A study on the cellular senescence-inducing function of aging markers acting on the 1st aging peak.

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Abstract

In a previous study, we discovered four types of aging marker genes, FSTL3, GDF15, MMP12, and CCDC80, whose expression in skin cells changes significantly during the 1st aging peak that occurs around the age of 40. We conducted a study to elucidate the mechanism by which these aging markers cause skin aging.

SA-β-gal activity was measured in aging marker-treated skin cells to evaluate whether the senescence of the cells in accelerated by the aging markers. SA-β-gal staining was increased when HDFn cells were treated with rh GDF15 and rh FSTL3. In HDFn cells treated with hydrogen peroxide (H_2O_2), rh GDF15 enhanced SA-β-gal activity by H_2O_2, whereas CCDC80 significantly reduced SA-β-gal activity caused by oxidative stress. This was quantitatively confirmed through the ONPG test. In addition, rh GDF15 increased the expression of SASP-related genes, and it was observed that SASP gene expression increased by H_2O_2 decreased by CCDC80. The fluorescence of DCFDA staining was explored to confirm whether ROS was generated by aging markers. Similar to the SA-β-gal assay results, rh GDF15 and rh FSTL3 enhanced DCFDA staining and, when treated with rh CCDC80, had the effect of reducing DCFDA intensity increased by oxidative stress. Through this, we confirmed that GDF15 and CCCD80 are involved in skin aging related to ROS generation, and we tried to confirm whether these phenomena are related to p16/p21 signaling. As a result, it was confirmed that GDF15 and CCDC80 affect the expression level of p21 protein.

Furthermore, it was confirmed that GDF15 has the effect of promoting melanin synthesis in relation to an increase in melanin, one of the important skin aging phenomena.
We found that the skin aging markers, GDF15 and FSTL3, promotes cell senescence by inducing ROS generation through the p21 signaling pathway. On the other hand, it was confirmed that CCDC80 can inhibit aging through the effect of inhibiting ROS generation. In addition, it was confirmed that melanin synthesis that darken the skin tone were promoted by the aging marker GDF15. Through this, the aging markers we discovered can be used as a new target for the development of ingredient that can comprehensively care for skin aging in various fields, from wrinkles and elasticity to whitening and cellular senescence.

Keywords: aging marker, senescence, cellular senescence, anti-aging, skin aging, cosmetics.

Introduction.

The final goal of cosmetic research is to prevent skin aging and restore aging skin. As part of this effort, in a previous study, we discovered four types of aging marker genes, FSTL3, GDF15, MMP12, and CCDC80, whose expression in skin cells changes significantly during the 1st aging peak that occurs around the age of 40. MMP12 as an elastase was confirmed to increase with aging of skin cells, and it can promote the degradation of elastin in the skin and cause a decrease in skin elasticity. However, in the case of FSTL3, GDF15 and CCDC80, except for MMP12 among the four aging markers, what function induces skin aging is not yet known.
Growth differentiation factor 15 (GDF15) is a stress molecule produced in response to mitochondrial, metabolic and inflammatory stress with a number of beneficial effects on metabolism. A recent study revealed upregulation of GDF15-induced cellular senescence in HAECs, confirmed by G0/G1 cell cycle arrest, decreased during cell proliferation and increased SA-β-gal staining. And GDF15 might play an important role in cellular senescence through a ROS-mediated p16 pathway [1]. Another study reported that increased expression of GDF15 in aged dermal fibroblasts affects skin pigmentation [2]. GDF15 is an emerging biomarker in several diseases and aging.

Follistatin-like 3 (FSTL3) is a glycoprotein that binds and inhibits the action of TGFβ ligands such as activin. It mediates cell differentiation and growth, acts as a biomarker of tumors and participates in cancer development and progression. The Physiological role of FSTL3 in adults remains to be determined. FSTL3 is critical for normal adult metabolic homeostasis [3]. In testis, FSTL3 deletion leads to increased AKT signaling and SIRT1 expression.

This shows that the interaction between TGFβ ligand and AKT signaling affects cell survival and anti-aging [4]. Studies on the role of FSTL3 in skin are mainly related to cancer, but considering TGF-b signaling, it can be considered as an aging marker that plays an important role in skin aging.

Coiled-coil domain containing 80 (Ccdc80) is a secreted protein highly enriched in mouse and human white adipose tissue (WAT) that plays an important role during adipocyte differentiation in vitro. Ccdc80 is a modulator of glucose and energy homeostasis [5]. Also, it has been reported that CCDC80 plays an anticancer role by inhibiting the migration of melanoma [6]. The role of CCDC80 in aging or skin aging has not been well studied.

In the present study, the mechanism of cellular senescence by aging markers was investigated. The GDF15 promotes skin cell aging through ROS generation, and CCDC80 suppresses ROS generation to suppress skin cell aging. This is done through p16 signaling associated with ROS generation. Gdf15 also promotes melanin synthesis, which leads to skin darkening. Therefore, the novel skin aging markers we found may be involved in comprehensive skin aging control.

Materials and Methods.

Cell cultures and reagents

Keratinocyte (HaCaT) cells, human primary fibroblast cells (HDFn, normal human dermal fibroblasts juvenile foreskin, C-12300, PromoCell, Heidelberg, Germany) and murine melanoma cell line (B16F10) 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) of 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. rh GDF15 (recombinant human GDF15 protein, OriGene, Rockville, Maryland, USA), rh FSTL3 (recombinant human FSTL3 protein, OriGene, Rockville, Maryland, USA) and rh CCDC80 (recombinant human CCDC80 protein, OriGene, Rockville, Maryland, USA) were used as recombinant aging marker proteins.

