



ORIGINAL ARTICLE

Synthesized Ceramide Induces Growth of Dermal Papilla Cells with Potential Contribution to Hair Growth

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Background: The ceramide is known to play an important role in the formation of intracellular lipids, and play a crucial role as a barrier for skin and hair cuticle. Recent study has revealed that ceramide has potential effect on hair growth in a mouse model. However, the role of ceramide in human dermal papilla cells (hDPCs) known to play an important role in hair growth is not well understood yet. **Objective:** The goal of this study was to investigate the effect of synthetic ceramides (oleyl and stearyl ceramides) on hair growth using hDPCs. **Methods:** hDPCs were treated with synthesized ceramides. hDPCs viability was evaluated by MTT assay. The expression of hair growth related factors were investigated by western blot, real-time polymerase chain reaction and growth factor array. The expression of β -catenin was confirmed by immunofluorescence. **Results:** Treatment with ceramides increased the expression of proteins affecting cell proliferation such as Bcl-2, BAX, phosphorylated-ERK and Cyclin D1. Also, ceramides treatment were increased the expression of several growth factors, including epidermal growth factor family, and promote the expression of Wnt/ β -catenin and BMP2/4 signaling. **Conclusion:** Our data suggest that synthetic ceramides stimulates hair growth by induction proliferation of hDPCs via modulation of Wnt/ β -catenin and BMP2/4 signaling. (*Ann Dermatol* 31(2) 164 ~

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-Keywords-

Ceramide, Dermal papilla cell, Hair, Wnt signaling pathway

INTRODUCTION

Hair aging is a complicated process involving various factors. The study of hair aging is divided into two main streams¹. One is an intrinsic biological process at cellular level that is directly linked to hair growth. For hair growth and cycling, interaction between human dermal papilla cells (hDPCs) and mesenchymal stem cells in hair follicles and various growth factors are essential². The other one is an extrinsic process that happens outside the scalp¹. Extrinsic process is highly related to the cuticle layer. The cuticle exists at the outermost part of the hair. It protects the hair shaft from external mechanical stress. The cuticle layer is damaged by external stress such as ultraviolet rays, smoking, and malnutrition. It is known that when the cuticle layer is damaged, aging process of the hair can be initiated easily³.

In terms of structure of hair cuticle which protects hair from extrinsic stimuli, ceramide is the most important intracellular lipid that constitutes the lipid layer of hair cuticle along with fatty acid and cholesterol⁴. Ceramide is a component of intercellular lipids that are present in hair cuticles to protect and strengthen internal components of hair. Moreover, ceramide is a crucial second signal molecule that regulates various signaling pathways, including cell cycle, cellular senescence, and apoptosis⁵.

Previous studies have shown that ceramide has an antagonistic effects for cell growth and cell survival⁶. However, recently it has been reported that ceramide can affect hair

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loss prevention and hair growth enhancement⁷. In particular, ceramide synthase 4 (CerS4) plays an important role in the maintenance of epidermal stem cell homeostasis and regulation of the hair cycle by regulating Wnt signal and BMP signal⁸. Although ceramide can affect Wnt/BMP balance *in vivo*⁸, the effect of ceramide on growth pathway such as Wnt/BMP balance *in vitro* has not been confirmed.

Therefore, the objective of this study was to investigate the effect of ceramide on hDPCs at cellular level. To investigate the effect of existing natural C8-ceramide (*N*-octanoyl sphingosine) and ceramide-like synthetic compound on hair growth, we evaluated the hair growth pathway including Wnt/ β -catenin and BMP signaling changes after stimulation with ceramide. Three types of ceramide were used in this experiment: C8-ceramide and two newly synthetic ceramides (oleyl and stearyl ceramide). These newly synthesized ceramides are different from C8-ceramide in that they have higher solubility through double bonds and carbon chain length.

MATERIALS AND METHODS

Ceramide material preparation

C8-ceramide was purchased from Cayman Chemical (Ann Arbor, MI, USA). Oleyl ceramide was synthesized by reacting one equivalent of 2-dodeceny succinic anhydride and oleyl amine in methylene chloride at room temperature (RT) for 30 minutes. The reaction mixture was then extracted with ethanol and water and recrystallized to a pale yellow paste, with a purity of 95% and a yield of 98%. Structure of the compound was confirmed by ¹H-NMR and LC-MSMS spectrometer. ¹H-NMR spectra were recorded on a ZEOL 400 MHz FT-NMR spectrometer in CDCl₃. LC-MSMS spectra were recorded on a Shiseido HTS HPLC system and an AB SCIEX API 3200 triple quadrupole mass spectrometer. LC-MSMS separations module with electrospray ionization was carried out in positive ion mode. Stearyl ceramide was synthesized in the same manner using stearyl amine (Fig. 1A). All ceramides were dissolved in ethanol to obtain concentration of 1 mM and store at -20°C.

