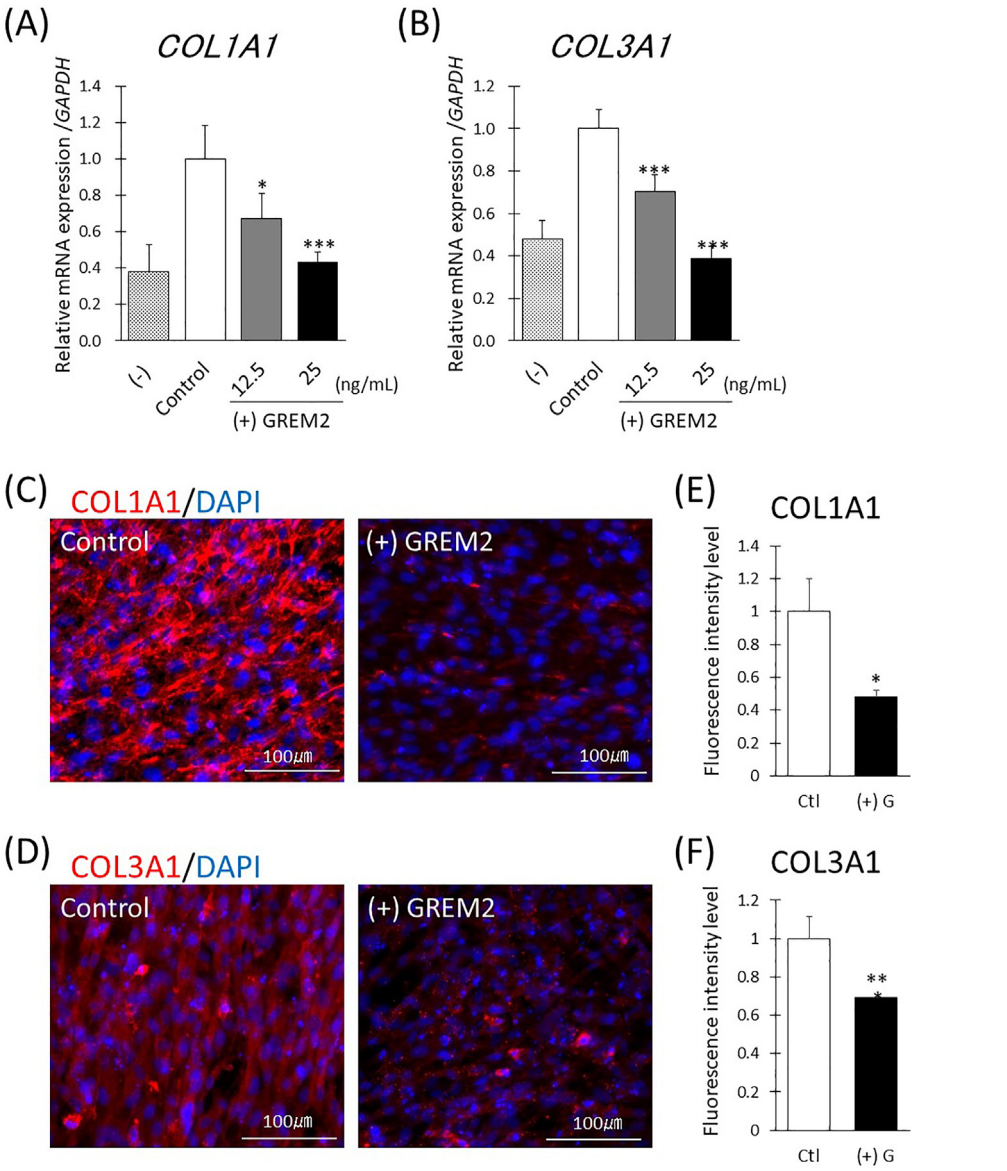

Fig. 6. The effect of GREM2 on the differentiation of SF8428 cells. SF8428 cells were cultured in the differentiation medium with GREM2 for 24 h. (A, B) mRNA was collected from cells, and the gene expressions of fibrogenesis markers, *type 1 collagen* (*COL1A1* in A) and *type 3 collagen* (*COL3A1* in B), were compared. The expression levels of both genes were significantly suppressed by GREM2 in a dose-dependent manner (n = 3, mean ± SD, ***$p < 0.001$, *$p < 0.05$). (C–F) SF8428 cells were cultured in the differentiation medium with GREM2 for four days to examine its effect on collagen fiber formation. Then cells were fixed with 4% PFA, and immunohistochemistry against COL1A1 (red in C) and COL3A1 (red in D) was performed. Blue, nuclei. In the cells cultured with GREM2, collagen fiber formation and the expressions of collagen genes were suppressed. Fluorescence intensities of COL1A1 (E) and COL3A1 (F) in immunohistochemical images were measured and compared between when cultured with and without GREM2. The expressions of both proteins were significantly suppressed when GREM2 was added (n = 3, mean ± SD, **$p < 0.01$, *$p < 0.05$).

25 ng/mL to the culture medium to generate 3D epidermis. We found that after seven-day culture with GREM2, the gene expressions of epidermal differentiation markers, *Keratin10* (*KRT10*) and *Involucrin* (*IVL*), were suppressed in a dose-dependent manner (Fig. 5A and B). In addition, histological analysis revealed that 3D epidermis cultured with GREM2 had an obviously thinner granular layer than those cultured with GREM2-negative control (Fig. 5C). We also performed immunohistochemistry against KRT10 and KRT14 to compare the expression levels in 3D epidermis cultured with or without GREM2. There was no difference in KRT14 expression, whereas KRT10 expression was significantly lower in the 3D epidermis cultured with GREM2 (Fig. 5D–F). These results suggest that GREM2 may suppress the differentiation of epidermal stem/progenitor cells.