Senescence-associated-β-galactosidase (SA-β-gal) staining and soluble enzyme assay

1×10^5 cells were seeded in a 6 -well plate. The cells were exposed to the recombinant aging marker proteins for 48h. Other cells were pre-treated with the recombinant aging marker for 24 hours, then treated with hydrogen peroxide (H_2O_2) for 24 hours. SA-β-gal activity was detected using a senescence cell histochemical staining kit (Sigma-aldrich, USA) as per the manufacturer's instructions. Stained cells were viewed under a microscope (Leica, Germany). For the ONPG (O-nitrophenyl-beta-D-galactopyranoside) enzyme assay of SA β-gal activity, equal numbers of cells were harvested in a micro-centrifuge tube following the experimental procedure of SensoLyte® NPG ß-Galactosidase Assay Kit Colorimetric (AnaSpec, Fremont, CA, USA). The absorbance was measured at 420 nm with an EPOCH2 ELISA reader (Bio-Tek, Winooski, VT, USA).

ROS detection

The Abcam DCFDA ROS detection kit (ab113851, Abcam, Cambridge, MA) uses 2',7'-dichlorofluorescein diacetate reagent (DCFDA), a fluorogenic dye measuring hydroxyl, peroxyl and other intracellular ROS. Following cell diffusion, DCFDA is deacetylated by cellular esterases to produce a nonfluorescent compound that will be oxidized by ROS to 2',7'-dichlorofluorescein (DCF). DCF is a strong fluorescent compound that can be detected by fluorescence spectroscopy at 495 nm and 529 nm spectra, respectively. The Abcam DCFDA kit includes: 20 mM DCFDA, 10x Buffer, 55 mM tert-butyl hydrogen peroxide (TBHP) solution. Additional materials are also required: bidistilled water and fetal bovine serum. Cells were treated with senescence marker protein, oxidative stress was applied, and then stained with DCFDA. Stained cells were observed using JuLI microscope (NanoEnTek, Korea).

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 (PrimeScriptTM 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^{TM}, Thermo Fischer Sci- entific) and QuantStudio^{TM} 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 1. The experiments were performed in duplicate for three independent repeats. Melting curve analysis was performed for each primer set

Table 1. Primer sequences for the quantitative PCR

Gene	Sequences	
Cdkn1a	Forward	GCAGACCAGCATGACAGATTT
	Reverse	GATGTAGAGCGGGCCTTTGA
Chkn2a	Forward	GGGTCGGGTAGAGGAGGTG
	Reverse	GCCCATCATCATGACCTGGA
Il-6	Forward	TTCTCCACAAGCGCCTTC
	Reverse	AGAGGTGAGTGGCTGTCTGT
Mmp3	Forward	ACAAAGGATACAACAGGGACCA
	Reverse	GGAACCGAGTCAGGTCTGTG
Cxcl1	Forward	CCCAAACCGAAGTCATAGCCA
	Reverse	CCTCCTCCCTTCTGGTCAGT
Cxcl2	Forward	CTTGCCAGCTCTCCTCCTC
	Reverse	AGGGGCGCTCCTGCT
Ccl8	Forward	ACTTGCTCAGCCAGATTCAGTT
	Reverse	GACCCATCTCTCCTTGGGGT
GAPDH	Forward	ACCCACTCCTCCACCTTTGA
	Reverse	CTGTTGCTGTAGCCAAATTGCT

Western blot analysis.

Cells were seeded at 1x10^{6} cells/ well on a 6-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 concentrations of 0.1% of the samples, and incubated the cells for 48 h. The cells were resuspended in 1X SDS loading buffer (50 mM Tris-Cl, pH 6.8, 2% sodium dodecyl sulfate) and incubated at 95 C for 10 min. The cell lysate was loaded onto a 5–20% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Wako, Tokyo, Japan) and separated in running buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS) for 50 min at 180 V. The separated proteins were transferred onto a nitrocellulose membrane using an Atto Western Blotting System (Atto Corp., Tokyo, Japan) with EZ Fast Blot (Atto Corp.) for 15 min at 0.25 A per mini gel. The membrane was blocked with 5% skim milk at 37 C for 20 min and then washed five times with wash buffer at room temperature for 5 min with shaking. Afterwards, the membrane was incubated with primary antibody at 37 C for 2 h. As the primary antiboy anti-CDKN2A/p16^{INK4a} antibody, anti-p21 antibody, (GeneTex, Irvine, USA) were used. After washing three times with wash buffer at room temperature for 20 min each, the membrane was incubated with a horseradish peroxidase-conjugated anti-mouse IgG 2nd antibody at 37 C for 1 h. The membrane was then washed three times with wash buffer at room temperature (20 min each). The protein bands were detected using EzWestLumi plus (Atto Corp., Tokyo, Japan) and analyzed using the Luminograph II (Atto Corp., Tokyo, Japan).

Melanin content assay

The B16F10 melanoma cells were seeded with 2×10^5 cells/well in 3 ml of medium in 6-well culture plates and incubated overnight to allow cells to adhere. The cells were exposed to various concentrations (50, 100, 150 and 100 ng/ml) of the recombinant aging marker proteins for 48 h. At the end of the treatment, the cells were washed with PBS and lyzed with 800 l of 1 N NaOH (Merck, Germany) containing 10% DMSO for 1 h at 60 ◦C. The absorbance at 420 nm was measured using an EPOCH2 ELISA reader (Bio-Tek, Winooski, VT, USA).

