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Monoterpenoid Loliolide Regulates Hair Follicle Inductivity of Human Dermal Papilla Cells by Activating the AKT/β-Catenin Signaling Pathway

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Loliolide is one of the most ubiquitous monoterpenoid compounds found in algae, and its potential therapeutic effect on various dermatological conditions via agent-induced biological functions, including anti-oxidative and anti-apoptotic properties, was demonstrated. Here, we investigated the effects of loliolide on hair growth in dermal papilla (DP) cells, the main components regulating hair growth and loss conditions. For this purpose, we used a three-dimensional (3D) DP spheroid model that mimics the in vivo hair follicle system. Biochemical assays showed that low doses of loliolide increased the viability and size of 3D DP spheroids in a dose-dependent manner. This result correlated with increases in expression levels of hair growth-related autocrine factors including VEGF, IGF-1, and KGF. Immunoblotting and luciferase-reporter assays further revealed that loliolide induced AKT phosphorylation, and this effect led to stabilization of β-catenin, which plays a crucial role in the hair-inductive properties of DP cells. Further experiments showed that loliolide increased the expression levels of the DP signature genes, *ALP*, *BMP2*, *VCAN*, and *HEY1*. Furthermore, conditioned media from loliolide-treated DP spheroids significantly enhanced proliferation and the expression of hair growth regulatory genes in keratinocytes. These results suggested that loliolide could function in the hair growth inductivity of DP cells via the AKT/β-catenin signaling pathway.

Keywords: Loliolide, hair follicle induction, dermal papilla, spheroids, AKT, migration

Introduction

Biologically active compounds in algae are an interesting source of therapeutic agents because they possess biological or pharmacological activity in vivo and are believed to have minor side effects, making them safe for use in humans. It has been reported that algae have functional properties including protection against photoaging, de-pigmentation, and anti-microbial activity due to the production of various chemical compounds [1]. Loliolide is the simplest and most common monoterpenoid hydroxy-lactone and is abundant in brown algae like *Padina tetrastromatica* and *Sargassum crassifolium*, and red algae like *Corallina pilulifera* [2, 3]. The structure of loliolide consists of the lactone ring of 11 carbon atoms, oxygen, and hydroxyl groups [3, 4]. Previous studies have shown loliolide to exhibit such biological properties as anti-oxidant activity, inhibition of hepatitis C virus activity, and anti-proliferative activity against cancer cells [2, 5]. In the skin, loliolide repairs cellular senescence, prevents apoptosis, and inhibits melanogenesis [6, 7]. However, little is known about the effect of loliolide on the hair-inductive properties of DP cells.

Hair is a unique mammalian characteristic involved in various biological functions like thermal regulation and protection from harmful environments [8]. The hair follicle

(HF) is a skin appendage that mainly consists of various lineages of epithelial cells surrounding the hair shaft with a mesenchymal cell aggregate of the dermal papilla (DP) at its proximal end [9]. Sophisticated and complicated crosstalk between mesenchymal cells and epithelial cells characterize hair growth cycling [10, 11]. During the hair cycle, DP cells signal to the epithelial cell via secreted molecules like wingless-int (WNT), sonic hedgehog (SHH), and bone morphogenetic protein (BMP) [12, 13]. In particular, canonical WNT signaling plays an essential role during the anagen-promoting process [14]. Moreover, global expression profiles of DP cells show the dynamic expression of secreted molecules like growth factors during the hair cycle that regulate neighboring epidermal cells to proliferate and differentiate via epithelial-mesenchymal interactions [11, 15]. The miniaturization of the hair follicle is observed in various types of alopecia. This can lead to loss of the hair-inductive properties in DP cells [16, 17]. Therefore, DP cells are often used as an in vitro model to study hair growth [18].

Although various therapeutic options for alopecia are available, none of them provide satisfying results because the pathogenesis and mechanisms of alopecia are heterogeneous and complicated. Minoxidil and finasteride are approved by the US Food and Drug Administration (FDA) for male pattern alopecia [19, 20]. However, these drugs generally need to be used continuously for the benefits to be maintained, and unpleasant side effects like migraines and depression sometimes occur. Finasteride is for use by men only because of certain side effects like birth defects and unwanted hair growth [19, 21]. Therefore, the development of a therapeutic candidate to treat hair loss and study the underlying mechanism are important.

Here, we determined the effects of loliolide on hair inductivity and the underlying mechanisms involved using a 3D-cultured DP system [22, 23]. The results suggest loliolide as a therapeutic candidate for alopecia.

Materials and Methods

Cell Culture, Plasmids, and Reagents

Human hair follicle dermal papilla (HDP) cells were purchased from PromoCell (Germany). The passage 3-7 cells were maintained in 5% CO_2 at 37°C in a follicle dermal papilla cell growth medium kit (PromoCell) and subcultured when the cells reached 70 to 80% confluency. For experiments, the cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, USA) supplemented with 5% (v/v) fetal bovine serum (FBS; Sigma-Aldrich, USA). The human HaCaT keratinocytes (Thermo Fisher Scientific) were cultured in Epilife (Invitrogen, USA) supplemented with Human Keratinocytes Growth Supplement (HKGS; Invitrogen). 293T cells (American Type Culture Collection, USA) were cultured in DMEM supplemented with 10% (v/v) FBS. TCF/LEF luciferase reporter plasmids were obtained from Promega (USA). LY294002 was purchased from Merck (Germany).

Cell Viability Analysis

The Water-Soluble Tetrazolium-1 (WST-1) assay (EZ-Cytox Cell Viability Assay Kit; ITSbio, Korea) was used as described in the manufacturer's protocol to determine cell viability. HDP cells (1×10^4 cells/well) were seeded on a 96-well, clear, flat-bottom ultra-low attachment microplate (Corning Inc., USA) to obtain spherical structures, and the cells were treated with the indicated doses of loliolide (1, 5, 10, 20, 50, and 100 μg/ml) for 48 h. Subsequently, the WST-1 solution was added to each well, and cell viability was examined by measuring absorbance at 450 nm using an iMark microplate reader (Bio-Rad Laboratories, USA).