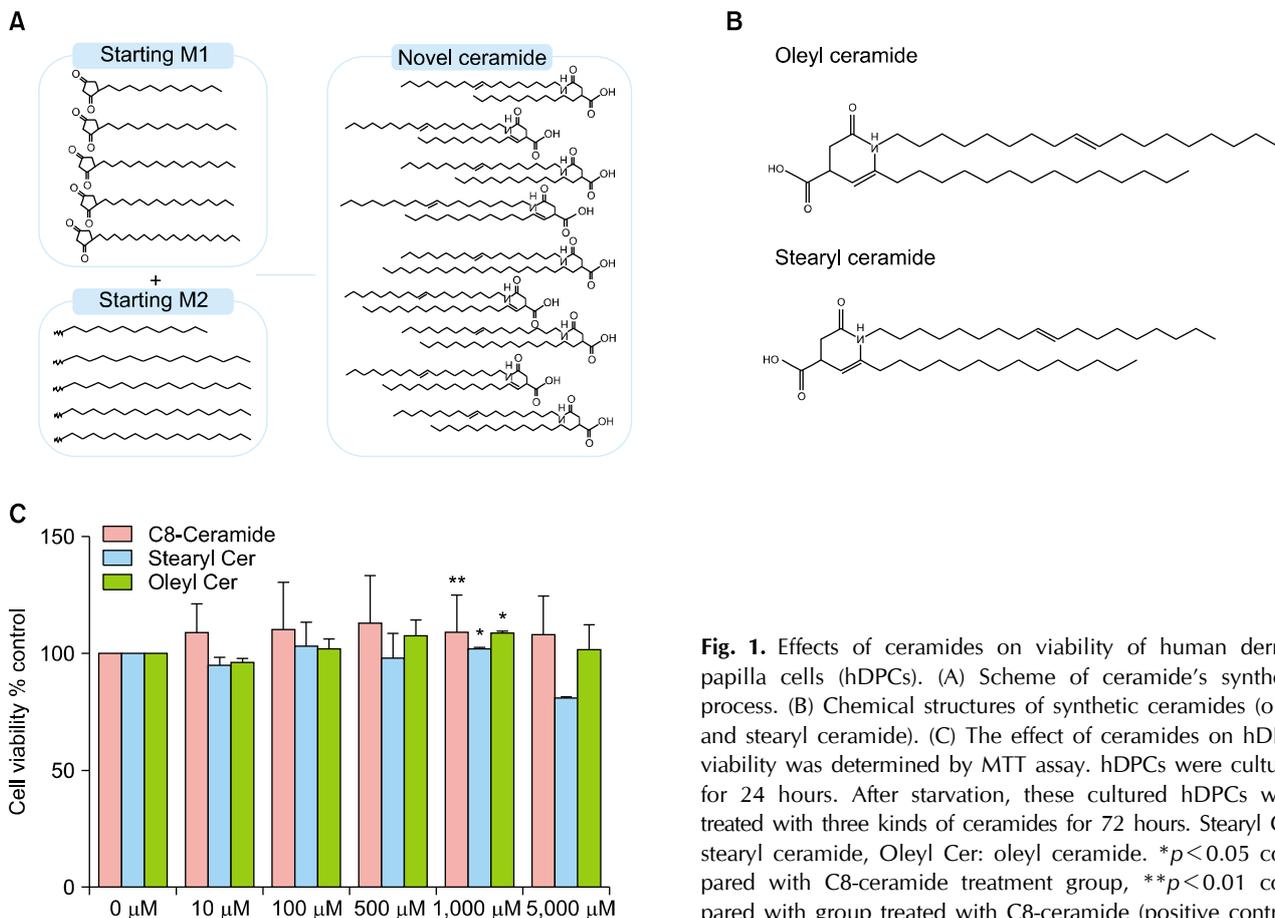


Fig. 1. Effects of ceramides on viability of human dermal papilla cells (hDPCs). (A) Scheme of ceramide's synthesis process. (B) Chemical structures of synthetic ceramides (oleyl and stearyl ceramide). (C) The effect of ceramides on hDPCs viability was determined by MTT assay. hDPCs were cultured for 24 hours. After starvation, these cultured hDPCs were treated with three kinds of ceramides for 72 hours. Stearyl Cer: stearyl ceramide, Oleyl Cer: oleyl ceramide. * $p < 0.05$ compared with C8-ceramide treatment group, ** $p < 0.01$ compared with group treated with C8-ceramide (positive control).

Cell culture

hDPCs were purchased and cultured in Follicle Dermal papilla cell growth medium (PromoCell GmbH, Heidelberg, Germany) supplemented with 10% fetal bovine serum and 1% penicillin and placed in an incubator at 37°C with 5% CO₂.

Cell viability assay (MTT assay)

The viability of hDPCs was evaluated by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, hDPCs were seeded into 24-well plates at density of 1.0×10^4 cell per well. After 48h of incubation for hDPCs to reach confluency, three kinds of ceramides (C8-ceramide, oleyl, and stearyl ceramides) at various concentrations of 10 μ M, 100 μ M, 500 μ M, 1,000 μ M and 5,000 μ M were used for treatment for 24 hours, 48 hours, and 72 hours under 1% penicillin conditions. After incubation, 100 μ l of MTT solution was added to each well and the hDPCs were then incubated at 37°C for 4 hours. After the medium was aspirated, 400 μ l DMSO was added to each well. The absorbance of the plate was measured at wavelength of 540 nm using an ELISA plate reader.

Real time polymerase chain reaction

RNA was isolated from cultured hDPCs using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) and cDNA synthesis was performed using a cDNA synthesis kit (Takara, Shiga, Japan) according to the manufacturer's instructions. The cDNA was used for real-time polymerase chain reaction (PCR) which was carried out with SYBR Green (Bio-Rad, Hercules, CA, USA). Sequences of primers used in real-time PCR are listed in Table 1.

Western blot analysis

The protocol for western blot analysis was described in a previous report⁹. Briefly, protein lysates from hDPCs were prepared using radio immunoprecipitation assay cell lysis buffer containing protease inhibitor cocktail. The cell lysate was collected by scraping after removed cell debris from lysed cells. Protein concentration was determined using a Bradford assay. Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and blotted onto polyvinylidene fluoride membrane. The membrane was blocked with 5% BSA/TBST reagent for 1 hour at RT. It was then incubated with primary antibodies against total ERK, total Akt, phosphorylated-ERK, Akt, and GSK3 β , Cyclin-D1, β -catenin (Cell Signaling Technology, Beverly, MA, USA), Bcl-2, BAX, and β -actin (Santa Cruz Biotechnology Inc., Dallas, TX, USA) overnight at 4°C on a rotary shaker. The blotted membrane was then visualized with an AI600 Bio-Image analysis system (GE Healthcare Life Science, Chicago, IL, USA).

Growth factor array

Synthetic ceramides were screened for their effects on the expression of growth factors by using Human Growth Factor Antibody Array G1 (RayBiotech Inc., Norcross, GA, USA) in accordance with the manufacturer's protocol.

