Discovery of a novel spot care cosmetic ingredient and a study of its functions that regulate the expression of SDF-1 in senescent fibroblasts and the dendritic changes of melanocytes caused by nerve fibers

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Abstract

Dark spot or age spots are hyperpigmented macules of skin that usually formed after long-term exposure to ultraviolet radiation, but they can be caused by other things, too. Skin conditions, pregnancy and certain medications or medical conditions may cause dark spots. To reduce dark spots, most cosmetic ingredients have used a strategy to reduce melanin production so far. In a recent study, it was revealed that dark spots are caused by the accumulation of a large number of senescent fibroblasts at the sites of pigmentation. The suppression of stromal-derived factor 1 (SDF-1) expression in senescence fibroblasts promotes melanocyte melanin production. Additionally, another study reported that the density of the nerve fiber affects the process of continuous regeneration even when the dark spot is removed.

In our previous study results, we discovered four aging markers that act on the 1st aging peak. Among these aging markers, it was confirmed that the expression of GDF15 was increased in aged skin fibroblasts. We tried to confirm that the aging marker GDF15 discovered by us

can also affect dark spots based on the previous study on the effect of aging fibroblasts on dark spots. Based on these studies, we developed ingredients using a new strategy to prevent the generation and recurrence of dark spots by acting on senescence fibroblasts and nerve fibers. To investigate the regulatory role played by senescent fibroblasts on melanocytes, SDF-1 and GDF15 mRNA expression levels were detected by qRT-PCR. In addition, to confirm whether the dendric extension of melanocytes is suppressed, a culture medium of nerve cells with or without a new ingredient was added to melanocytes. To improve dark spots, it is ultimately necessary to decrease the melanin contents in the pigmented area; thus, we evaluated whether our new ingredient could inhibit melanin synthesis. As a result, the expression of SDF-1 mRNA was inhibited in UVA-induced senescent fibroblasts. In addition, it was confirmed that the increased GDF15 in senescent fibroblasts activates melanogenesis in melanocytes. Moreover, this new ingredient increased the expression of SDF-1 and inhibited the expression of GDF15 in senescent fibroblast. Additionally, the dendrites of melanocytes had been reduced by a culture medium of nerve cells when the new ingredient added to melanocytes. Furthermore, the ingredient decreased the expression of MITF, TRP1, and inhibited melanin contents in melanocytes.

We found that GDF15, one of the aging markers we screened for, is related to the aging phenomenon in which dark spots on the skin increase with aging. Our new ingredient has a dark spot-specific care activity by reducing the effects of senescent fibroblasts and nerve fibers on melanocytes. Through this effect, as a result, our new ingredient can reduce the melanin content in the dark spot area as well as inhibit dark spot regeneration by nerve cells.

Keywords: senile lentigo. Senescent fibroblast, nerve fiber

Introduction.

Dark spots or aged spots are hyperpigmented macules of the skin that usually form after long-term exposure to ultraviolet radiation [1], but they can be caused by other things, too. Skin conditions, pregnancy and certain medications or medical conditions may cause dark spots. Skin pigmentation is primary related to melanocytes functionality, and the surrounding cells

and extracellular matrix proteins contribute to cutaneous homeostasis [1]. There is increasing evidence of a crucial role of senescent fibroblasts and the senescence-associated secretory phenotype in melanogenesis. A few studies have examined that a large number of senescent fibroblasts accumulated in senile lentigo area and that the loss of SDF1 in aged fibroblasts may lead to skin pigmentation through crosstalk with melanocytes [2]. Also, it has been found that nerve branches extend more toward superficial layers in senile lentigo, and the increased density of the nerve fibers in the SL cutis may contribute to the persistence and regeneration of dark spot [3].

In our previous study results, we discovered four aging markers that act on the 1st aging peak. We tried to confirm that the aging marker GDF15 which was increased in aged skin fibroblasts can also affect dark spots and explore the connection between GDF15 and SDF1.

The aim of this study was to discover a powerful strategy fot dark spot care ingredients that prevents the generation and recurrence of dark spots by acting on senescence fibroblasts and nerve fibers.

Materials and Methods.

Ingredients

L-(+)-Ergothioneine (EGT, 497-30-3, Sigma), L-Ascorbic acid (AA, 50-81-7, Sigma) and hexylresorcinol (HR, 136-77-6, Sigma) were purchased from Sigma. The purified recombinant protein of mouse SDF1(TP723771, OriGene) and the GDF15(NM_004864) Human recombinant protein (TP723131, OriGene) were purchased from OriGene Technologies, Inc.

Cell cultures and reagents

Human primary fibroblast cells (HDFn, normal human dermal fibroblasts juvenile foreskin, C-12300, PromoCell, Heidelberg, Germany), the murine melanoma cell line(B16-F10, CRL-6475, ATCC, USA), and the murine neuroblastoma cell line(Neuro-2a, CCL-131, ATCC, USA) were cultured in Dulbecco's modified Eagle's medium high glucose (DMEM high glucose, SH30243.01, Hy-80 clone, Hyclone, Logan, UT, USA) containing 10% (v/v) fetal bovine serum (FBS, SH30084.03, Hyclone, Logan, UT, USA) and 1% antibiotic-

antimycotic agents (Anti-anti, 15240-062, Gibco, Grand Island, NY, USA). Cell cultures were maintained at sub confluence in a 95% air, 5% CO2 humidified atmosphere at 37°C.