Three-Dimensional (3D) Culture of HDP Cells

Three-dimensional (3D) culture of HDP cells was performed as previously described [24]. To obtain one spherical structure, HDP cells (4×10^4 cells/well) were seeded on a 96-well, clear, round-bottom ultra-low attachment microplate (Corning Inc.) and treated with loliolide (10, 20, and 50 μg/ml) for 48 h. The diameters of spheroids were quantified using phase contrast images.

Luciferase Reporter Assay

293T cells were stably transfected with T cell-specific transcription factor and lymphoid enhancer-binding factor (TCF/LEF) luciferase reporter plasmids in combination with the pSV-β-galactosidase (pSV-β-gal) plasmid using Lipofectamine 3000 (Invitrogen). The pSV-β-gal plasmid was used as a control for transfection efficiency. After 48 h of treatment, the cells were lysed using a passive lysis buffer (Promega), and the lysates were incubated with D-luciferin (Sigma-Aldrich) to determine luciferase activity. Luciferase activity was measured using a Glomax 96 Microplate Luminometer (Turner BioSystems, USA). β-gal activity was analyzed using the Luminescent β-galactosidase Detection Kit II (Clontech Laboratories Inc., USA). Relative luciferase activity was determined by normalizing the levels to β-gal activity.

Quantitative RT-PCR (qRT-PCR) Analysis

Total RNA was extracted from HDP spheroids using Trizol reagent (Invitrogen). cDNA was synthesized from 1 μg of total RNA using a M-MLV reverse transcriptase (Invitrogen). Quantitative RT-PCR (qRT-PCR) was carried out using a SYBR Green PCR Master Mix (Thermo Fisher Scientific) with a Step OnePLus Real-Time PCR system (Applied Biosystems, USA) according to the manufacturer's protocol. The primers used for the amplification of specific genes are listed in Table 1. Each mRNA expression level was calculated using the $2^{-\Delta\Delta Ct}$ method and was normalized to the expression level of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and cyclophilin housekeeping genes.

Table 1. List of primer sequences used in qRT-PCR.

Targets for qRT-PCR	Sequence of primer
Vascular endothelial growth factor (VEGF)	F: 5′-GGAGAGATGAGCTTCCTACAG-3′
	R: 5′-TCACCGCCTTGGCTTGTCACA-3′
Insulin-like growth factor 1 (IGF-1)	F: 5′-AGGAAGTACATTTGAAGAACGCAACT-3′
	R: 5′-CCTGCGGTGGCATGTCA-3′
Keratinocyte growth factor (KGF)	F: 5′-ATCAGGACAGTGGCAGTTGGA-3′
	R: 5′-AACATTTCCCCTCCGTTGTGT-3′
Wnt family member 5A (WNT5A)	F: 5′-TTGAAGCCAATTCTTGGTGGTCGC-3′
	R: 5′-TGGTCCTGATACAAGTGGCACAGT-3′
Lymphoid enhancer binding factor 1 (LEF1)	F: 5′-AATGAGAGCGAATGTCGTTGC-3′
	R: 5′-GCTGTCTTTCTTTCCGTGCTA-3′
Alkaline phosphatase (ALP)	F: 5′-CAAACCGAGATACAAGCACTCCC-3′
	R: 5′-CGAAGAGACCCAATAGGTAGTCCAC-3′
Versican (VCAN)	F: 5′-GGCAATCTATTTACCAGGACCTGAT-3′
	R: 5′-TGGCACACAGGTGCATACGT-3′
Bone morphogenetic protein 2 (BMP2)	F: 5′-GGAACGGACATTCGGTCCTT-3′
	R: 5′-CACCATGGTCGACCTTTAGGA-3′
HES-related with YRPW motif protein 1 (HEY1)	F: 5′-GCGCACGCCCTTGCT-3′
	R: 5′-GCCAGGCATTCCCGAAA-3′
Interleukin 1 alpha (IL-1α)	F: 5′-CTTCTGGGAAACTCACGGCA-3′
	R: 5′-GTGAGACTCCAGACCTACGC-3′
Cyclophilin	F: 5′-CGCGTCTCCTTTGAGCTGTT-3′
	R: 5′-ACCACCCTGACACATAAACCC-3′
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	F: 5′-CGGAGTCAACGGATTTGGTCGTAT-3
	R: 5′-AGCCTTCTCCATGGTGAAGAC-3′

Immunoblotting

Total cell lysates were prepared, and protein extracts were fractionated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting. For nuclear and cytoplasmic fractionation of total cell lysate, NE-RER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific) were used. The primary antibodies used for immunoblotting analysis were as follows: antibodies against Lamin C (ab8984), β-tubulin (ab6046) purchased from Abcam (UK); antibodies targeting AKT (9272), p-AKT (S473) (9271), glycogen synthase kinase 3β (GSK-3β) (9315), p-GSK-3β (9323), extracellular signal regulated kinase (ERK) (9102), p-ERK (9101), stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) (9252), p-SAPK/JNK (9251), p-p38 (9211), β-catenin (9562), and p-β-catenin (9561) from Cell Signaling Technology (USA); antibodies targeting p38α (sc-728), VEGF (sc-7269), IGF-1 (sc-365440) and KGF (sc-74116) from Santa Cruz Biotechnology (USA), and the antibody against β-actin (a5441) from Sigma-Aldrich. After incubation with the primary antibodies, the membranes were incubated with goat anti-mouse or goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology). The stained bands were visualized using the Pierce ECL western blotting substrate (Thermo Fisher Scientific). β-actin for total protein levels and Lamin C and β-tubulin for nuclear and cytoplasmic protein levels were used as loading controls.