Immunofluorescence staining

Cultured hDPCs were seeded on 4-well chamber slides at density of 5×10^3 cells per well (SPL Life Science, Pochon, Korea). After starvation, hDPCs were treated with C8-ceramide, oleyl, and stearyl ceramides at concentration of 1,000 μ M for 24 hours. After aspirating the medium, cells were rinsed with 1X PBS three times for 5 mi-

Table 1. List of primers and real-time polymerase chain reaction conditions

Species	Primer name	Forward	Reverse
Human	β -catenin	TTTAAGCCTCTCGGTCTGTG	CAAATACCCTCAGGGGAACA
	Wnt3	CCTCAAGGACAAGTACGACA	GGCACCTTGAAGTAGGTGTA
	Wnt5a	AATTCTGGCTCCACTTGTG	CAATTACAACCTGGGCGAAG
	Wnt7a	TCTGTAACAAGATCCCAGGC	CATTGCGGAAGTAAAAGTGA
	AP1	TGTCCGAGAAGTAAAGCCAA	AAAAGTCCAACGTTCCGTTT
	Lef1	CAGTGACGAGCACTTTTCTC	CGTGATGGGATATACAGGCT
	Axin2	CAGTGGATGCTGGAGAGTGA	TGCCAGTTTCTTTGGCTCTT
	BMP2	CAGGTCCTTTGACCAGAGTT	CCATGGTCGACCTTTAGGAG
	BMP4	ATTCCCGTCCAAGCTATCTC	CTACGGAATGGCTCCATAGG
	GAPDH	GAAGGTGAAGGTCGGAGTCAA	GCTCCTGGAAGATGGTGATG

β -catenin: beta-catenin, Wnt3: wingless type MMTV integration site family, member 3, Wnt5a: wingless type MMTV integration site family, member 5A, Wnt7a: wingless type MMTV integration site family, member 7a, AP1: activator protein 1, Lef1: lymphoid enhancer-binding factor-1, Axin2: axin-related protein 2, BMP2: bone morphogenetic protein 2, BMP4: bone morphogenetic protein 4, GAPDH: glyceraldehydes 3-phosphate dehydrogenase.

minutes each time. Then hDPCs were fixed with 4% paraformaldehyde for 15 minutes and permeabilized with 0.1% Triton X-100 for 10 minutes. After washing with 1X PBS, cells were blocked with 1% BSA in 1X PBS for 1 hour at RT on a shaker. They were incubated with anti-rabbit β -catenin (Cell Signaling Technology) primary antibody diluted at 1:100 in blocking buffer and incubated at 4°C overnight. On the next day, they were incubated with Alexa Fluor 488 labeled goat anti-rabbit secondary antibody (Invitrogen) diluted at 1:200 in PBST at RT for 1 hour in the dark. These stained cells were mounted with mounting medium containing DAPI to counterstain nuclei. Cells were then observed with a fluorescence microscope (Axiovert 200; Zeiss, Oberkochen, Germany).

Statistical analysis

All statistical data analyses were carried out using Graphpad Prism 5 Software. One-way analysis of variance (ANOVA) was used to evaluate significant difference at $p < 0.05$.

RESULTS

Viability of hDPCs after treatment with ceramides

To investigate the effect of synthetic ceramides (Fig. 1B) on viability of hDPCs, we performed MTT assay. Firstly, optical concentrations of oleyl and stearyl ceramides and C8-ceramide as a positive control were determined. hDPCs were treated with various concentrations (0 μ M, 10 μ M, 100 μ M, 500 μ M, 1,000 μ M, and 5,000 μ M) of oleyl and stearyl ceramides, and C8-ceramide for 72 hours. Results showed that oleyl and stearyl ceramides inhibited cell viability at 5,000 μ M (Fig. 1C). However, viability of hDPCs was increased after treatment with C8-ceramide (110%), oleyl ceramide (109%), and stearyl ceramide (102%) at 1,000 μ M. Based on these results, 1,000 μ M was chosen as the proper concentration of ceramides for further experiments.

Ceramides regulates Bcl-2/BAX and ERK/Cyclin-D1 in hDPCs

We examined expression levels of Bcl-2 and BAX known as apoptosis regulator genes by western blot analysis. Results showed that Bcl-2 expression was significantly enhanced by treatment with ceramides compare to that in the control group. However, treatment with ceramides showed no significant effect on BAX expression (Fig. 2A). When the ratio of Bcl-2 to BAX was compared, there was no significant difference between C8-ceramide treatment group and the control group. However, in groups treated with the two newly synthesized ceramides, the ratio of Bcl-2 to BAX was increased 1.22-fold in the group treated

with oleyl ceramide and 1.53-fold in the group treated with stearyl ceramide. Such increases were statistically significant.

ERK is also an important factor involved in cell survival and proliferation^{10,11}. We examined the effect of ceramides on phosphorylated-ERK in order to determine if our synthetic ceramides could affect mitogen-activated protein kinase (MAPK) signaling involved in the growth of hDPCs. Protein expression level of phosphorylated-ERK was increased following treatment with ceramides (Fig. 2B). PD98059, an ERK inhibitor, is known to regulate the proliferation of hDPCs. hDPCs were pretreated with ERK inhibitor PD98059 (10 μ M) for 1 hour and then incubated with ceramides for 24 hours.

Treatment of PD98059 reduced the overall activation of ERK compared to the untreated group. In Fig. 2C, phosphorylated-ERK expression was abolished in the PD98059 treated group compared to the untreated group. Also, we tested whether the ceramides affected phosphorylation tendency to aggravate. As a results, in the ceramide-treated group, the expression of phosphorylated-ERK was suppressed compared with Fig. 2B, but the expression was increased as compared with the control group.

Our results revealed that the expression level of Cyclin-D1 was increased after treatment with C8-ceramide, oleyl, and stearyl ceramide (Fig. 2D). These results indicate that our synthetic ceramides could regulate proliferation of hDPCs by regulating Bcl-2/BAX ratio and ERK/Cyclin-D1.