Cell viability assay (MTT assay)

Cells were seeded at $5x10^3$ cells/ well on a 96-well plate and cultured for 24 h under cell culture conditions. Then, we discarded the medium, washed the cells with a solution of phosphate buffered saline (PBS, 21-040- CV; Corning, USA), placed the cells in new serum-free media, treated the cells with different concentrations of the samples, and incubated the cells for 24 and 48 h. Then, we added 100 µL of MTT solution (0.5%, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-2H- tetrazolium bromide) to each well and incubated the cells for another 2 hours. After removing the culture media, we added 100 µL of 100% dimethyl sulfoxide (DMSO), shook the cells for 10 minutes, and measured the absorbance at 590 nm with a microplate reader (EPOCH2 microplate reader, EPOCH2NSC, BioTek, USA).

In vitro model of senescent fibroblasts

HDFn cells were seeded at $1x10^5$ cells/ well on a 6-well plate and cultured for 24 h under cell culture conditions. Then, we discarded the medium, washed once with PBS and irradiated with UVA light (Bio-sun system, Vilber Lourmat, Inc.) at a total dose of 50 mJ/cm^2. Following irradiation, the medium was replaced with a culture medium with or without the sample ingredients for 24 h.

Quantitative real-time polymerase chain reaction (qRT PCR)

RNA extraction was performed on the cells using an RNA extraction kit (TaKaRa Mini-BEST Universal RNA extraction kit, 9767A, Takara Bio, Inc., Otsu, Japan), and the samples were purified following the manufacturer's protocol. cDNA was synthesized from the isolated RNA (1µg) using a T.R. reagent kit (PrimeScript™ RT reagent Kit with gDNA Eraser, RR047A, Takara Bio, Inc., Otsu, Japan). Using a SYRB Green Realtime PCR Master Mix (Power SYBRTM Green PCR Master Mix, 4367659, Applied Biosystems™, Thermo Fischer Sci- entific) and QuantStudio™ 3 Real-Time PCR instrument, (A28132, Thermo Fisher Scientific), the gene expression levels were standardized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primers used in this study are

presented in Table 2. The experiments were performed in duplicate for three independent repeats. Melting curve analysis was performed for each primer set

Table 2. Primer sequences for the quantitative PCR

Gene	Sequences	
h-SDF1	Forward	TGCCAGAGCCAACGTCAAG
	Reverse	CAGCCGGGCTACAATCTGAA
h-GDF15	Forward	AGATCAAGACGAGCCTGCAC
	Reverse	ACAGTGGAAGGACCAGGACT
h-GAPDH	Forward	ACCCACTCCTCCACCTTTGA
	Reverse	CTGTTGCTGTAGCCAAATTGCT
m-MITF	Forward	GACCAGAGCAGGGCAGAGAGTGAGT
	Reverse	TGGGAAGGTTGGCTGGACAGGAGTT
m-MC1R	Forward	GCTGCGTTATCACAGCATCG
	Reverse	AGAAAGTGACGAGGCAGAGC
m-TYR	Forward	GCACTGGTGGGAGCTGTTAT
	Reverse	AGCAAGCTGTGGTAGTCGTC
m-GAPDH	Forward	CATCACTGCCACCCAGAAGACTG
	Reverse	ATGCCAGTGAGCTTCCCGTTCAG

Gene knockdown (SDF1 siRNA Transfection)

Human SDF1 siRNA was incubated with lipofectamine RNAiMAX in serum-free DMEM for 5 min following the manufacturer's protocol. All HDFn cells were supplemented with the siRNA by directly adding it to the cell culture media followed incubation for 48h. The siRNA sequences were as follows: si-SDF1 5′-CGGCUGAAGAACAACAACATT- 3′, 5′-UGUUGUUCUUCAGCCGTT- 3′.

Measurement of melanin production in B16F10 melanocytes

B16f10 cells were seeded into 6-well plates and allowed to adhere overnight. The cell were then treated with conditioned medium in the presence or absence of the skincare ingredients for 3 days. After centrifugation, the pellets were lysed with 100μL of 1N NaOH containing

10% DMSO solution and heated at 60℃ for 10 min. Absorbance was then measured at 490 nm using an EPOCH2 microplate reader.

Measurement of the dendrite length of B16F10 cells

B16F10 cells were seeded at $1x10^3$ cells/ well on a 6-well plate and cultured for 24h. Media from N2A cells treated with or without samples were added to B16F10 cells. After 24 h, the B16F10 cells were observed using a microscope (DMi1, Leica microsystems), and the dendrite length was measured by a ruler.