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 recombinatn aging marker protein on skin cell senescence.

To measure the effect of aging markers on skin cellular senescence, the cells were treated of the recombinant aging marker protin, rh GDF15 (100 ng/ml), rh FSTL3 (150 ng/ml) and rh CCDC80 (200 ng/ml). As a result of SA-β-gal staining, it was confirmed that the dyed blue color increased when treated with rh GDF15 and rh FSTL3. This suggests that rh GDF15 and rh FSTL3 promote cellular senescence (Fig. 1A, Upper panel). Furthermore, the effect of aging markers on cellular aging caused by oxidative stress was measured. As a result, SA-β-gal activity increased by H_2O_2 was enhanced when treated with rh rh GDF15, and weakened when treated with rh CCDC80 (Fig. 1A, Lower panel). In addition, to quantitatively measure SA-β- activity the ONPG test was performed. Results similar to those of SA-β-activity were also found in the ONPG test. The O.D value increased by H_2O_2 was further increased by rh GDF15 and decreased by rh CCDC80 (Fig. 1B). From these results, it was confirmed that rhGDF15 promoted cellular senescence and rh CCDC80 inhibited cellular senescence. However, the case of rh FSTL3 showed a different result from the staining result. This should be studied further later.

Effects of regulating SASPs gene expression by the aging markers.

The expression level of SASPs gene was measured in order to find out whether aging markers affect gene expression related to actual cellular senescence. First, rhGDF15 was treated in HDFn cells and gene expression levels were measured for each time point (0h, 3h, 6h, 9h, 15h and 24h). As a result, expression levels of CXCL2, MMP3. IL-6, and Ccl8 genes were increased by rh GDF15 (Fig. 2A). In addition, it was observed that rh CCDC80 suppressed the increase in gene expression of CXCL1, MMP3 and IL-6 by H_2O_2 (Fig. 2C). From the above results, it can be hypothesized that rh GDF15 promotes cellular senescence and rh CCDC80 inhibits cellular senescence caused by oxidative stress.

Effects of the recombinant aging marker protein on ROS generation.

It was speculated that the mechanisms of the aging-promoting effect of the aging marker GDF15 and the aging-inhibiting effect of CCDC80 are related to oxidative stress, and ROS generation was measured to confirm this. The fluorescence of DCFDA staining was explored to confirm whether ROS was induced by the aging markers. When HDFn cells were treated

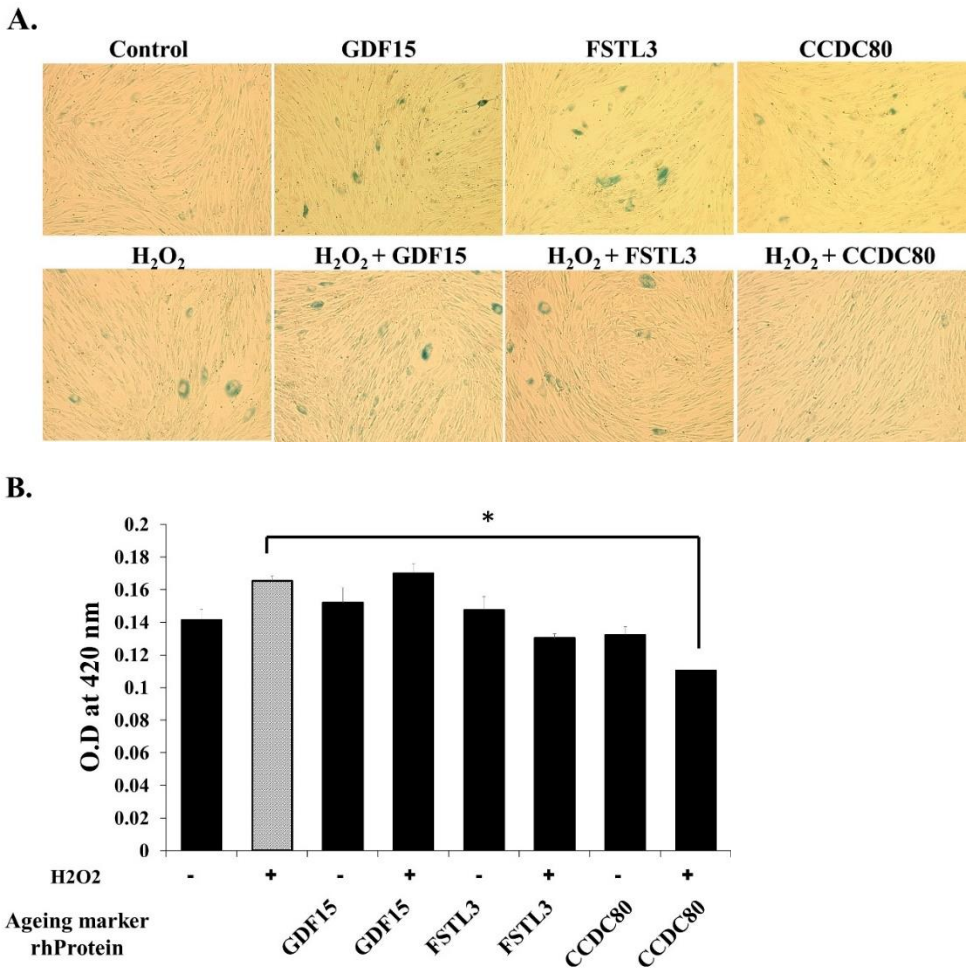

Figure 1. Effect of Aging markers on cell senescence.