Ceramide treatment upregulates growth factors including epidermal growth factor family

Growth factors were assessed using growth factor membrane microarray (RayBiotech Inc.) according to the manufacturer's instructions. As shown in Fig. 3A, treatment with oleyl and stearyl ceramides increased growth factors levels overall. Epidermal growth factor (EGF) family members such as amphiregulin (AR), EGF, EGF receptor (EGFR), and insulin-like growth factor 1 (IGF-1) are known to stimulate nuclear translocation of β -catenin.

We confirmed that the IGF-1, EGF and EGFR expressions were increased in response to ceramides-treatment (Fig. 3B). AR expression was decreased by C8-cer treatment, but oleyl and stearyl ceramide-treatment increased AR expression.

Ceramides activates β -catenin pathway in hDPCs

We focused on the β -catenin pathway involved in hair growth. The level of phosphorylated-Akt was significantly increased by treatment with ceramides compared to that in the control group (Fig. 4A). Our results also showed that oleyl and stearyl ceramides had a reversal effect on

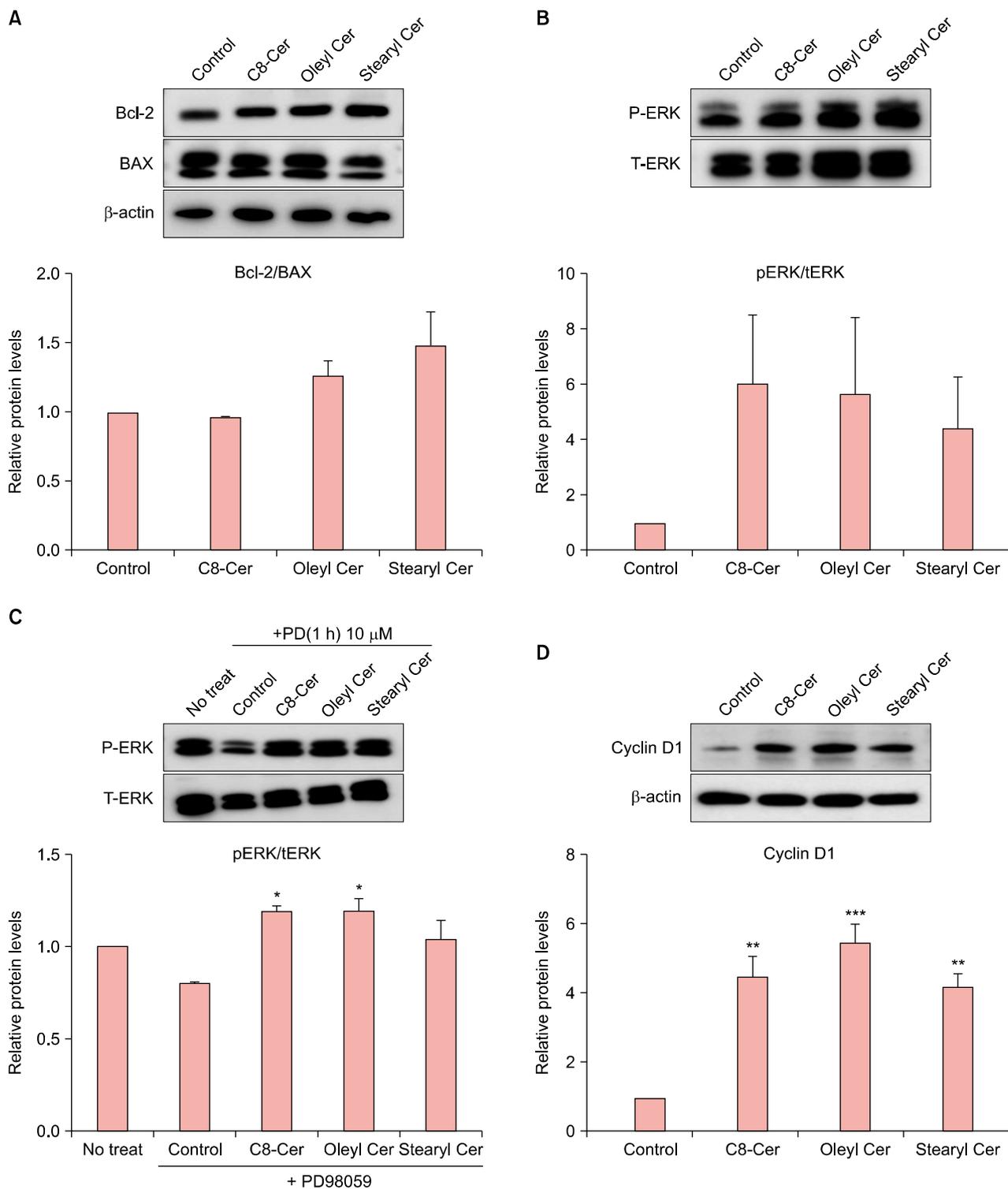


Fig. 2. Ceramide increases expression levels of Bcl-2/BAX and ERK/Cyclin-D1. (A) Expression levels of various factors known to affect proliferation were examined by western blot. Bcl-2 expression in groups treated with oleyl and stearyl ceramides was increased compared to those of the control group and C8-ceramide group. (B~D) Phosphorylated-ERK was normalized relative to total ERK. Cyclin-D1 was normalized relative to β -actin. Western blot results showed upregulated levels of Cyclin-D1 and phosphorylated ERK after treatment with ceramides compared to the control group. Results are expressed as mean \pm standard deviation. C8-Cer: C8-ceramide, Stearyl Cer: stearyl ceramide, Oleyl Cer: oleyl ceramide. * $p < 0.05$ compared with the control, ** $p < 0.01$ compared with the control, *** $p < 0.001$ compared with the control.