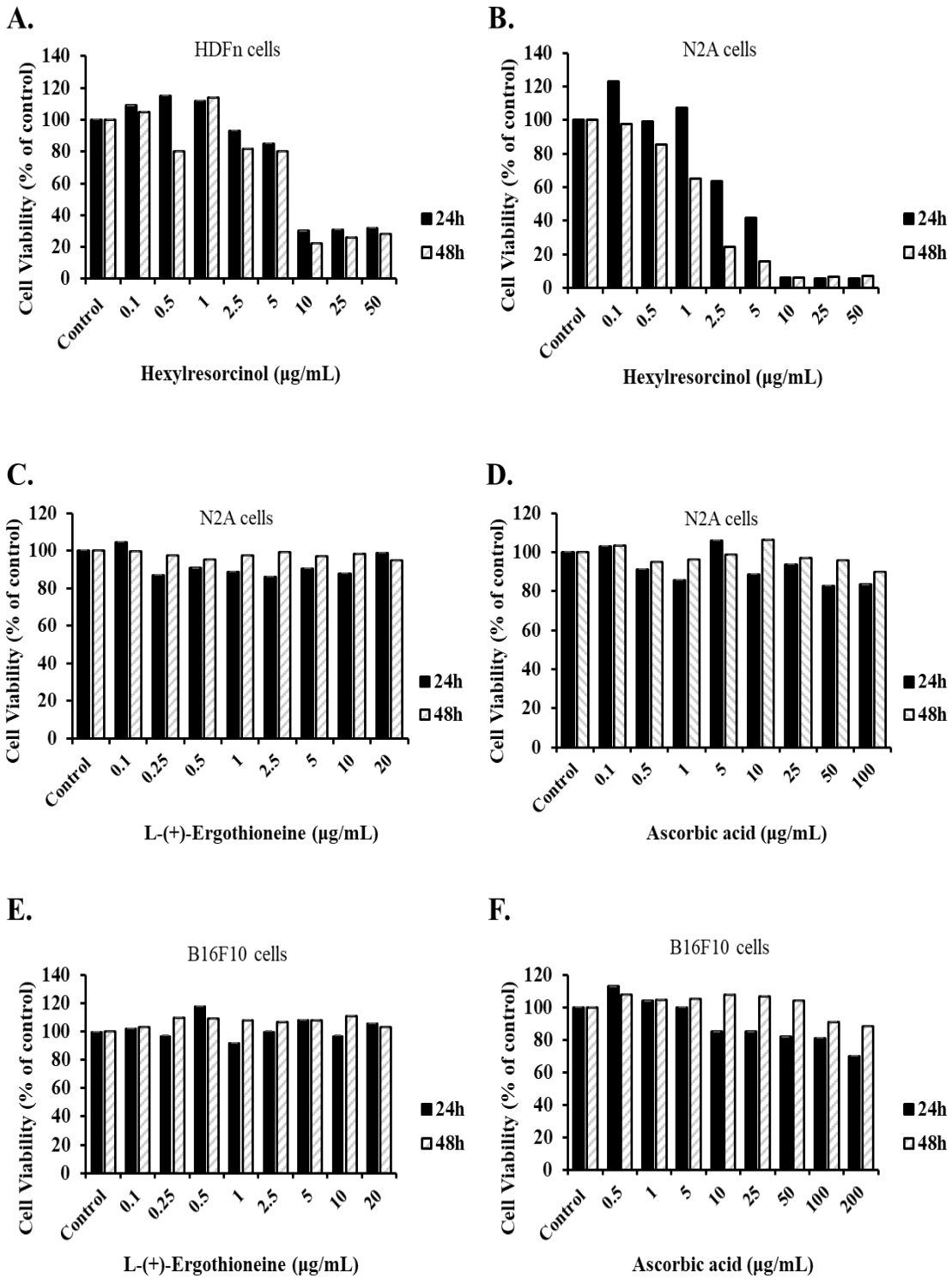
Data analysis

Data are expressed as the mean ± SD. Data were analyzed using the analysis of variance (ANOVA) or student's t-test. Dunnett's test was used for post hoc analysis to determine the difference from the negative control. P-values of 0.05 or less were considered significant.

Results.

Effects of the skincare ingredients on cell viability

The MTT assay was performed to determine the cytotoxicity of EGT, AA, HR treatment in HDFn, N2A, and B16F10 melanocytes. No significant cytotoxicity of EGT was observed up to 20 μg/mL in Neuro-2A cells (Fig 1C) and B16F10 melanocytes (Fig 1E). No significant cytotoxicity of AA was observed up to 100 μg/mL in Neuro-2A cells (Fig 1D) and B16F10 melanocytes (Fig 1F). However, above 1 μg/mL, HR resulted in significant cytotoxicity in HDFn, N2A, and B16F10 cells at 24, and 48h. Thus, a HR concentration of 0.5 μg/mL or less was used for the subsequent experiments.


A.
HDFn cells
Cell Viability (% of control)
140
120
100
80
60
40
20
0
Control
0.1
0.5
1
2.5
5
10
25
50
24h
48h
Hexylresorcinol (μg/mL)
B.
N2A cells
Cell Viability (% of control)
Control
0.1
0.5
1
2.5
5
10
25
50
24h
48h
Hexylresorcinol (μg/mL)
C.
N2A cells
Cell Viability (% of control)
120
100
80
60
40
20
0
Control
0.1
0.25
0.5
1
2.5
5
10
20
24h
48h
L-(+)-Ergothioneine (μg/mL)
D.
N2A cells
Cell Viability (% of control)
Control
0.1
0.5
1
5
10
25
50
100
24h
48h
Ascorbic acid (μg/mL)
E.
B16F10 cells
Cell Viability (% of control)
Control
0.1
0.25
0.5
1
2.5
5
10
20
24h
48h
L-(+)-Ergothioneine (μg/mL)
F.
B16F10 cells
Cell Viability (% of control)
Control
0.5
1
5
10
25
50
100
200
24h
48h
Ascorbic acid (μg/mL)

G.

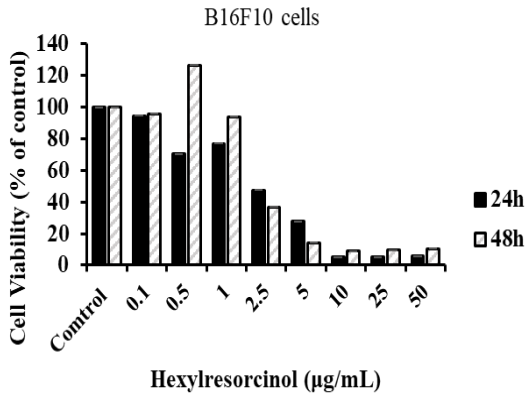


Figure 1. Effects of the skincare ingredients on cell viability.

The human primary fibroblasts (HDFn), murine neuroblastoma cell (Neuro-2a) and murine melanoma(B16F10) cells were incubated with different concentrations of L-(+)-Ergothioneine (NGT), L-Ascorbic acid(AA) and hexylresorcinol(HR), and the cell viability was accessed by MTT assay.