SA-β-gal was used to mark the aging cells; the cytoplasm of positive cells was stained blue. Result of (A) the SA-β-gal staining and (B) ONPG test for investigation of the effects of senescence marker proteins and effects of aging marker proteins on cells subjected to oxidative stress. Data are shown as mean±S.D. of three experiments. * <0.05 compared to control; SA-β-gal, senescence-associated β-galactosidase.

with rh GDF15 (100 ng/ml), rh FSTL3 (150 ng/ml) and rh CCDC80 (200 ng/ml) for 48 hours, green fluorescence was observed in the cells treated with rh GDF15 (Fig, 3A). This indicates that ROS was generated by rh GDF15. To evaluate the effect of aging markers on ROS generation in H_2O_2, oxidative stress was applied to HDFn cells using H_2O_2 after 24 hours of pretreatment with aging markers proteins. When H_2O_2 is treated, ROS generation is increased and the green fluorescence intensity is increased. When rh GDF15 was pre-treated, the

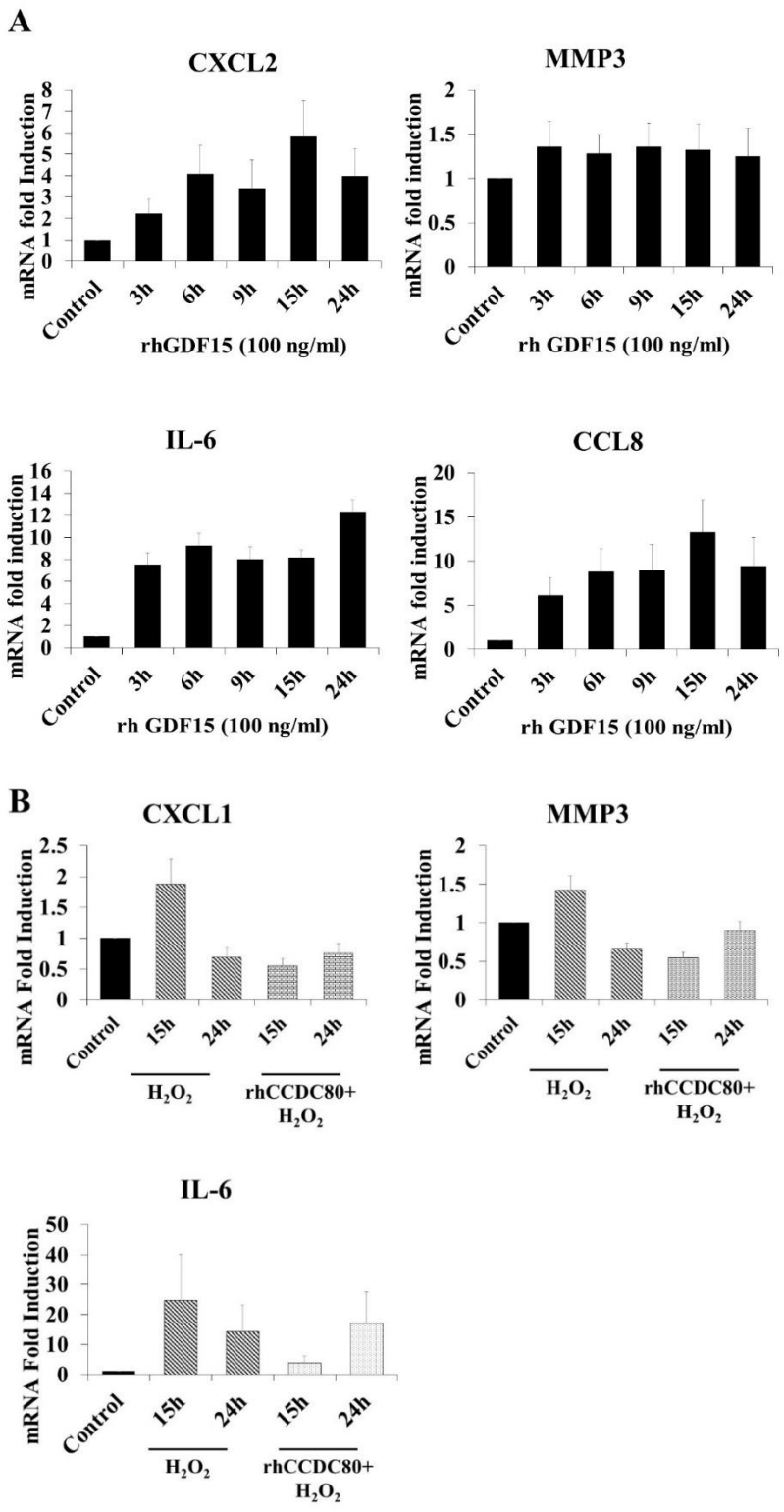

Figure 2. Effect of Aging markers on mRNA expression of SASP components.