Conditioned Media Preparation

HDP spheroids were grown in 60 mm ultra-low attachment culture dishes (Corning Inc.) and treated with 20 μg/ml loliolide for 48 h. After treatment, the medium was changed to serum-free Epilife to treat HaCaT cells with conditioned media (CM). The cells were incubated for 20 h, and CM from DMSO-treated HDP cells (CM0) and loliolide (20 μg/ml)-treated cells (CM20) was prepared.

Enzyme-Linked Immunosorbent Assay (ELISA)

VEGF, IGF-1 and KGF concentrations in conditioned medium were measured by ELISA method. Samples were coated on 96-well plate (SPL) with a carbonate coating buffer for 24 h at 4°C. Then, the coating buffer was removed and blocking buffer, 5% skim milk in PBST, was added and incubated for 2 h at 37°C. After blocking, primary antibodies against each growth factor and secondary antibodies were incubated for 2 h at 37°C. Antibodies were washed out and *o*-phenylenediamine dihydrochloride/hydrogen peroxide was added as a substrate for 30 min at 37°C in dark conditions. The reaction was ceased by adding sulfuric acid for 10 min at 37°C in dark condition. Concentrations were

examined by measuring absorbance at 490 nm using an iMark microplate reader and a standard curve comprised of recombinant human VEGF, IGF-1 and KGF (R&D System, USA).

BrdU Cell Proliferation Assay

Cell proliferation was determined according to the manufacturer's protocol for the BrdU cell proliferation assay (BrdU Cell Proliferation Assay 200 Test Kit; Merck). HaCaT cells (1×10^4 cells/well) were seeded on 96-well plates (SPL, Korea) and maintained in complete medium for 24 h. After incubation, the cells were treated with CM0, CM20, or serum-free Eplilife (Epi) for another 48 h. BrdU was added before the end of the loliolide treatment period. After incubation, the anti-BrdU monoclonal antibody was added to detect the BrdU label. The goat anti-mouse IgG, peroxidase conjugate, and TMB peroxidase substrate were added and incubated. Cell proliferation was determined by measuring absorbance at 450/595 nm using an iMark microplate reader.

Chemotaxis Migration

To determine chemotactic migration, we used a commercially available chemotaxis chamber with a polycarbonate membrane filter with 8.0 μm pores and 6-well culture plates (SPL). The bottom wells were filled with serum-free Epilife, CM0, and CM20 as a chemoattractant. HaCaT cells (8×10^5) in serum-free Epilife were inserted into the chambers and maintained for 48 h. The chambers were removed, fixed with 4% paraformaldehyde in PBS for 30 min, and stained with 0.2% crystal violet in 25% methanol for 30 min. To remove cells that did not migrate, the upper side of the membrane was scraped using a cotton swab. Chemotaxis was assessed by counting the number of migrated cells in five random microscope fields.

Statistical Analysis

All data are presented as mean ± standard deviation (SD), and normally distributed data have been evaluated using a two-tailed Student's *t*-test as indicated in the figure legends. All analyses were performed with triplicate independent experiments. Results are considered statistically significant when $*p < 0.05$ and $**p < 0.005$.

Results

Loliolide Increases Cell Viability of HDP Spheroids

The molecular structure of loliolide is represented in Fig. 1A. To begin our evaluation of loliolide, we first investigated its effects on the cell viability of HDP spheroids using a WST-1 assay. As shown in Fig. 1B, loliolide significantly increased the cell viability of HDP spheroids up to 100 μg/ml, and it was highest at a dose of 20 μg/ml. No significant toxicity was observed because the viability remained >100% compared to the DMSO-treated control. Overall, these results indicate that loliolide has a potential proliferative effect on HDP spheroids.

Loliolide Enhances Formation of HDP Spheroids

The sphere formation of the DP cells is related to their hair-inductive properties, and DP sphere size is closely related to hair shaft diameter [15, 23]. Moreover, decreases in DP sphere size are observed during hair follicle miniaturization in alopecia conditions [16, 17]. Therefore, we attempted to determine whether loliolide increases the size of HDP spheroids by comparing the diameters of HDP spheres using a microscope with phase-contrast images. As shown in Fig. 1C, loliolide significantly increased the size of HDP spheroids up to 20 μg/ml and the value was decreased at a dose of 50 μg/ml. These results were correlated with an increase in cell viability, suggesting that loliolide stimulates HDP proliferation and spheroid formation.

Loliolide Upregulates Expression of Growth Factors in HDP Spheroids

Recent reports demonstrated that DP cells are a reservoir of growth factors that regulate hair growth via autocrine and paracrine factors [25, 26]. Therefore, we next determined whether the increased proliferation and sphere formation abilities of HDP spheroids are accompanied by gene expression of growth factors secreted by the cells. Using qRT-PCR, we determined the expression levels of vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), and keratinocyte growth factor (KGF). As shown in Fig. 1D, loliolide increased mRNA levels of VEGF. Other growth factors like *IGF-1* and *KGF* expression were also upregulated in loliolide-treated HDP spheroids. Likewise, expression levels of the growth factors were highest at a dose of 20 μg/ml. We found that the protein levels of these growth factors increased through loliolide treatment (Fig. 1E). The values were highest at a dose of 20 μg/ml, which correlates with an increase in mRNA expression of growth factors in HDP spheroids. Together, these results suggest that loliolide upregulates the expression of growth factors in HDP spheroids. We next carried out an additional experiment to examine whether those growth factors have any effects on the growth of HDP spheroids after neutralization of these factors. The results showed that the spheroid size of HDP cells was decreased after neutralization in DMSO-treated control groups. However, the increased spheroid size of HDP cells after treatment with 20 μg/ml of loliolide was slightly lessened after neutralization (Fig. S1A). These results indicated that the effect of loliolide on cell growth was not markedly regulated by the autocrine effect of growth factors.