Effects of the skincare ingredients to promote SDF1 in senescent fibroblast and improve skin pigmentation

To determine the effect of hexylresorcinol on SDF1 expression of UVA irradiation, HDFn cells were exposed to UVA (50 mJ/cm^2) and SDF1 gene expression was measured at 24h post-UVA irradiation. Our data showed that UVA exposure resulted in a significant decrease in SDF1 gene expression of senescent fibroblasts (Fig 2A). However, 0.05, 0.1, and 0.5 μg/mL of hexylresorcinol increased SDF1 gene expression by 32%, 47%, and 46% respectively in senescent fibroblasts. To investigate the effect of SDF1 on pigmentation, the B16F10 cells were treated with different concentrations of recombinant mouse SDF1 protein. The melanin contents were lower than those of controls in a dose dependent manner (Fig 2B). Additionally, the mRNA level of MITF, MC1R, and TYR were downregulated by the SDF1 protein especially after 6 h of incubation with SDF1 proteins (Fig 2C-E). Consistent with these results, SDF1 can decrease melanogenesis by down-regulating the MITF/MC1R/TYR

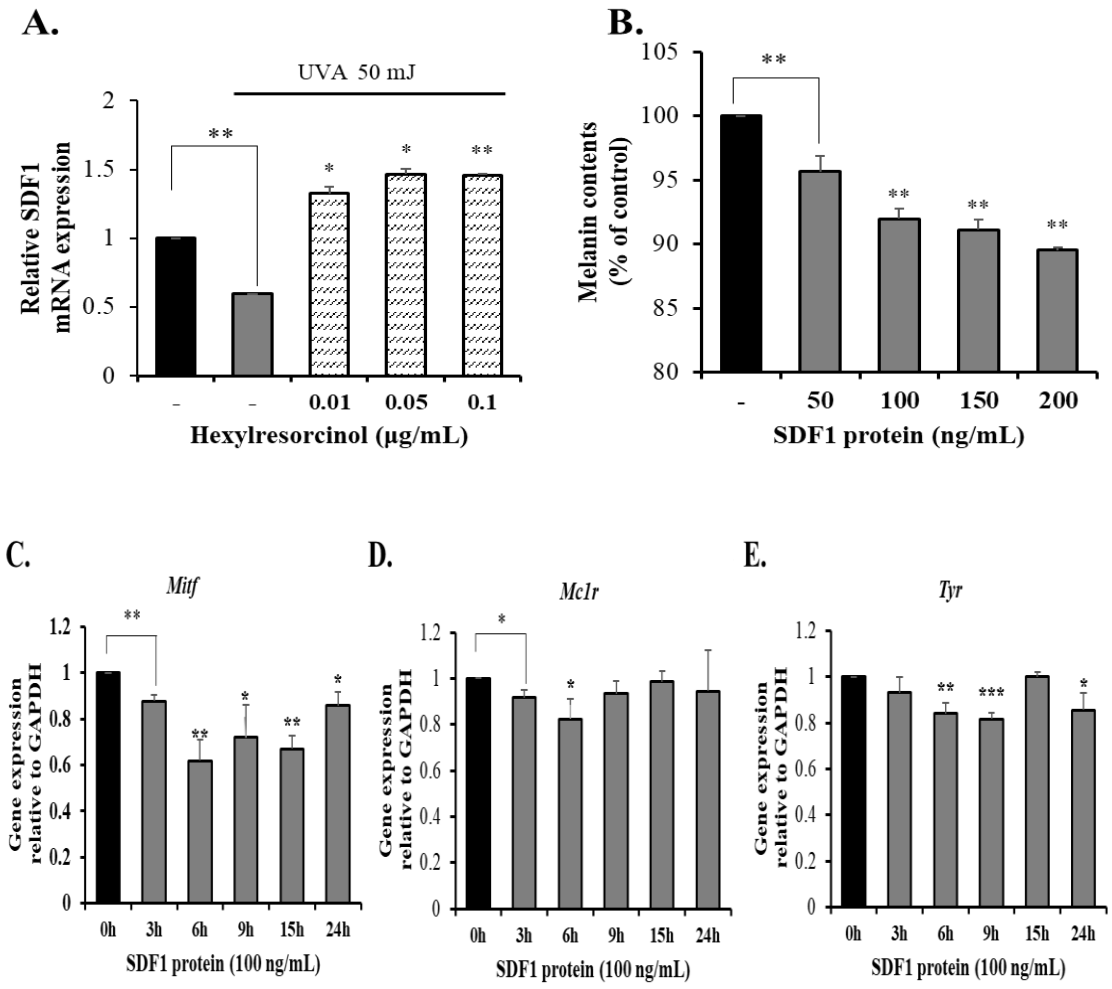


Figure 2. Effects of the skincare ingredients to promote SDF1 in senescent fibroblasts and improve skin pigmentation.

HDFn cells were exposed to UVA (50mJ/cm^2) in the presence or absence of HR (0.01-0.5 μg/mL) for 24 h(A). The content of melanin is expressed as a percentage of the recombinant SDF1 protein untreated control cells (B). The mRNA levels of MITF (C), MC1R (D), TYR (E) gene were determined using qRT-PCR and normalized to mRNA levels of GAPDH. All data were expressed as a percentage of control from three independent experiments with the mean ± standard deviation and analyzed using Student's t-tests, *$p < 0.05$, **$p<0.005$, ***$p<0.001$

signaling in melanocytes. Moreover, hexylresorcinol treatment restored the SDF1 expression to levels comparable to control. These findings indicate that hexylresorcinol can restore SDF1 in senescent fibroblasts and correct the uneven pigmentation.