(A) The HDFn cells were exposed to rh GDF15. Total RNA was extracted on the indicated time after treatment. CXCL1, CDKN1a, CDKN2a, IL-6, MMP3 and CCL8 mRNA levels were then determined by quantitative RT-

PCR analysis. (B) The HDFn cells treated with rh CCDC80 for 24 hours were treated with H_2O_2 to give oxidative stress, and total RNA was extracted 15h and 24h later. CXCL1, MMP3 and IL-6 mRNA levels were then determined by quantitative RT-PCR analysis. The levels of the indicated mRNA were normalized to that of GAPDH mRNA and shown as fold induction by 0h. Data are shown as mean±S.D. of three experiments. * <0.05 compared to control

intensity of green fluorescence was increased compared to when H_2O_2 was treated alone. Conversely, when rh CCDC80 was pretreated, the fluorescence intensity was decreased compared to when H_2O_2 was treated alone (Fig. 3B). As a result, it was confirmed that rh GDF15 induces ROS generation and promotes ROS generation under oxidative stress, and rhCCDC80 has the effect of inhibiting ROS generation due to oxidative stress. This means that GDF15 and CCDC80 are involved in ROS generation and affect cell senescence.

Effects of GDF15 and CCDC80 on p16 signaling pathway

First, gene expression levels of CDKN1a (p21) and CDKN2a ($p16^{INK4a}$) related to the p16/p21 signaling pathway were measured at the mRNA level. As a result, rh GDF15 increased the gene expression levels of CDKN1a (p21) and CDKN2a ($p16^{INK4a}$) in a time-dependent manner in HDFn cells. On the other hand, by rh CCDC80, it was confirmed that the gene expression level of CDKN2a ($p16^{INK4a}$) was significantly increased only at 24 hours (Fig. 4A). Furthermore, when the protein expression levels of CDKN2a ($p16^{INK4a}$) and CDKN1a (p21) were confirmed through western blot, the results were similar to those of mRAN expression (Fig. 4B). The rh GDF15 increased the prtoein expression levels of CDKN1a (p21) and CDKN2a (p16INK4a). On the other hand, the protein expression level of CDKN2a (p16INK4a) was increased by rh CCDC80, but CDKN1a (p21) was decreased. Through this, we were able to confirm that GDF15 and CCDC80 affect cell senescence through opposing actions on p21.

Effects of the recombinant aging marker protein on skin pigmentation

As the skin ages, it darkens and becomes dull. To confirm whether the aging marker is involved in this aspect of skin aging, the amount of melanin synthesis by the aging marker protein was confirmed. B16F10 cells were treated with recombinant aging marker protein by concentration (50, 100, 150, and 200 ng/ml), and the amount of melanin synthesis was measured. As a result, the amount of melanin synthesis increased by rh GDF15 (50 and 100

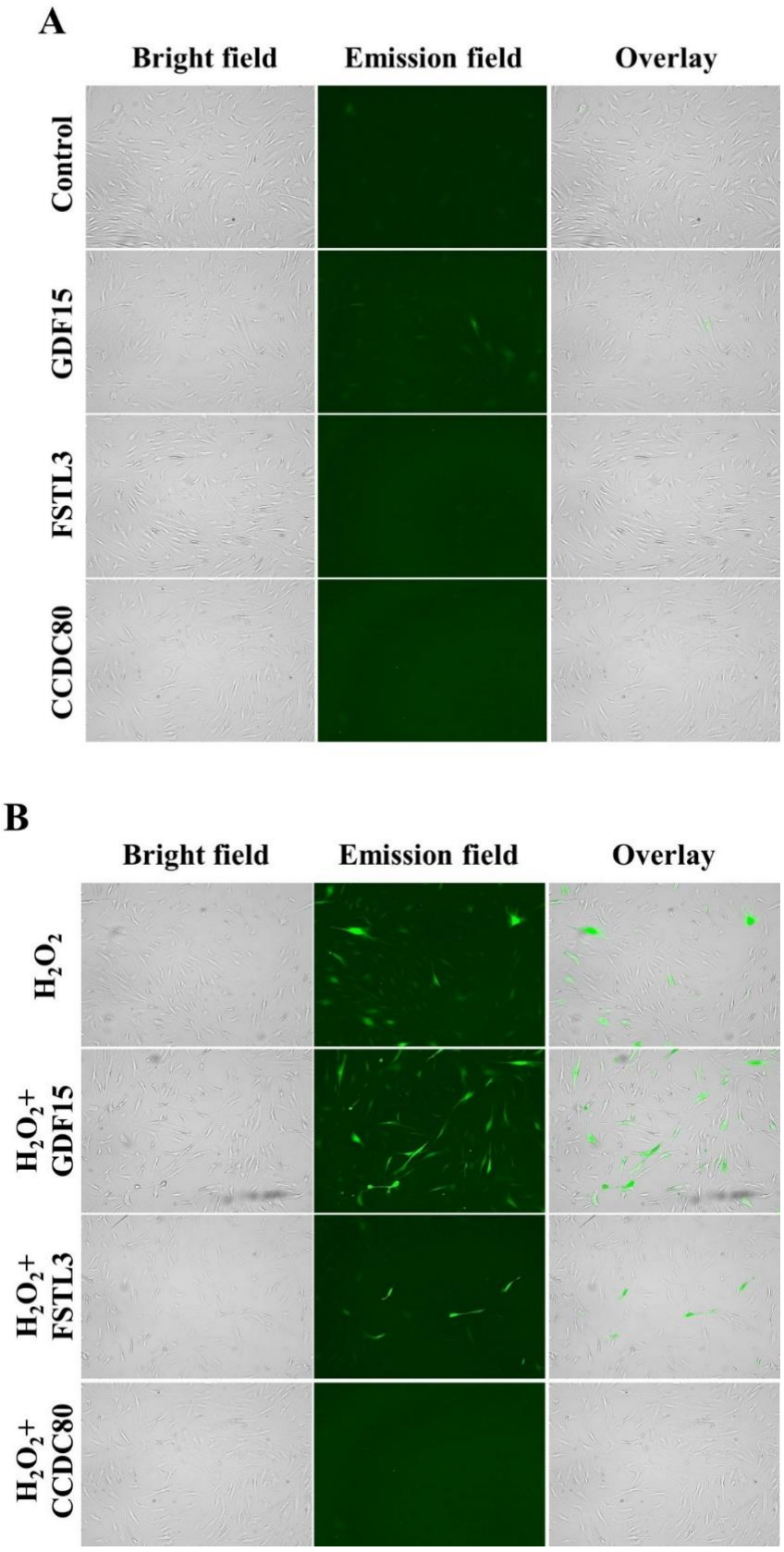
A
Bright field
Emission field
Overlay
Control
GDF15
FSTL3
CCDC80
B
Bright field
Emission field
Overlay
H2O2
H2O2+ GDF15
H2O2+ FSTL3
H2O2+ CCDC80