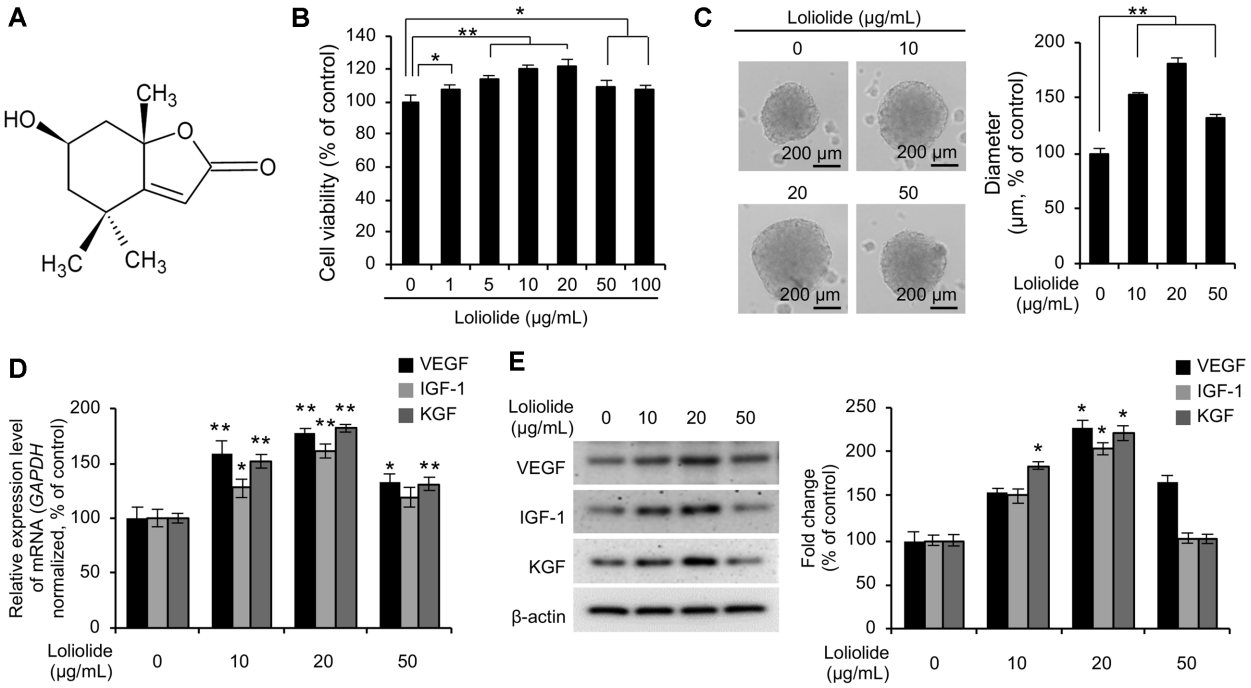

Fig. 1. Effect of loliolide on HDP spheroid viability.

(**A**) Chemical structure of loliolide drawn by ACD/Chemsketch. (**B**) Cells were treated with loliolide at the indicated doses for 48 h. Cell viability was determined using a WST-1 assay. (**C**) Cells were treated with 10, 20, and 50 μg/ml of loliolide for 48 h, and phase-contrast images of spheroids were captured. (**D**) After treating with loliolide for 48 h, the mRNA expression of growth factors in HDP spheroids was analyzed by qRT-PCR. *GAPDH* served as an endogenous control. (**E**) After treating with loliolide for 48 h, the protein expression of growth factors in HDP spheroids was determined using immunoblotting assays with specific antibodies. β-actin served as a loading control. Quantification of the protein levels was performed using ImageJ. The data represent the means of three independent samples ± SD. *$p < 0.05$ and **$p < 0.005$ versus DMSO-treated control. Scale bars: C, 200 μm.

Loliolide Activates AKT Signaling Pathways in HDP Spheroids

AKT and mitogen-activated protein kinase (MAPK) signaling have a pivotal role in promoting cell proliferation in various types of cells. In DP, proliferation increased via stimulating the ERK and phosphoinositide 3-kinase (PI3K)/AKT pathway [26, 27]. In addition, previous studies reported that loliolide activates the AKT signaling pathway in various cell types [6, 28]. Based on this knowledge, we sought to determine if the growth promoting effect of loliolide can be mediated by the MAPK and AKT pathways in HDP spheroids. Using immunoblotting assays with specific antibodies, the expression levels of a protein associated with the AKT and MAPK pathways and the phosphorylation status were analyzed. As shown in Fig. 2A, loliolide significantly increased the phosphorylation status of AKT in HDP spheroids, but the total AKT protein level was not changed. Subsequently, the phosphorylation of GSK3β at serine 9, a known downstream effector of AKT, was increased by loliolide treatment. However, MAPK-related signaling molecules like ERK, JNK, and p38 were not activated by loliolide treatment (Fig. 2B). Taken together, our data indicate enhanced proliferation and sphere formation mediated by loliolide via the AKT/GSK3β signaling pathways in a dose-dependent manner in HDP spheroids. Then, we further tested whether the loliolide-induced growth-promoting effect was not dependent of the autocrine effect of the growth factors using a PI3K inhibitor LY294002. We found that the expression levels of those growth factors were not increased after LY294002 treatment in loliolide-treated HDP cells (Fig. S1B). Therefore, these results suggested that the effect of loliolide on the increasing expression of growth factors was mediated by AKT signaling.