Effects of the skincare ingredients to inhibit neurosecretion and suppresses melanogenesis

In order to confirm whether EGT, AA and HR have the effect of suppressing the secretory activity in nerves and reducing melanin production, conditioned medium from N2A cells cultured with or without EGT (1, 5, and 10 μg/mL), AA (5, 10, and 25 μg/mL), HR (0.05, 0.1, and 0.5 μg/mL) was added to B16F10 melanocytes. As a result, it was found that the length of B16F10 melanocyte dendrites was elongated by conditioned media from N2A cells, while the length of B16F10 dendrite was decreased by conditioned media from N2A that cultured with different concentration of EGT, AA and HR (Fig 3A). The conditioned media from N2A cells treated with different concentrations of EGT (1, 5, 10 μg/mL), AA (5, 10, 25 μg/mL), HR (0.05, 0.1, 0.5 μg/mL) were less likely to darken the color compared to the conditioned media from N2A cells without the added ingredients (Fig 3B). Moreover, the mRNA expression of MITF, MC1R, and TYR was also downregulated by conditioned media from N2A added with different concentration of EGT, AA and HR (Fig 3C-E). It is thought that the amount of some melanocyte activator secreted from N2A cells was suppressed by EGT, AA and HR, and a number of melanin and melanogenesis relative mRNAs produced by melanocytes was reduced. Based on these results, it is expected that EGT, AA and HR can effectively suppress the cause of dark spots from the depths of the skin by reducing the effect of nerves on melanocytes.

The skincare ingredients act directly on B16F10 melanocytes to inhibit the effects from N2A cells

B16F10 melanocytes were treated with conditioned medium obtained from N2A cells and then cultured with different concentration of EGT (1, 5, 10 μg/mL), AA (5, 10, 25 μg/mL),

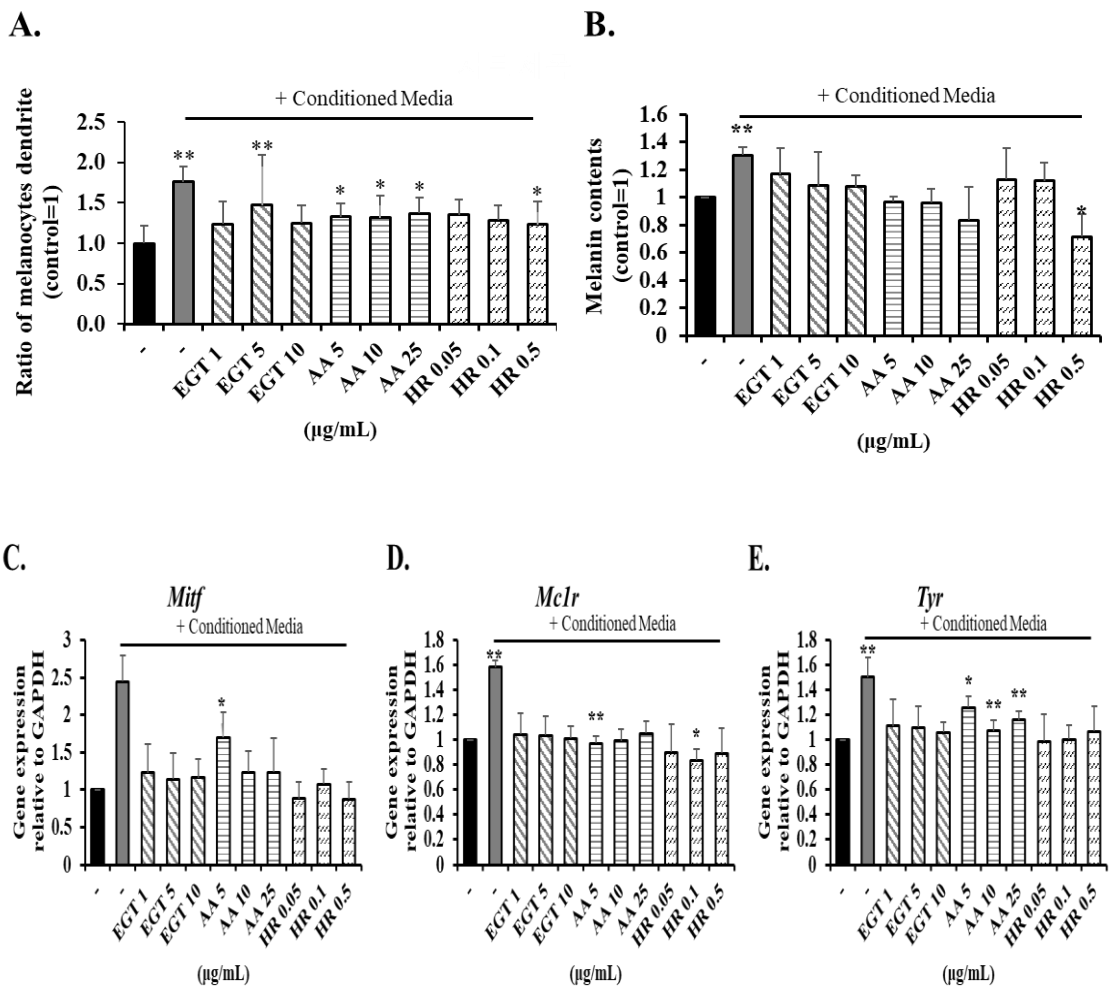


Figure 3. Effects of the skincare ingredients to inhibit neurosecretion and suppresses melanogenesis.

B16F10 were cultured with conditioned medium from N2A cells treated with or without EGT (1, 5, 10 μg/mL), AA (5, 10, 25 μg/mL), HR (0.05, 0.1, 0.5 μg/mL). Total length of melanocyte dendrites per cell was measured on the pictures using ruler (A). B16f10 cells were incubated with conditioned media from N2A cells added with various concentrations of EGT, AA, HR and melanin content was detected (Fig 3B). The mRNA levels of MITF (C), MC1R (D), TYR (E) genes were determined in the same way and normalized to mRNA levels of GAPDH. All data were expressed as a percentage of control from three independent experiments with the mean ± standard deviation and analyzed using Student's t-tests, *$p < 0.05$, **$p<0.005$, ***$p<0.001$.