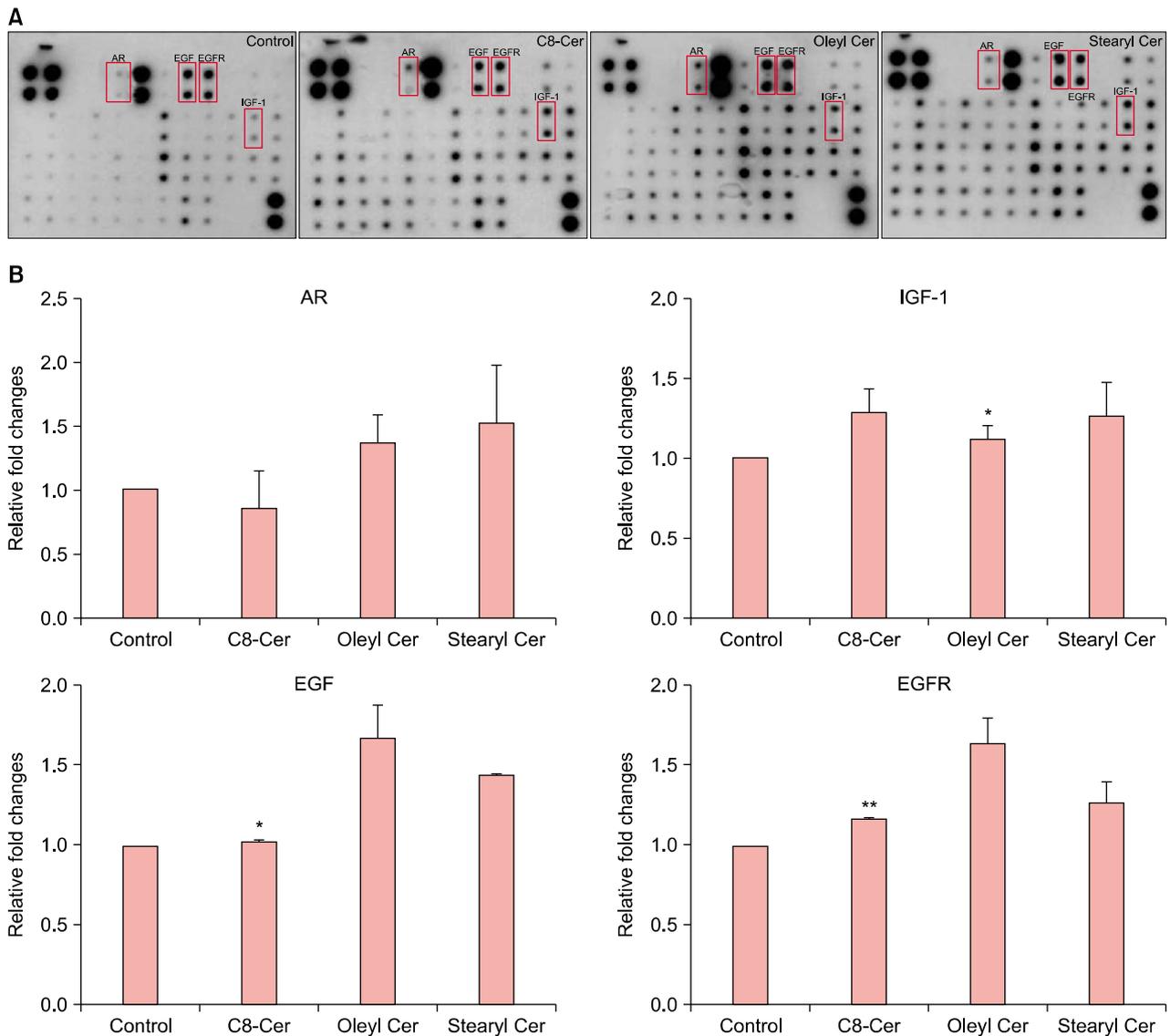


Fig. 3. (A) Ceramides upregulate growth factor protein levels. Human growth factor antibody array was used to detect growth factor expression in human dermal papilla cells (hDPCs). (B) Ceramide treatment significantly increased the expression of growth factors in hDPCs. Results are expressed as mean \pm standard deviation. C8-Cer: C8-ceramide, Stearyl Cer: stearyl ceramide, Oleyl Cer: oleyl ceramide, AR: amphiregulin, IGF-1: insulin-like growth factor 1, EGF: epidermal growth factor, EGFR: receptor.. * $p < 0.05$ compared with the control, ** $p < 0.01$ compared with the control.

LY294002, an Akt inhibitor. They induced a decrease in phosphorylation of Akt (Fig. 4A). Our results showed that protein expression level of phosphorylated-GSK3 β was increased by treatment with ceramides including C8-ceramide, oleyl, and stearyl ceramides (Fig. 4B).

Western blot results showed that β -catenin expression was increased in groups treated with ceramides compared to that in the control group (Fig. 4B). Additionally, our immunofluorescent staining results confirmed the expression of β -catenin in the nucleus. Interestingly, the expression of β -catenin in the nucleus was very weak in the control

group untreated with ceramides whereas the expression of β -catenin in the nucleus was significantly increased in groups treated with synthetic ceramides. In particular, its expression in the nuclei and cytoplasm in groups treated with synthetic ceramides (oleyl and stearyl ceramides) was significantly increased compared to that in the control group (Fig. 4C). These results suggest that our synthetic ceramides could induce β -catenin expression.