Figure 3. Effect of Aging markers on cell senescence.

Intracellular ROS was measured by DCFDA staining after recombinant aging markers protein and H_2O_2. Representative fluorescence images of (A) cells treated with recombinant aging markers protein alone and (B) oxidative stress applied after pretreatment with recombinant aging markers protein. The green fluorescence indicates ROS concentration in the cells.

ng.ml). This suggests that GDF15 not only promotes cellular aging but also increases melanin synthesis, thereby causing overall aging of the skin.

Discussion.

Normal somatic cells undergo a limited number of divisions in culture and then reach a state of irreversible proliferation inhibition, which is called replicative senescence, and this cellular senescence ultimately contributes to 'Aging' [7]. Cellular senescence is induced by several factors such as DNA damage, telomere shortening, and ROS generation. Senescent cells do not proceed with the cell cycle further and are inhibited, and cell proliferation does not resume again and has resistance to apoptotic signals. Because of the senescent cells are in a stable state and are stably accumulated in the body as they age. Cells increase the expression of cell cycle inhibitors, $p21^{WAF1,}$ and $p16^{INK4a}$. $P21^{WAF1}$ is a transcriptional target of p53 and causes proliferation inhibition. $p16^{INK4a}$ prevents further cell cycle progression by preventing phosphorylation and inactivation of Rb. In addition, the expression level of a protein that can change the microenvironment by reconstituting the cell-matrix or inflammation when the cell is aging is also increased in senescent cells [8, 9].

The final goal of cosmetic research is to prevent skin aging and restore aging skin. In general, the anti-aging effect of cosmetic ingredients refers to the effect of increasing collagen synthesis or inhibiting collagen degrading enzymes. However, aging of the skin is a more complex phenomenon and cannot be taken care of by simply controlling the amount of collagen production. Therefore, we wanted to develop a new strategy that can more fundamentally prevent and treat skin aging. As part of this effort, in a previous study, we discovered four types of aging marker genes, FSTL3, GDF15, MMP12, and CCDC80, whose