HR (0.05, 0.1, 0.5 μg/mL). The conditioned media significantly increased the dendrite length of B16F10 cells, but when treated with different concentrations of skincare ingredients can inhibit the elongations of dendrite length of B16F10 cells (Fig 4A). Meanwhile, melanin productions was increased by conditioned media compared to the control group, and when cultured with EGT (1, 5, 10 μg/mL), AA (5, 10, 25 μg/mL), and HR (0.05, 0.1, 0.5 μg/mL) can decrease melanin synthesis (Fig 4B). The gene expression of MITF, MC1R, and TYR was also upregulated by conditioned media, but downregulated by EGT, AA, and HR treatment (Fig 4C-E). Therefore, the EGT, AA, HR effects on melanocytes are to prevent dendrite elongation and excessive formation of melanin by N2A cells and also decrease the relative melanogenesis gene expression like MITF, MC1R, and TYR.

The connection of the ageing marker GDF15 and SDF1 on age-related pigmentation

To identify GDF15 upregulation and SDF1 downregulation in senescent fibroblasts, HDFn cells was induced with UVA irradiation (50mJ/cm^2). The GDF15 mRNA expression level weas increased in UVA-irradiated senescent fibroblasts. In contrast, the expression of SDF1 was decreased (Fig 5A). To investigate the effect of GDF15 on pigmentation, different doses of recombinant GDF15(50, 100, 150, and 200 ng/ml) protein was cultured with B16F10 melanocytes for 3 days. In the presence of GDF15 proteins, the melanin contents levels were significantly increased in the B16F10 melanocytes, especially at a concentration of 100 ng/ml (Fig 5B). In addition, the mRNA expression level of the melanogenesis-associated genes, MITF, MC1R, and MLANA were upregulated at 15h (Fig 5C-E). These results suggest that senescent fibroblast-derived GDF15 has a stimulatory effect on skin pigmentation. SDF1 deficiency in senescent fibroblast leads to skin pigmentation. Thus, to explore the connection between SDF1 and GDF15, 25pmol of SDF-1 siRNA were incubated with HDFn cells for 48 h. Consistent with these results, SDF1-knockdown in fibroblasts exhibited an increased mRNA expression level of GDF15 (Fig 5F-G). Taken together, the results show that in ageing-related pigmentation, senescent fibroblasts exhibit SDF1 deficiency as a result of

promoting GDF15, which then finally stimulates melanogenesis-associated gene expression and synthesis melanin in melanocytes. UVA exposure resulted in a significant increase of GDF15 gene expression in senescent fibroblasts. However, 0.05, 0.1, and 0.5 μg/mL hexylresorcinol decreased GDF15 gene expression in senescent fibroblasts (Fig 5H). These

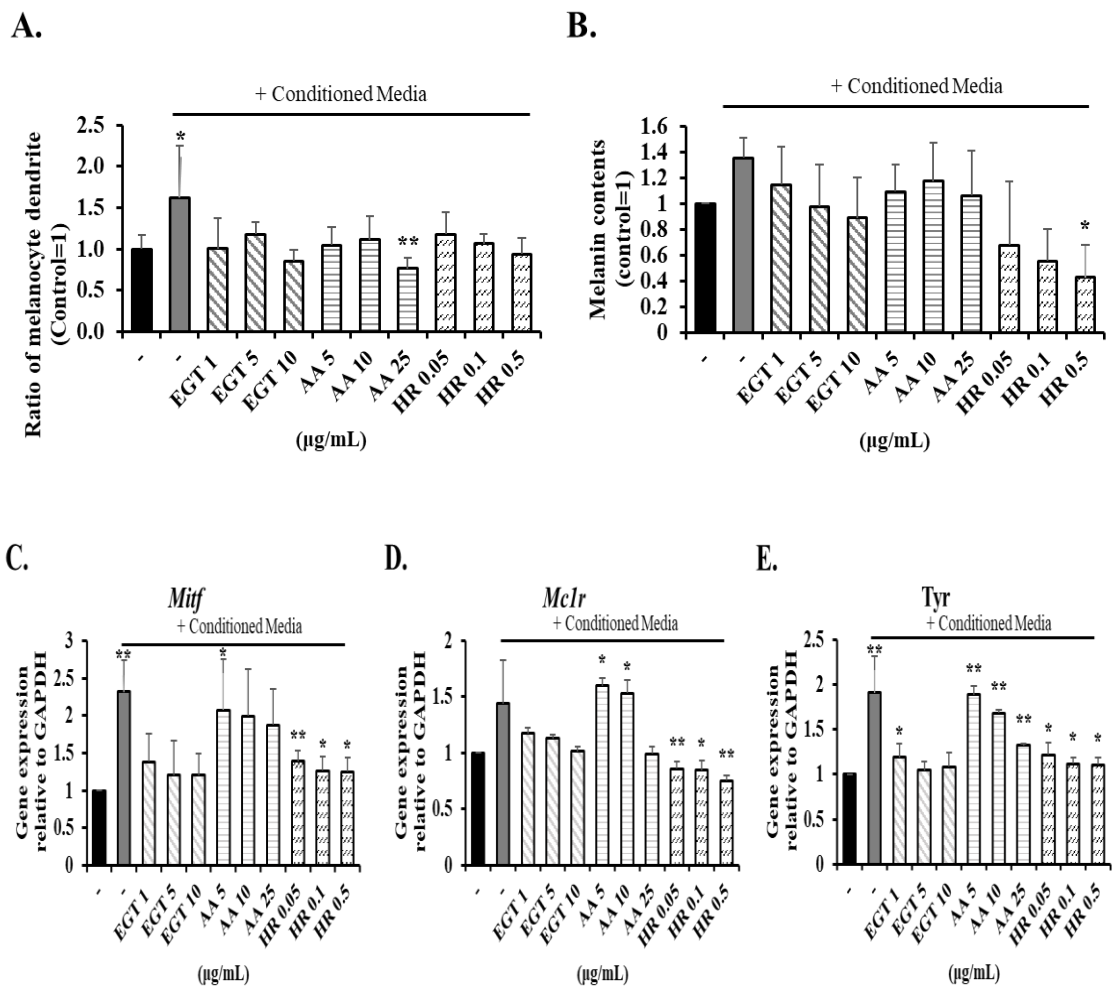


Figure 4. The skincare ingredients act directly on B16F10 melanocytes to inhibit the effects from N2A cells.