Wnt/ β -catenin and BMP signaling in hDPCs

Wnt signaling is known as the master regulator of hair

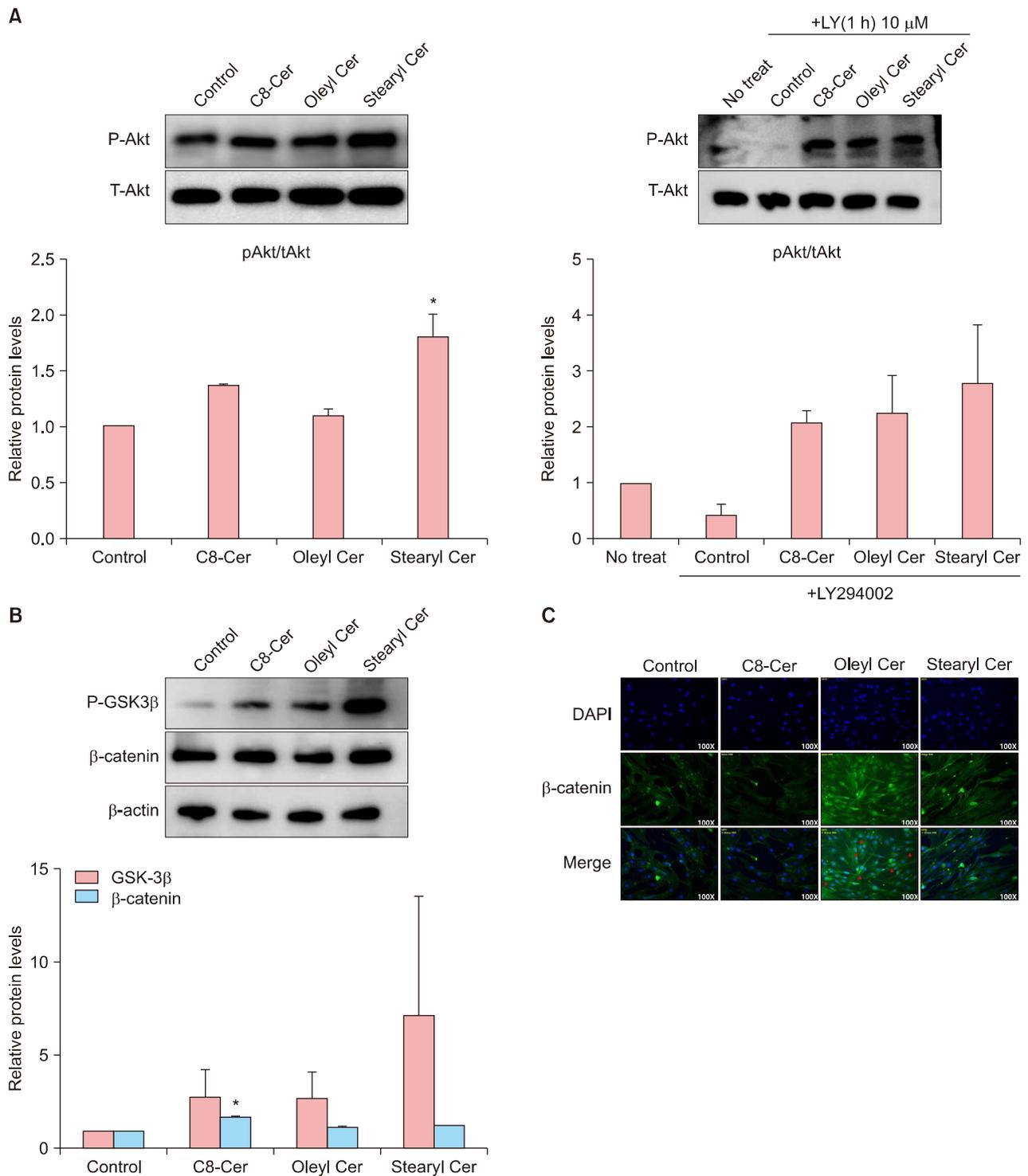


Fig. 4. Effects of ceramide treatment on B-catenin pathway. Phosphorylation of Akt/GSK3 β and β -catenin expression was determined by western blot. Graph was schematized data of western blot band. (A, B) Phosphorylation of Akt/GSK3 β and β -catenin expression was increased by ceramide treatment compare to those in the control. (C) β -catenin expression in the nucleus of human dermal papilla cells was determined by immunofluorescence staining. Oleyl and stearyl ceramides significantly increased β -catenin expression in nuclei compare to C8-ceramide treatment or control. DAPI (blue) was used to counterstain nuclei ($\times 100$). Results are expressed as mean \pm standard deviation. C8-Cer: C8-ceramide, Stearyl Cer: stearyl ceramide, Oleyl Cer: oleyl ceramide. * $p < 0.05$ compared with the control.

growth and hair cycle while β -catenin is a key transcription factor activated by Wnt signaling¹². BMP signaling is known to regulate stem cell activation during hair regeneration¹³. To determine whether our synthetic ceramides might affect Wnt/ β -catenin and BMP signaling, we conducted real-time PCR analysis. As shown in Fig. 5, treatment with stearyl ceramide increased Wnt signaling such as mRNA expression level of wnt3a (2.1-fold), wnt5a (1.8-fold), and wnt10b (6.1-fold) (Fig. 5A). Treatment with stearyl ceramide also significantly increased β -catenin signaling, including mRNA expression level of β -catenin (5.5-fold), AP-1 (3.2-fold), Lef-1 (3.8-fold), and Axin2 (2.2-fold) compared to the control group (Fig. 5B). Moreover, treatment with oleyl ceramide increased mRNA expression level of wnt3a, wnt5a, and wnt7a (Fig. 5A), as well as β -catenin, AP-1, Lef-1, and Axin compared to the control group (Fig. 5B).

The mRNA expression of BMP2 and BMP4, which are known as BMP target gene, were downregulated by oleyl ceramide treatment. Stearyl ceramide treatment also induced a decrease in BMP4 expression (Fig. 5C). Our data support that our synthetic ceramides could regulate Wnt/ β -catenin pathway by inhibiting BMP target genes.