Loliolide Activates the WNT/β-Catenin Signaling Pathway in HDP Spheroids Via AKT

WNT/β-catenin signaling, one of the most important signal pathways in the hair follicle, plays a crucial role in the hair-inductive properties of DP cells [14, 29]. We

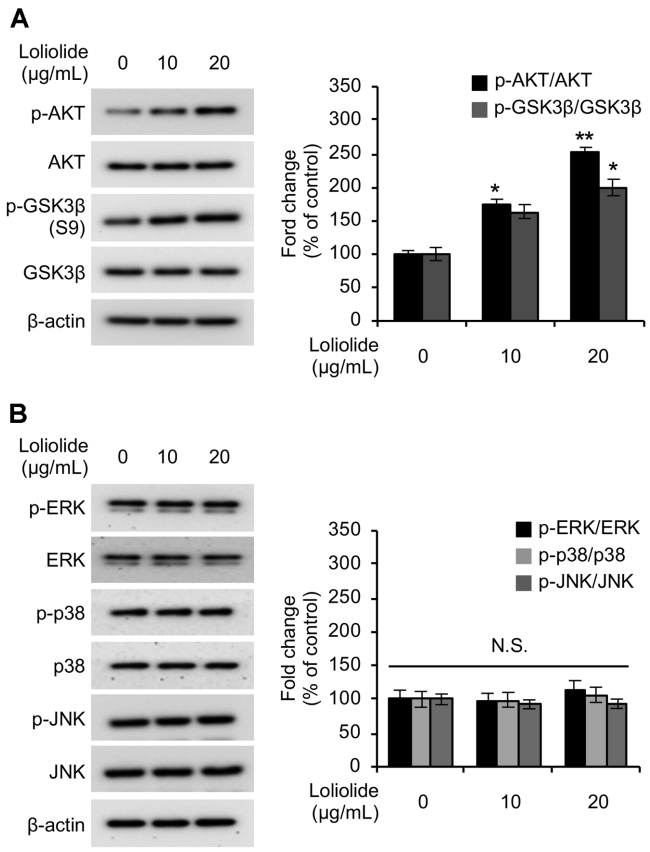

Fig. 2. Effect of loliolide on AKT and MAPK signaling activation.

Cells were treated with 10 and 20 μg/ml of loliolide for 48 h, (**A**) AKT/GSK3β and (**B**) MAPK (p38, ERK, JNK) signaling phosphorylation were determined using immunoblotting assays with specific antibodies. β-actin served as a loading control. Quantification of the phosphorylation level was performed using ImageJ software and normalized to total protein levels. The data represent the means of three independent samples ± SD. $*p < 0.05$ and $**p < 0.005$ versus DMSO-treated control. N.S. means none significant.

investigated whether the WNT/β-catenin signaling pathway is activated by loliolide. First, the phosphorylation status of β-catenin was investigated in the control and loliolide-treated HDP spheroids. As expected, the immunoblot assay revealed that loliolide inhibited phosphorylation of β-catenin (Fig. 3A). This was followed by β-catenin translocalization to the nucleus in loliolide-treated HDP spheroids compared to DMSO-treated controls (Fig. 3B). We examined the transcriptional activity of β-catenin after loliolide treatment using a luciferase reporter assay. As shown in Fig. 3C, loliolide increased the luciferase activity in a dose-dependent manner in HDP spheroids. Likewise, the expression of WNT/β-catenin downstream target genes like *WNT5A* and *LEF1* was significantly upregulated at the transcriptional level in loliolide-treated HDP spheroids compared to DMSO-treated control (Fig. 3D). To clarify the relationship between AKT and WNT signaling in loliolide-treated HDP spheroids, we used loliolide treatment with or without LY294002. We found that loliolide-mediated upregulation of *WNT5A* and *LEF1* mRNA expression were abrogated by LY294002 treatment. Together, these results suggest that loliolide activates WNT/β-catenin signaling via the AKT pathway in HDP spheroids.

Loliolide Promotes the Expression of DP Signature Genes in HDP Spheroids

Our results prompted us to investigate the gene expression levels of hair induction-related genes called DP signature genes [13]. We investigated whether loliolide treatment affects the gene expression level of the DP signature genes like alkaline phosphatase (ALP), versican (VCAN), BMP2, and hairy/enhancer related with YRPW motif protein 1 (HEY1). Using qRT-PCR analysis, we found that loliolide treatment significantly upregulated DP signature gene expression at the transcriptional level in a dose-dependent manner (Fig. 4). These results show that loliolide treatment effectively reinforces the hair-inductive properties in HDP spheroids.

Conditioned Media of Loliolide-Treated HDP Spheroids Is Responsible for Migration and Proliferation of HaCaT Cells and Hair Follicle Growth Promotion

The hair cycle is closely regulated by epithelial-mesenchymal interactions [11]. Outer root sheath (ORS) keratinocytes play an essential role in development, regeneration, and elongation of the HF as a linkage component between the HF and the epidermis [30]. Growth factors and cytokines secreted from DP cells instruct surrounding epithelial components to proliferate and differentiate [13, 31]. Thus, we examined whether conditioned media (CM) from loliolide-treated HDP spheroids can exert an effect on the ORS-like HaCaT keratinocytes. As shown in Fig. 5A, we found that loliolide increased the secretion levels of VEGF, IGF-1 and KGF from HDP spheroids. Conditioned media from loliolide (20 μg/ml)-treated cells (CM20) enhanced the chemotactic migration of HaCaT cells compared to cells grown in conditioned media from DMSO-treated cells (CM0) and Epilife supplement-free medium (Epi) (Fig. 5B). These data indicate that CM20 contains various chemokines responsible for migration of HaCaT. Moreover, CM20 significantly enhanced the proliferation of HaCaT cells (Fig. 5C).