B16F10 melanocytes were cultured with conditioned media from N2A cells, then treated with different concentrations of EGT (1, 5, 10 μg/mL), AA (5, 10, 25 μg/mL), and HR (0.05, 0.1, 0.5 μg/mL). Total length of melanocyte dendrites per cell was measured on the pictures using a ruler (A). Melanin content of B16F10 cells and cell pellets after 72 h of treatment with EGT, AA, and HR and conditioned media (Fig 3B). The mRNA levels of MITF (C), MC1R (D), TYR (E) genes were determined in the same way and normalized to mRNA levels of GAPDH. All data were expressed as a percentage of control from three independent experiments with the mean ± standard deviation and analyzed using Student's t-tests, *$p < 0.05$, **$p<0.005$, ***$p<0.001$.

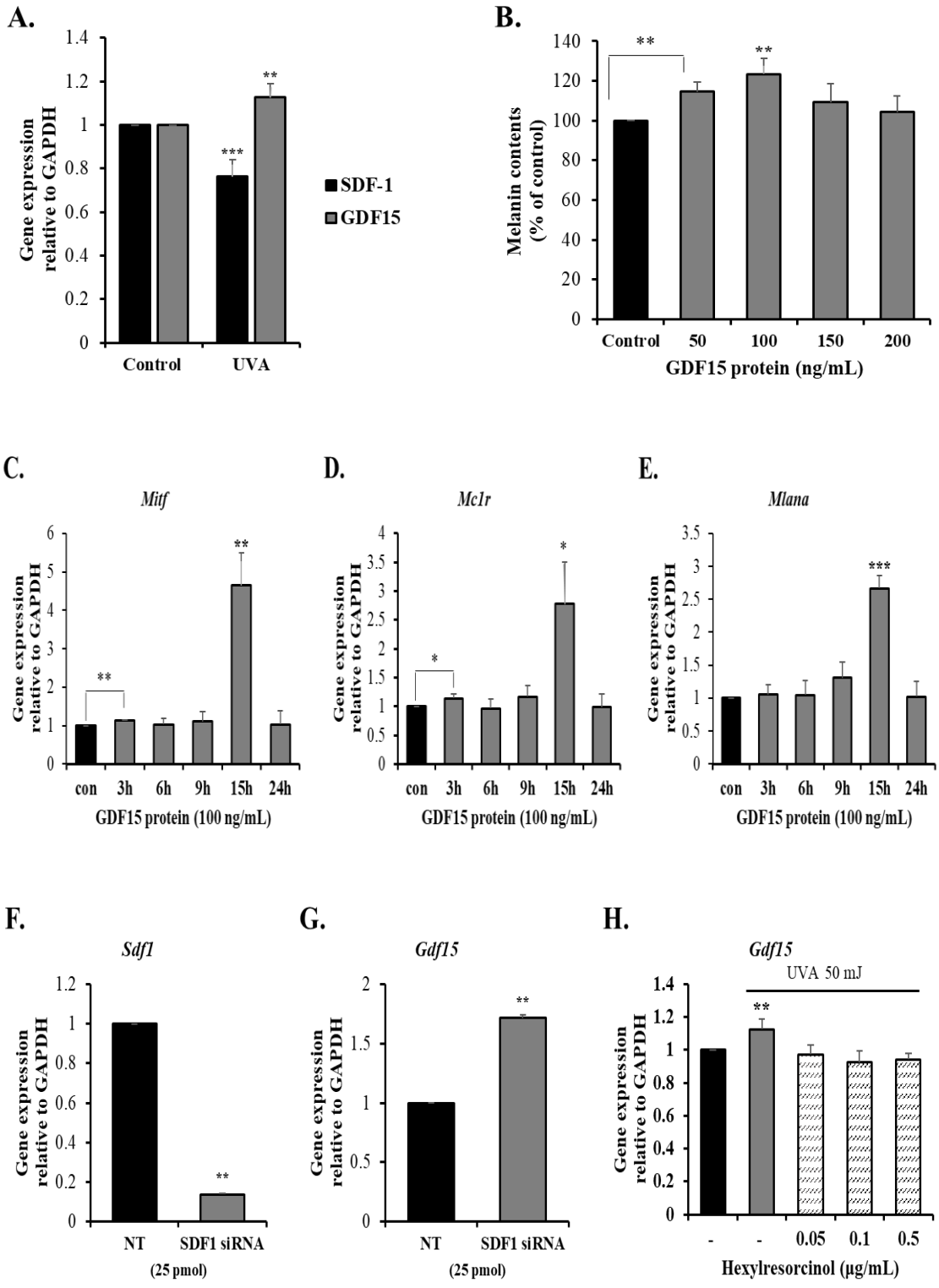


Figure 5. The connection obetween GDF15 and SDF1 on age-related pigmentation.

findings show that hexylresorcinol can inhibit GDF15 in senescent fibroblast. They provide further support for the therapeutic potential of hexylresorcinol in restoring SDF1 and decreasing GDF15 to correct skin-related dark spots. GDF15, and SDF1 expression levels in HDFn cells were analyzed in normal, and UVA-induced senescent fibroblasts by qRT-PCR (A). The production of melanin is expressed as a percentage of the recombinant GDF15 protein untreated control cells (B). The mRNA levels of MITF (C), MC1R (D), TYR (E) genes were determined using qRT-PCR and normalized to mRNA levels of GAPDH. HDFn cells were exposed to UVA (50mJ/cm^2) in the presence or absence of HR (0.01-0.5 μg/mL) for 24 h (H). All data were expressed as a percentage of control from three independent experiments with the mean ± standard deviation and analyzed using Student's t-tests, *$p < 0.05$, **$p<0.005$, ***$p<0.001$.