3.4. GREM2 suppressed dermal stem/progenitor cell differentiation

Since GREM2 may negatively affect dermal differentiation (Fig. 3), we next examined the effect of GREM2 on the differentiation of dermal stem/progenitor cells *in vitro*. SF8428 cells were used as a dermal stem/progenitor cell model, and differentiation was induced with TGF-β1. To evaluate the differentiation levels when cells were cultured with or without GREM2, we examined the gene expression levels of collagens, *COL1A1* and *COL3A1*, which are known to increase in association with fibroblast differentiation. At the same time as the addition of TGF-β1 to the culture, human recombinant GREM2 protein was added at a concentration of 12.5 or 25 ng/mL, and cells were cultured for 24 h. We found that the gene expression levels of *COL1A1* and *COL3A1* were significantly

suppressed in a dose-dependent manner of GREM2 (Fig. 6A and B). In addition, we examined the protein levels of COL1A1 and COL3A1 in cells four days after differentiation induction and found that both COL1A1 and COL3A1 levels were significantly lower when cells were cultured with GREM2 than when cultured without GREM2 (Fig. 6C–F).

Furthermore, we tested whether GREM2 also has a negative effect on the dermal stem/progenitor cell differentiation into other lineages. We induced differentiation into adipocytes and osteoblasts in SF8428 cells and examined the effect of GREM2. The gene expression levels of adipocyte differentiation markers, *PPARG* and *CEBPA*, and also osteoblast differentiation markers, *RUNX2* and *ALPL*, were found to be significantly lower when GREM2 was added, suggesting that GREM2 suppresses the differentiation of SF8428 cells into adipocytes and osteoblasts (Fig. S2). Based on these findings, GREM2 may suppress dermal stem/progenitor cell differentiation.