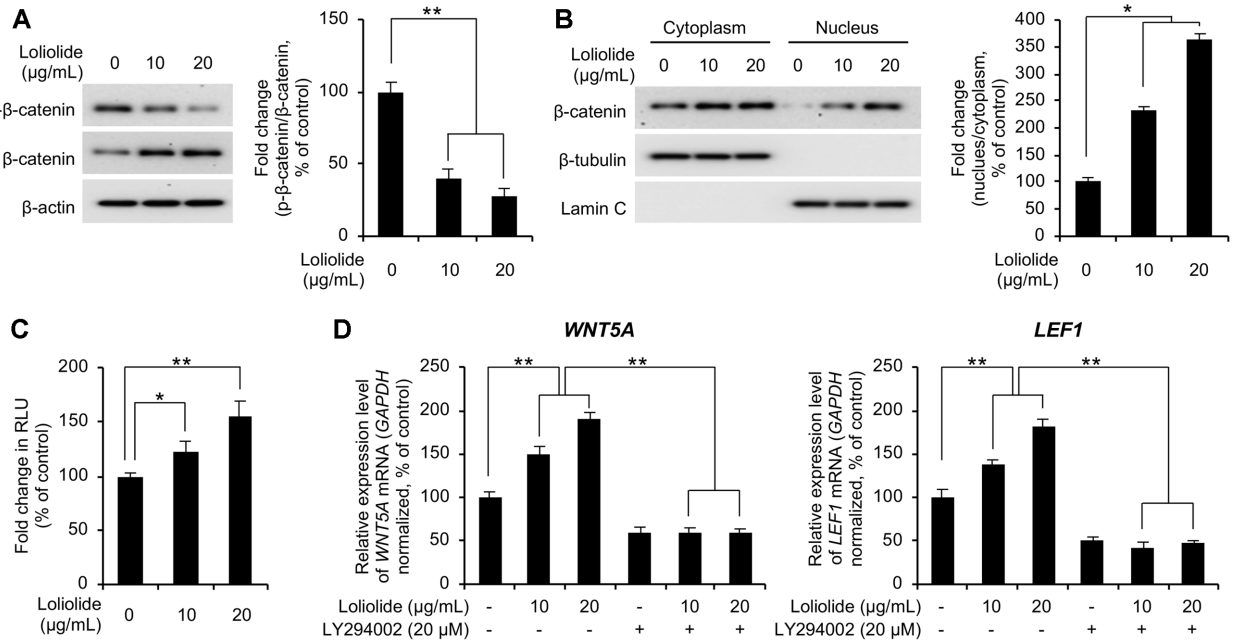

Fig. 3. Effect of loliolide on WNT signaling activation.

(**A**) β-catenin stabilization and (**B**) translocation were analyzed using an immunoblotting assay with specific antibodies after treating with 10 and 20 μg/ml loliolide for 48 h. β-actin, Lamin C, and β-tubulin served as loading controls for total protein, nuclear fraction, and cytoplasmic fraction, respectively. Quantification of the phosphorylation level was carried out using ImageJ software. (**C**) TCF/LEF transcriptional activity was determined using a luciferase assay by normalizing the β -galactosidase activity after treating with 10 and 20 μg/ml of loliolide. (**D**) Cells were treated with 10 and 20 μg/ml of loliolide with or without 20 μM LY294002 for 48 h, and the mRNA levels of WNT/β-catenin target genes were assessed using a qRT-PCR. *GAPDH* served as an endogenous control. The data represent the means of three independent samples ± SD. *$p < 0.05$ and **$p < 0.005$ versus DMSO-treated control.

Because CM20 increased the proliferation and migration of HaCaT cells, qRT-PCR analysis was performed to determine whether the expression of hair growth promotion-associated genes was affected. CM20-treated HaCaT cells showed significantly increased gene expression of *VEGF*, *IGF-1* and decreased interleukin-1α (*IL-1α*) when compared to CM0 (Fig. 5D). Taken together, these results demonstrate that loliolide-treated HDP spheroids promote hair growth via paracrine effects.

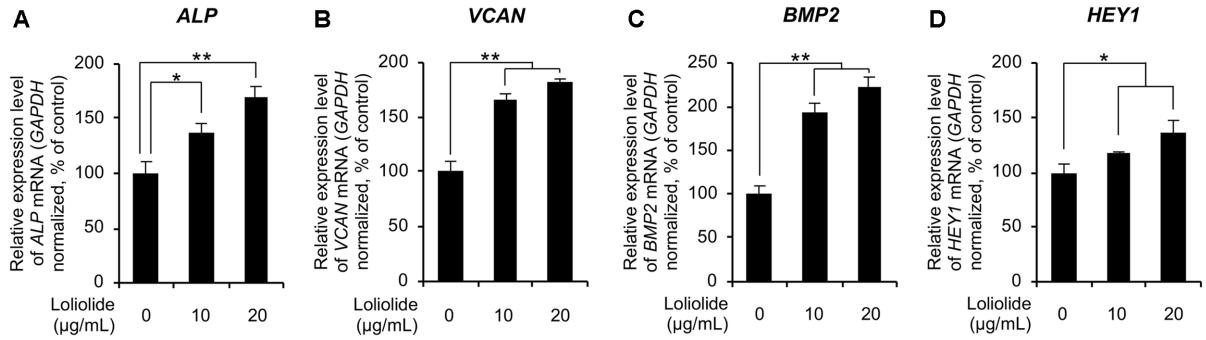

Fig. 4. Effects of loliolide on the expression of DP signature genes in HDP spheroids.

(**A-D**) Cells were treated with 10 and 20 μg/ml loliolide for 48 h, and mRNA levels of the genes *ALP*, *VCAN*, *BMP2*, and *HEY1* were analyzed using a qRT-PCR. *GAPDH* served as an endogenous control. The data represent the means of three independent samples ± SD. *$p < 0.05$ and **$p < 0.005$ versus DMSO-treated control.