Discussion.

Dark spot skin areas were found to have more senescent fibroblasts [2] in the derma and a high density of nerve branches that extend more toward the superficial layers which result in more elongated and branched melanocytes compared to normal skin [3]. In addition, the dendritic processes that extend in various directions can contribute to the accumulation of numerous melanosomes in neighboring keratinocytes. In this study, we revealed that Hexylresorcinol is a novel ingredient to regulate the effects of senescent fibroblast on melanogenesis (Fig 2, 5). SDF1 plays an inhibitory role in controlling skin pigmentation. In UVA-irradiated aging skin, SDF-1 level was markedly reduced (Fig 2A) and SDF1 inhibited melanin contents (Fig 2B) and expression of melanogenesis associated mRNAs of MITF (Fig 2C), MC1R (Fig 2D), and TYR (Fig 2E) in melanocytes.

GDF15 has been found in aging and age-related disorders and has been demonstrated as a possible biomarker for aging and age-related comorbidity [4]. This study identified GDF15 as an ageing marker of the 1st aging peak produced by senescent fibroblasts to contribute to the development of ageing pigmentation. We showed that 50 mJ/cm^2 of UVA irradiation induces GDF15 compared with that of normal fibroblasts (Fig 5A), and that the treatment of rhGDF15 protein led to increased melanin contents (Fig 5B) and melanogenesis associated mRNA of MITF (Fig 5C), MC1R (Fig 5D), MLANA (Fig 5E) in melanocytes. Also, we demonstrated for first time that SDF1 knockdown in fibroblasts can stimulate GDF15

overexpression (Fig 5G). Thus, it may be concluded that ageing skin induced by UVA downregulates SDF1 resulting in the overexpression of GDF15 and finally activates the melanogenesis related genes of MITF, MC1R, TYR to promote a large amount of melanin production in melanocytes. Nevertheless, SDF1 stimulative actions and GDF15 inhibitory actions of hexylresorcinol suggest that it is a possible therapeutic agent for the development of skin-lightening agents that can treat dark spots (Fig 2A, and 5H).

Nerve branches extend more toward the superficial layers in dark spots, and it return increased occupancy ratio of nerve fibers in the dark spot areas may contribute to persistence and regeneration of dark spots. Recent studies have shown that the dark spot areas seen in melasma exhibit increased deposition of melanin in the epidermis and dermis. No increase in the number of melanocytes in those areas was noted, but the melanocytes were larger, more dendritic, and showed increased melanogenesis [5]. However, it is currently unclear whether the nerve fibers with more frequently in contact with melanocytes induce dark spot formation. Our study showed that the length of dendrites was significantly increased by the conditioned media obtained from Neuro-2A cells cultured with B16F10 melanocytes (Fig 3A, and 4A). These results indicated that nerve fibers stimulate the extension of melanocyte dendrites enabling more surrounding keratinocytes to come into contact with melanocytes and promoting the transfer of melanosomes to keratinocytes. Our anti-dark spot ingredients of EGT (1, 5, 10 μg/ml), AA (5, 10, 25 μg/ml), HR (0.05, 0.1, 0.5 μg/ml) not only suppress N2A cells from secreting substances that enhance the elongation of melanocyte dendrites, but also act directly on melanocytes, preventing the dendrite extension effect by N2A cells (Fig 3A, and 4A). Besides, they also affect the melanin synthesis (Fig 3B, and 4B) and melanogenesis associated gene of MITF (Fig 3C, and 4C). MC1R (Fig 3D, and 4D), TYR (Fig 3E, and 4E) are upregulated by conditioned medium from N2A cells while the EGT (1, 5, 10 μg/ml), AA (5, 10, 25 μg/ml), HR (0.05, 0.1, 0.5 μg/ml) can decrease the production of melanin and downregulate the melanogenesis related mRNA gene expression (Fig 3, 4). These results suggest that the interaction between melanocytes and nerve cells could induce spot formation by activating the MITF/MC1R/TYR signaling pathway to product more melanin; however, our anti-dark spot ingredients of EGT, AA, and HR have the ability to reduce the formation of spots and prevent the recurrence of spots by inhibiting the interaction between melanocytes and nerve cells.

Conclusion.

This study examines that hexylresorcinol can inhibit the mRNA expression of the aging marker GDF15 and restore the mRNA expression of SDF1 to reduce the effects of senescent fibroblasts on melanocytes. Furthermore, L-(+)-Ergothioneine, L-Ascorbic acid, and hexylresorcinol can prevent the dendrite extension effect by N2A cells and inhibit the expression of melanogenesis related genes of MITF, MC1R, TYR to reduce melanin formation in melanocytes, ultimately suppressing aged related dark spots. Through these effects, as a result, our dark spot care ingredients can reduce the melanin content in dark spot areas and inhibit dark spot regeneration by nerve cells. Thus, this study showed that EGT, AA, and HR have value as stronger dark spot care ingredients.

Acknowledgments.

Conflict of Interest Statement. NONE.

References. All the references must be quoted numerically in the order in which they appear in the manuscript. This must be done in between square brackets [1].

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