DISCUSSION

Ceramide is central component of cellular membrane. It is a key player in many signaling pathways involved in cell cycle arrest and differentiation¹⁴. Previous studies about effects of ceramide on hair have focused on developing cosmetics to protect hair shaft¹⁵. However, recent studies have suggested that ceramide promotes hair lengthening, leading to increased anagen follicles in mice and human organ culture system⁷. These results indicate that ceramide might not only play a role as hair shaft protector, but also act as hair growth stimulator. Consistent with previous studies, our results showed that the two newly synthesized ceramides could stimulate hair growth at cellular levels. In the present study, we demonstrated that synthetic ceramides could increase proliferation of hDPCs accompanied by increased Bcl-2/BAX and phosphorylated-ERK/Cyclin-D1 expression levels. We also observed that expression levels of β -catenin stimulated genes such as phosphorylated-Akt/GSK3 β and AR/EGF/EGFR/IGF-1 were increased after treatment with ceramides. In addition, oleyl and stearyl ceramides could upregulate the expression of Wnt/ β -catenin signaling and downregulate BMP2 and

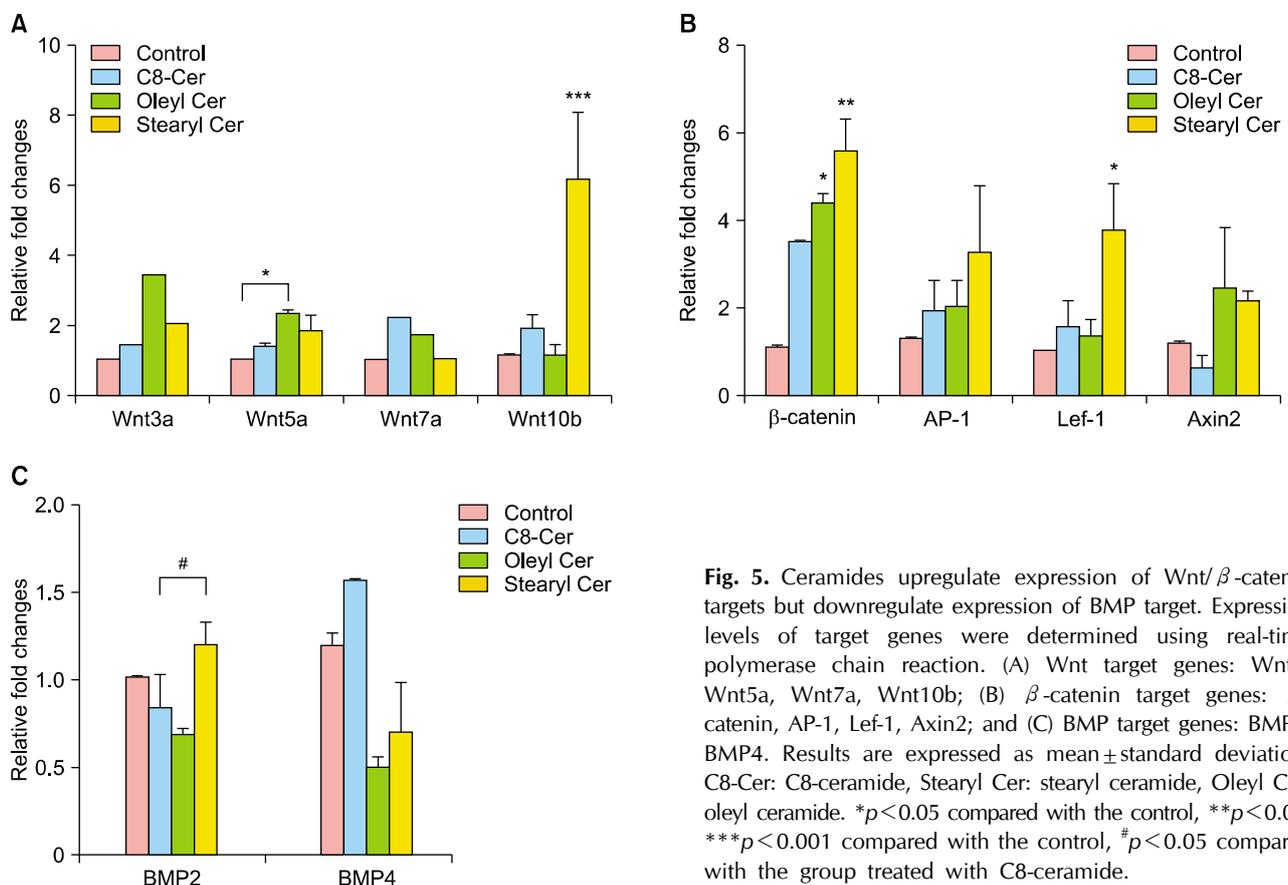


Fig. 5. Ceramides upregulate expression of Wnt/ β -catenin targets but downregulate expression of BMP target. Expression levels of target genes were determined using real-time polymerase chain reaction. (A) Wnt target genes: Wnt3, Wnt5a, Wnt7a, Wnt10b; (B) β -catenin target genes: β -catenin, AP-1, Lef-1, Axin2; and (C) BMP target genes: BMP2, BMP4. Results are expressed as mean \pm standard deviation. C8-Cer: C8-ceramide, Stearyl Cer: stearyl ceramide, Oleyl Cer: oleyl ceramide. * p <0.05 compared with the control, ** p <0.01, *** p <0.001 compared with the control, # p <0.05 compared with the group treated with C8-ceramide.

BMP4 (BMP2/4) expression. Taken together, our results suggest that synthetic ceramides have hair growth stimulating potentials.

Studies about hair growth have shown that the proliferation of hDPCs is accompanied by increased duration of anagen phase¹⁶. Hair follicle size is determined by the volume of hDPCs while hDPCs volume depends on the number of cells¹⁷. Hence, we evaluated the effect of synthetic ceramides on hDPCs proliferation. hDPCs were treated with various concentrations of ceramides. Our results confirmed that synthetic ceramides at 1,000 μ M could increase viability of hDPCs. These results indicate that these synthetic ceramides might contribute to the proliferation of hDPCs.