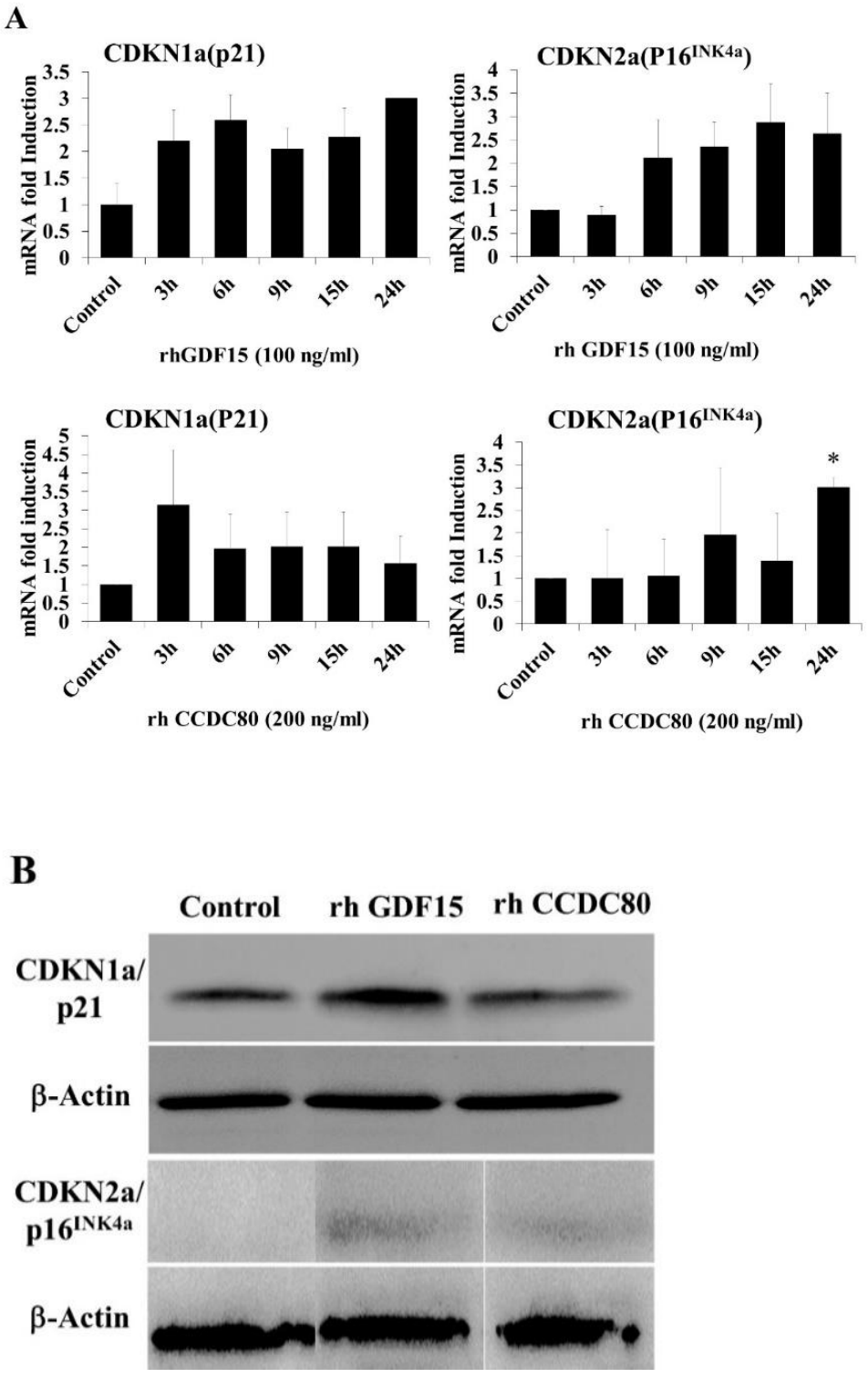

Figure 4. Effect of Aging markers on p16 signaling pathway.

Analysis of mRNA(A) and protein(B) expression levels of The CDKN1a (p21) and CDKN2a ($p16^{INK4a}$)

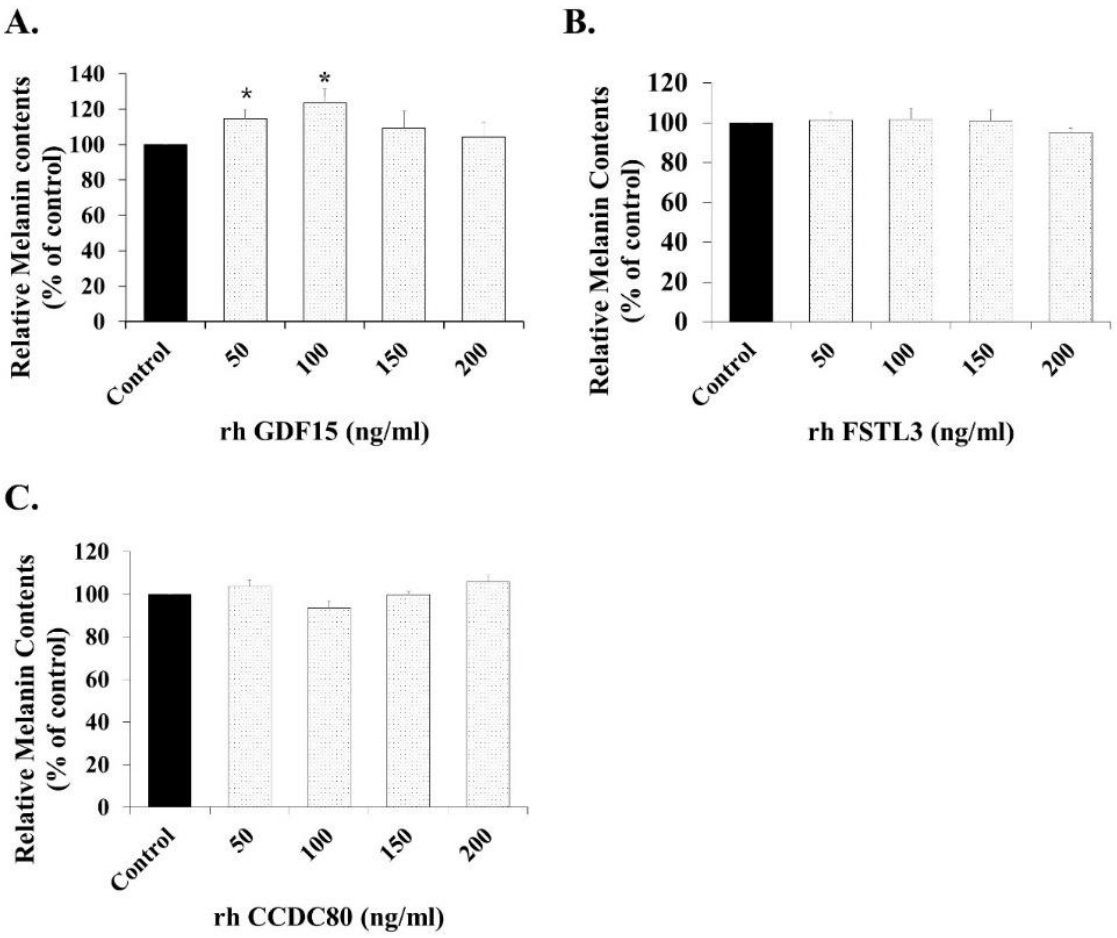

Figure 5. Effect of Aging markers on the regulation of pigmentation.