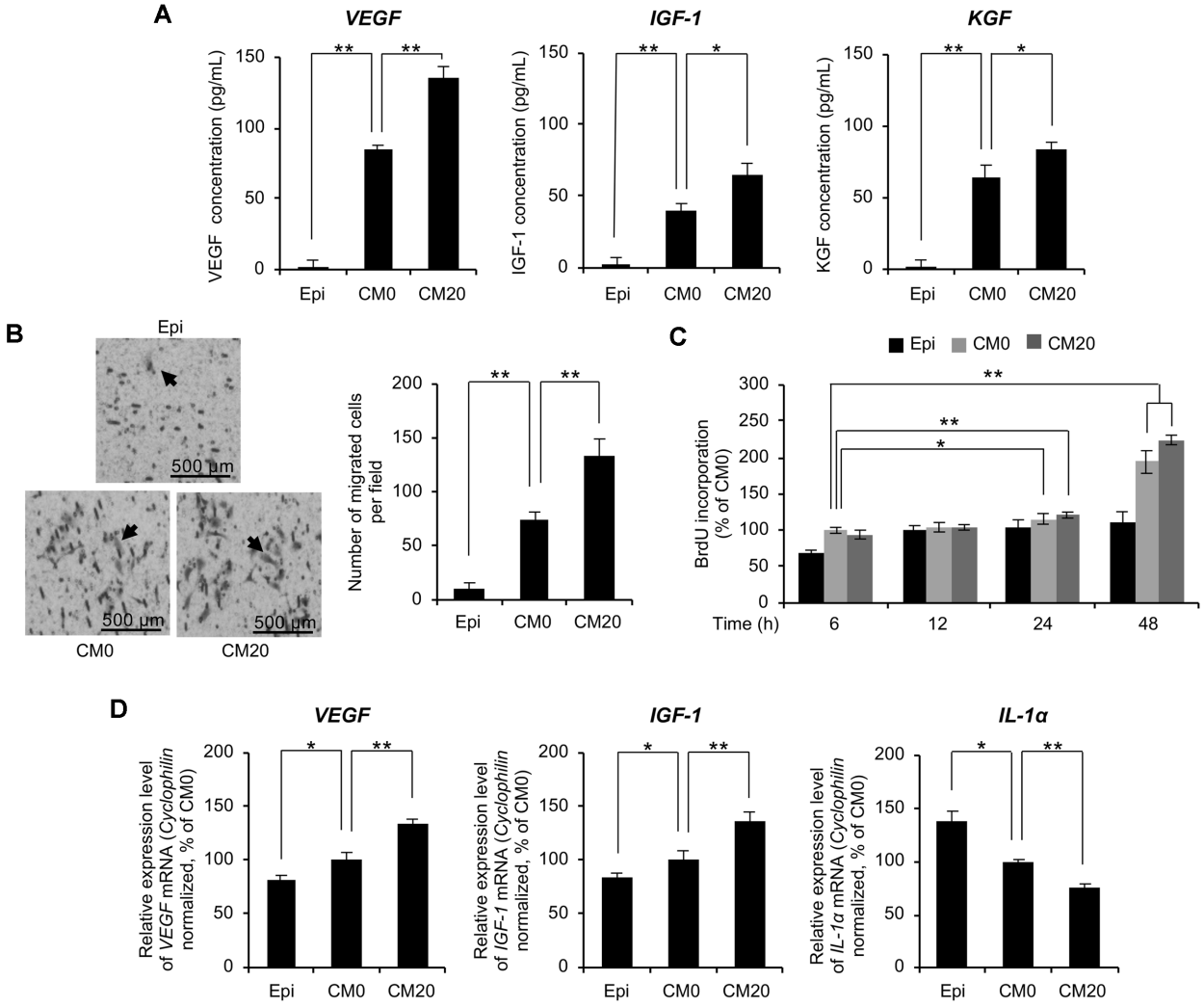

Fig. 5. Effects of conditioned media from HDP spheroids treated with loliolide on HaCaT cells.
(**A**) VEGF, IGF-1 and KGF concentrations in Epi, CM0 and CM20 from HDP spheroids were analyzed by ELISA. Concentrations were examined by measuring absorbance and a standard curve comprised of recombinant human VEGF, IGF-1 and KGF. (**B**) Cells were treated with Epi, CM0 and CM20 for 48 h, and migration was analyzed using a chemotactic migration assay. Black arrows indicate migrated cells. Quantification of cell numbers that migrated was done using ImageJ software. (**C**) Cells were treated with Epi, CM0 and CM20 for the indicated time periods (h), and proliferation was determined using the BrdU incorporation assay. The data were normalized by the value of HaCaT cells treated with Epi, CM0 and CM20 for 6 h. (**D**) Cells were treated with Epi, CM0 and CM20 for 48 h, and expression of genes related to hair growth promotion in HaCaT cells was analyzed using a qRT-PCR. *Cyclophilin* served as an endogenous control. The data represent the means of three independent samples ± SD. *$p < 0.05$ and **$p < 0.005$ versus DMSO-treated control. Scale bars: B, 500 μm.

Discussion

Hair loss (alopecia) is a common disorder in men and women of all ages. Although the pathogenesis and mechanisms of alopecia are complicated, most patients show abnormal DP cell activity, including decreased proliferation, senescence, and abnormal gene expression [13]. Therefore, it is important to understand the etiopathogenesis and molecular mechanisms in DP cells in the context of hair loss and also to develop natural candidates with fewer side effects that give more than one mode of action to treat the disorder.

Algae have traditionally been used for hair growth. Many studies reported that various chemical compounds from algae like *Grateloupia elliptica* and *Ecklonia cava* promote anagen progression by stimulating DP and ORS cells [32,

33]. Although there is no direct evidence of loliolide having any effect on hair growth, one study showed that an algae extract mixture containing loliolide promoted hair growth in mice [34]. Although the result did not provide direct evidence of loliolide's effect on hair growth, they found that loliolide was one of the major components of the mixture, as revealed by gas chromatography with mass spectrometry [34]. Loliolide is the simplest monoterpenoid hydroxylactone, and its anti-oxidant activity has led to widespread dermatologic applications in preventing melanogenesis and premature senescence without serious toxic effects or even skin irritation in humans at moderate doses [4, 6, 7]. Also, previous studies have focused on AKT pathway activation, also known as protein kinase B (PKB), by loliolide treatment [6, 28]. AKT is the serine/threonine-specific kinase involved in the proliferation of various cell types like DP cells [26, 27]. Moreover, various therapeutic candidates for alopecia treatment have demonstrated hair growth-promoting effects via AKT activation in DP cells [20, 27, 35]. Based on this knowledge, we conducted experiments to examine the effects of loliolide on the hair inductive properties of HDP cells.