Apoptosis is mainly processed through the intrinsic death pathway¹⁸. The most important regulators of apoptosis process are members of the Bcl-2 gene family. Bcl-2 protein is known to possess anti-apoptotic ability to obstruct a variety of apoptotic signals. BAX protein is another member of the Bcl-2 family. It plays a pro-apoptotic role by stimulating apoptosis function, unlike Bcl-2 protein¹⁹. It has been reported that cellular Bcl-2/BAX ratio represents regulation of cell survival²⁰. Also, Bcl-2/BAX ratio was considered important in the hair cycle. It is known that the expression of Bcl-2 is dependent on the cycling of hair follicle, and apoptosis is the central program of the hair cycle²¹. Our results revealed that BAX expression did not change while Bcl-2 expression was significantly increased in groups treated with synthetic ceramides. Bcl-2/BAX ratio was increased in oleyl or stearyl ceramide treated group compared to that in the control group.

Ceramide activation may either activate or inhibit the MAPK pathway depending on the cell type²². MAPK signal is known to regulate various cellular responses such as cellular proliferation, cell differentiation, development, and apoptosis²³. ERK is known as one of MAPK signaling pathways involved in cell survival and growth¹⁰. Also, ERK pathway was known stimulate hDPCs proliferation²⁴. Cyclin-D1 is also a key protein that promotes cell cycle (G1-S phase) progression. An increase in cyclin-D1 expression is required for cell proliferation to occur²⁵.

In this study, we examined the effect of synthetic ceramides on phosphorylation of ERK and Cyclin-D1 expression. Results showed that treatment with oleyl and stearyl ceramides increased phosphorylation of ERK and induced expression of Cyclin-D1 protein. Previous studies have shown that high concentration of ceramide in cells induces apoptosis⁶. However, our results showed Bcl-2/BAX ratio and ERK/Cyclin-D1 levels were increased by treatment with our synthetic ceramides. Although the underlying apoptotic effect of ceramide on hDPCs is not fully

understood yet, our results show that synthetic ceramides may stimulate proliferation of hDPCs by regulating Bcl-2/BAX ratio and ERK/Cyclin-D1 pathway.

Hair growth involves various transcription factors. EGF family members are known to stimulate nuclear translocation of β -catenin and upregulate the expression of Wnt10b and β -catenin levels. Especially, IGF-1 is known as an important effect on hair growth and extended the anagen phase generally²⁶. The Wnt/ β -catenin signaling pathway is known as a master regulator for hair growth and hair follicle morphogenesis¹². We investigated Akt/ β -catenin signaling pathway to elucidate the molecular mechanism involved in the effect of ceramides relate to β -catenin activation. To activate β -catenin translocation into nucleus, activation of Akt is needed. Akt is involved in signal transduction pathways such as inflammatory mediator, glycogen metabolism, and proliferation apoptosis²⁷. Activation of Akt results in phosphorylation of several substrates such as GSK-3 β . GSK-3 β is an active enzyme negatively regulated by Wnt pathway and Akt-induced phosphorylation. In the Wnt pathway, GSK-3 β phosphorylation and inactivation will lead to the accumulation and nuclear translocation of β -catenin²⁸. Activated β -catenin induces Wnt signaling activation. Therefore, β -catenin is an essential molecule in Wnt signaling. As shown in Fig. 4, ceramides-treatment increased phosphorylated GSK3 β via activation of Akt. Finally, β -catenin expression in the nucleus was increased by ceramide-treatment. Our findings are consistent with results of Kwack et al.¹⁶ reporting that minoxidil can increase phosphorylation levels of Akt/GSK3 β and β -catenin expression.

The balance between Wnt/ β -catenin pathway and BMP pathway is important for hair growth cycling²⁹. Axin2 and Lef-1 are directed target genes of the β -catenin pathway. Axin2 plays a role in regulating β -catenin level. Cytoplasmic protein is activated when Axin2 binds to β -catenin. Lef-1 is known as the binding site for β -catenin to accelerate directional differentiation of hair follicle stem cells³⁰. Wnt3a and Wnt7a are inductive signals to maintain hDPCs in an anagen state³¹. Wnt5a is a key target gene of Shh in hair follicle morphogenesis³². Wnt10b, also known as hair follicle's growth stimulator, promotes hair follicle regrowth by upregulating Wnt/ β -catenin signaling pathway related factors including Axin2 and Lef-1³³. Previous studies have also reported that Wnt10b/ β -catenin signaling causes regeneration of hair follicles and proliferation of hair matrix cells^{33,34}. As shown in Fig. 5, treatment with synthetic ceramides upregulated expression of β -catenin target genes such as Lef-1, AP-1, and Axin2. Especially, Wnt10b and β -catenin expression levels were significantly increased by treatment with stearyl ceramide. These findings indicate

that treatment with synthetic ceramides has potential to affect hair growth.

BMP signaling can promote telogen. It has been reported that inhibition of BMP signaling induces Wnt signaling and initiates hair growth early¹³. BMP2/4 activation can elaborately regulate the development of hair follicle bulge stem cell activation and growth during hair cycle³⁵. As shown in Fig. 5, treatment with oleyl ceramide downregulated expression levels of BMP target genes (BMP2/4). These results suggest that downregulating BMP2/4 expression could help hair growth via early activation of Wnt signaling.

In conclusion, our study demonstrated that synthetic ceramides had positive effects on hair cell proliferation by regulating Bcl-2/BAX and ERK/Cyclin-D1 in hDPCs. Moreover, synthetic ceramides have potential to stimulate hair growth via Wnt/ β -catenin and BMP2/4 signaling in hDPCs. Our results provide novel information that synthetic ceramides such as oleyl and stearyl ceramides might have potential to help hair growth via cell proliferation by regulating gene expression in hDPCs. Although these findings are just laboratory results, our results explained the possibility of stimulating hair growth by treatment with ceramide. Furthermore, our synthetic ceramides might be used as new therapeutic agents to promote hair growth.

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CONFLICTS OF INTEREST

The authors have nothing to disclose.

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