Analysis of melanin content in B16F10 melanoma cells incubated for 48 h with (A) rh GDF15, (B) rh FSTL3 and (C) rh CCDC80. Data are shown as mean±S.D. of three experiments. * <0.05 compared to control.

expression in skin cells changes significantly during the 1st aging peak that occurs around the age of 40. MMP12 as an elastase was confirmed to increase with the aging of skin cells, and it can promote the degradation of elastin in the skin and cause a decrease in skin elasticity. However, in the case of FSTL3, GDF15, and CCDC80, except for MMP12 among the four aging markers, what function induces skin aging is not yet known. Therefore, we conducted this study to investigate the function of aging markers in skin aging by elucidating the correlation between these aging markers and cellular senescence.

In this study, it was confirmed that among the aging markers involved in the first peak of skin aging, GDF15 induces cellular senescence and CCDC80 inhibits cellular senescence.

Senescence-associated (SA) β-galactosidase activity, known as a marker of cellular senescence, is indicated by increased expression of lysosomal β-galactosidase in senescent cells. GDF15 increased the SA-β-gal staining intensity in HDFn cells. (Fig. 1A).

In the ONPG test, ONPG value did not increase with GDF15 alone, but when oxidative stress induced cellular senescence, the ONPG value increased in the experimental group pretreated with GDF15 compared to when H_2O_2 was treated alone (Fig1. B). This suggests that GDF15 can induce cellular senescence alone or promote cellular senescence by several factors,ROS generation. On the other hand, CCDC80, when treated alone, contrary to GDF15, SA- β -gal activity or ONPG values were not affected, but when oxidative stress induced senescence, SA- β -gal activity and ONPG values were decreased (Fig. 1A, B). Through this, it was found that GDF15 aggravates cellular senescence caused by ROS generation, and CCDC80 attenuate it on the contrary. Next, it was confirmed through an increase in SASPs gene expression that GDF15 induces cellular senescence (Fig. 2).

As a result of the SA-b-gal assay, it can be estimated that there is a correlation between GDF15 and ROS generation of CCDC80. To confirm this, a DCFDA test that can confirm ROS generation by fluorescence was performed. ROS was generated when GDF15 alone was treated, which observed that ROS generation occurred more strongly under oxidative stressed cells (Fig. 3A). On the other hand, CCDC80 inhibited ROS generated by oxidative stress (Fig. 3B). Through this, it was expected that aging markers GDF15 and CCDC80 could control skin aging through opposing mechanisms related to ROS generation.

Based on the above results, we hypothesized that GDF15 and CCDC80 would regulate skin aging through opposing actions. p16 signaling was confirmed to check whether they are involved in the same signaling process and regulate ROS generation to induce or inhibit skin aging. Among many signaling mechanisms, CDKN1A (p21) and CDKN2A ($p16^{INK4a}$) were identified. The long-term activation of the checkpoint gene CDKN1A (p21) induces mitochondrial dysfunction and production of reactive oxygen species (ROS). In addition, it is known for its ability to suppress ROS generation when konkout CDKN2a. When GDF15 was treated alone, CDKN1A (p21) and CDKN2A ($p16^{INK4a}$) mRNA and protein expression levels in HDFn cells all increased. CCDC80 decreased the expression level of CDKN1A

(p21) while increasing the expression level of CDKN2A ($p16^{INK4a}$) (Fig. 4A, B). Through this, we found that GDF15 and CCDC80 are involved in skin cell aging through opposing roles in ROS generation and signaling processes related to cellular senescence. The expression level of MMP3 increases in p21-knockout (p21-/-) mice. Looking at the expression level of the MMP3 gene confirmed in FIG. 3, the expression level of the MMP3 gene increased by GDF15, and the MMP3 gene increased by H2O2 was observed to decrease by CCDC8. It is likely that p21 is involved in the regulation of this MMP3 gene expression. Among the various phenomena related to skin aging, as a result of testing the amount of melanin production to confirm the relationship between the aging markers and the skin becoming dull with age, it was confirmed that the amount of melanin synthesis of B16F10 increased by GDF15. As a result of another study by our research group, increased GDF15 in aged fibroblasts activates melanin biosynthesis of melanocytes, as well as reveals that there is a correlation with SDF-1, which is involved in the recently revealed mechanism of dark spots.

Collectively, the skin aging markers we found through previous studies may be involved in cellular senescence, regulating the overall signs of skin aging. In particular, CCDC80, one of the markers of aging, its expression is significantly reduced in senescent cells, and it has an anti-aging effect through the opposite action to GDF15, which induces aging. Therefore, CCDC80 will play an important role as a target for the development of a new anti-aging ingredient.

Conclusion.

We developed a technology to dramatically prevent aging by using aging markers that act during the 1st aging peak, when the skin is rapidly aging. We found that the skin aging markers, GDF15 and FSTL3, promotes cell senescence by inducing ROS generation through the p16 signaling pathway. On the other hand, it was confirmed that CCDC80 can inhibit aging through the effect of inhibiting ROS generation. In addition, it was confirmed that melanin synthesis that darken the skin tone were promoted by the aging marker GDF15. Through this, the aging markers we discovered can be used as a new target for the

development of ingredient that can comprehensively care for skin aging in various fields, from wrinkles and elasticity to whitening and cellular senescence.

Acknowledgments.

Conflict of Interest Statement. NONE.

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