In this study, we provide the evidence that loliolide increases hair growth-inductive property in HDP cells using a three-dimensional spheroid culture system to mimic in vivo [22, 23]. Mouse models are believed to be useful tools for studies focused on investigating the mechanisms of hair loss and identifying therapeutic candidates; however, mice do not exhibit vellus to terminal hair type change and suffer from androgenetic alopecia that occurs commonly in human [36]. To overcome such limitations, researchers have found that DP cells grown in 3D spheroid structures can restore their intact transcriptional signature and reproducible hair inductivity, and that enhancement of the 3D sphere formation is directly related with the hair growth inductivity of HDP cells [22, 23, 37]. Moreover, the spheroids are sufficient to induce hair growth *de novo* when transplanted [22, 23]. Therefore, an alternative strategy for in vitro culture to mimic an in vivo DP-like condition has demonstrated that a 3D spheroid culture system is relevant in evaluating the effect on hair growth [37].

Here, we demonstrated that loliolide increased the cell viability and size of HDP spheroids. According to previous reports, DP cell numbers are increased during the anagen phase and affect the size and morphology of hair by providing a physical niche to influence the number of progenitor cells [31, 38]. Also, decreased hair growth by DP leads to the miniaturization of the hair follicle in alopecia [39]. Moreover, spherical structures are very important for hair induction by the dermal papilla [22]. Therefore, these results suggested that loliolide is a potent stimulator of HDP spheroid growth and that loliolide-mediated increase of DP spheroid formation could be associated with hair inductive potential. Additionally, we revealed that loliolide could increase the expression of *VEGF*, *IGF-1*, and *KGF*, which are increased and regulate cellular proliferation during hair growth [25, 26, 40]. However, our further experiments showed that although the HDP cells produced and responded to their own growth factors in control status, neutralization of growth factors did not exert a significant inhibitory effect with loliolide on the growth of HDP spheroids. These results may indicate that the loliolide-induced growth promoting effect of the cells was not markedly affected by the autocrine effect of the growth factors.

As mentioned above, our data showed that loliolide increased the phosphorylation status of AKT, followed by phosphorylation of GSK3β. This corroborates previous reports that loliolide could activate the AKT pathway. To clarify whether the loliolide-induced growth promoting effect was not dependent of the autocrine effect of the growth factors, we inhibited AKT activation. We examined the inhibition of AKT activation abolished increasing growth factor expressions. These results suggest that the growth promoting effect of loliolide on HDP spheroids would be mediated by AKT signaling pathways rather than growth factor-dependent pathways. Meanwhile, MAPK signaling is also important to growth, but it was not affected by loliolide [26]. GSK3β is known as a key mediator of WNT signaling pathways that play an important role in hair follicle morphogenesis and hair growth [13, 14]. Our data showed that loliolide could induce stabilization and translocation of β-catenin to the nucleus in HDP spheroids, enhancing the transcriptional activity of β-catenin. Moreover, expression of WNT signaling target genes like *WNT5A* and *LEF1* was enhanced by loliolide treatment. Inhibition of WNT target gene expression by LY294002, an AKT inhibitor, clarified WNT signaling activation by loliolide treatment mediated via the AKT/GSK3β signaling pathways. These results suggest that WNT signaling is involved in the effects of loliolide via AKT/GSK3β signaling. Also, loliolide-induced growth promoting effects would be mediated by activating the AKT/GSK3β and the WNT signaling pathways rather than increasing the autocrine effects of growth factors on HDP spheroids.

DP cells express a specific molecule known as DP signature genes during hair growth [13]. The activity of ALP has been used to identify DP cells and reaches the maximal

level in early anagen. VCAN is a widespread extracellular matrix component with a unique pattern of distribution in the hair cycle [13]. BMP2 preserves hair-inductive properties [41]. HEY1, known as Notch target gene and also induced by β-catenin, is expressed in DP cells and anagen hair bulbs [42]. DP spheroids restore their hair-inductive properties when grown in 3D spheroids and, at the molecular level, the expressions of typical signature genes are considered as an indicator of hair inductivity of DP cells in vitro [43]. Loliolide caused the expression of DP signature genes including *ALP*, *VCAN*, *BMP2*, and *HEY1* in HDP spheroids. This suggests that loliolide could be related to hair induction by HDP spheroids. Also, other signals like BMP and Notch might be involved in the effects of loliolide on HDP spheroids [41, 42].

DP cells modulate the growth of hair follicles by exerting effects on themselves and epithelial components surrounding them. Epithelial components proliferate and differentiate to generate new follicles, especially in response to growth factors and cytokines secreted from DP cells like IGF-1 and KGF [13, 31]. Therefore, epithelial-mesenchymal interactions play a crucial role in hair follicle development. Here, we found that loliolide stimulated secretion of VEGF, IGF-1 and KGF from HDP spheroids. Also, conditioned media from loliolide-treated DP spheroids (CM20) enhanced the migration and proliferation of ORS-like HaCaT keratinocytes [44]. In addition, we found that CM20 treatment could increase the expression of genes related to hair growth promotion like *VEGF* and *IGF-1* and decrease *IL-1α* which induces catagen in HaCaT. These observations show that loliolide induces chemokine secretion from HDP spheroids, stimulating the migration and proliferation of neighboring follicular matrix keratinocytes and leading to hair growth enhancement. To summarize, loliolide ultimately enables enhancement of hair induction via AKT-mediated WNT signaling activation in 3D spheroid HDP cells. Also, loliolide might be responsible for the effects of algae-derived chemicals that are used to facilitate hair growth. Thus, our findings indicate that loliolide can potentially treat alopecia.

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Conflicts of Interest

The authors have no financial conflicts of interest to declare.

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