4. Discussion

In the human skin, senescent cells accumulate with age or when cells receive damage [6–8], and SASP factors secreted from these cells impair the tissue structure or the capacity to maintain homeostasis, which promotes aging [14–17]. Previously, we reported an increase in GREM2 expression level in keratinocytes and fibroblasts when cellular senescence was induced. We also showed that GREM2 expression was higher in the skin from the elderly than from the young and that the individual differences GREM2 expression varied more largely in the elderly [18]. Moreover, we showed that GREM2 suppressed the differentiation of human adipose-derived stromal/stem cells (ASCs) [29]. These findings suggest that GREM2 expression increases with age and that GREM2 plays a role as a SASP factor in affecting the homeostasis of the skin and may directly affect stem cells in the skin. However, little is known about the function of GREM2 in the epidermis and dermis. In the present study, we investigated the expression and function of GREM2 in the epidermis and dermis.

First, we examined the expression levels of GREM2 and differentiation markers in the skin samples randomly chosen from human elderly subjects (n = 14) and analyzed the correlations between the expression levels. We found negative correlations between the expression levels of GREM2 and a differentiation marker both in the epidermis and dermis (Figs. 1C and 3C), suggesting that GREM2 is involved in the differentiation of the epidermis and dermis. On the other hand, no correlation was observed between GREM2 expression level and the number or proliferation of stem/progenitor cells based on partial correlation analysis. Although negative involvement of GREM2 on differentiation was suggested, the evidence supporting that GREM2 maintains stem cells undifferentiated was not obtained. Next, we tested whether GREM2 has any effects on models of skin stem/progenitor cells (epidermal and dermal stem/progenitor cells) that have great proliferation and differentiation abilities *in vitro*. GREM2 did not affect cell proliferation of an epidermal stem/progenitor cell model, HDK1 cells, nor a dermal stem/progenitor cell model, SF8428 cells (Fig. S3), whereas it suppressed the expressions of differentiation markers. Epidermal differentiation markers, *KRT10* and *IVL*, were suppressed in HDK1 cells by GREM2 (Fig. 5), and the expressions of dermal differentiation markers, COL1A1 and COL3A1, were suppressed in SF8428 cells (Fig. 6). In addition, GREM2 suppressed the differentiation of SF8428 cells into adipocytes and osteoblasts (Fig. S2). These findings suggest that GREM2 downregulates the differentiation of epidermal and dermal stem/progenitor cells into mature functional cells. Previous studies from other groups have shown that GREM2 is involved in proliferation and differentiation of stem cells. For example, GREM2 promotes the proliferation of myocardial progenitor cells and the differentiation of stem cells into myocytes [25–27]. Additionally, GREM2 suppresses the osteoblast differentiation of bone marrow-derived mesenchymal stem cells [28]. Little was known about GREM2 function in the skin, and here, we showed that GREM2 functions in suppressing the differentiation of skin stem/progenitor cells. Based on our findings, when GREM2 expression is increased by aging, the differentiation of skin stem/progenitor cells may be downregulated and immaturely differentiated cells, which become a part of tissue, may then cause a reduction in tissue function.

Recently, removal of senescent cells has been reported to prevent or ameliorate age-related diseases or dysfunction of tissues [58–60]. In the mouse skin, removal of senescent cells restored the thickness of subcutaneous adipose tissue [58] or fur density [59]. This suggests that SASP factors secreted from senescent cells accumulating with age may also have a noxious influence on the homeostasis of the skin. In the present study, we focused on GREM2 as a SASP factor and investigated its function. GREM2 was suggested to affect the stem/progenitor cells in the skin and inhibit differentiation into constituent cells in the epidermis and dermis, and age-related increase in GREM2 may contribute to reduced function of the entire tissues in the elderly. Therefore, regulating the expression level of GREM2, one of the SASP factors in the skin, may prevent or ameliorate age-related dysfunction of the skin. Further investigation on the functional mechanism of GREM2 in the skin will elucidate how it is involved in the regeneration of the skin or age-related diseases.

Declaration of competing interest

All authors declare no conflict of interest.

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Supplementary data

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