의학박사 학위논문

포스포디에스테라아제 3 억제제의 모낭세포생존 및 모발 성장에 대한 효과

The effects of phosphodiesterase 3 (PDE3) inhibitor on hair follicle cell viability and hair growth

2020년 2월

서울대학교 대학원

의학과 피부과학 전공

김 동 영

포스포디에스테라아제3 억제제의
모낭세포생존 및 모발 성장에 대한 효과

지도교수 권 오 상

이 논문을 의학박사 학위논문으로 제출함

2019 년 10월

서울대학교 대학원

의학과 피부과학전공

김 동 영

김동영의 박사 학위논문을 인준함

2020 년 1월

위 원 장 _____ (인)

부 위 원 장 _____ (인)

위 원 _____ (인)

위 원 _____ (인)

위 원 _____ (인)

The effects of phosphodiesterase 3 (PDE3) inhibitor on hair follicle cell viability and hair growth

by

Dong Young Kim, M.D.

(Directed by Ohsang Kwon, M.D., Ph.D.)

A Thesis Submitted to the Department of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Medicine (Dermatology) at the Seoul National University College of Medicine

January 2020

Approved by thesis committee:

Professor	_____	Chairman
Professor	_____	Vice Chairman
Professor	_____	
Professor	_____	
Professor	_____	

Abstract

The effects of phosphodiesterase 3 (PDE3) inhibitor on hair follicle cell viability and hair growth

Dong Young Kim

Department of Dermatology, College of Medicine

The Graduate School

Seoul National University

Phosphodiesterase 3 (PDE3) inhibitor increases the intracellular level of cyclic adenosine monophosphate to cause vasodilation. Topical application of PDE3 inhibitor, for example cilostazol, is reported to improve local blood flow and enhance wound healing; however, its effect on human hair follicles is unknown.

The purpose of this study was to determine the effect of PDE3 inhibitor on hair growth.

We investigated the expression of PDE3 in human dermal papilla cells (DPCs), outer root sheath cells (ORSCs), and hair follicles. The effects of PDE3 inhibitor on DPC and ORSC proliferation were evaluated using BrdU and WST-1 assays. The expression of various growth factors in DPCs was investigated by growth factor antibody array. Additionally, hair shaft elongation was measured using *ex vivo* hair follicle organ cultures, and anagen induction was evaluated in C57BL/6 mice. Finally, the effects of PDE3 inhibitor on vessel formation and activation of

the mitogen-activated protein kinase pathway were evaluated.

We confirmed high mRNA and protein expression of PDE3 in human DPCs. PDE3 inhibitor not only enhanced the proliferation of human DPCs but also regulated the secretion of several growth factors responsible for hair growth. Furthermore, it promoted hair shaft elongation *ex vivo*, with increased proliferation of matrix keratinocytes. PDE3 inhibitor also accelerated anagen induction by stimulating vessel formation and upregulating the levels of phosphorylated extracellular signal-regulated kinase, c-Jun N-terminal kinase, and P38 after its topical application in C57BL/6 mice.

Finally, our results show that PDE3 inhibitor promotes hair growth and may serve as a therapeutic agent for the treatment of alopecia.

.....

keywords : Hair, Hair growth, Hair follicle cell viability, Phosphodiesterase, Phosphodiesterase inhibitor

Student Number : 2014-21987

Abbreviations

PDE3 phosphodiesterase 3

hDPCs human dermal papilla cells

hORSCs human outer root sheath cells

HFs hair follicles

DP-CM conditioned medium derived from DPCs

cAMP cyclic adenosine monophosphate

DAPI 4',6-diamidino-2-phenylindole

GAPDH glyceraldehyde 3-phosphate dehydrogenase

RT-PCR Reverse transcription-polymerase chain reaction

MAPK mitogen-activated protein kinase

JNK c-Jun N-terminal kinase

ERK extracellular signal-regulated kinase

IGF insulin-like growth factor

IGFBP insulin-like growth factor binding protein

M-CSF macrophage colony-stimulating factor

M-CSFR M-CSF receptor

PDGF platelet-derived growth factor

PDGF-R PDGF receptor

List of Figures

Contents

Introduction

Hair follicle is structured organ which is composed of characteristic cells including outer root sheath cells (ORSCs) and dermal papilla cells (DPCs), stem cells, and matrix keratinocytes. Actively growing hair follicles eventually become anchored in the subcutis, and periodically regenerated by spontaneously undergoing repetitive cycles of growth (anagen), apoptosis-driven regression (catagen), and relative quiescence (telogen) [1].

Hair follicle formation and hair growth are delicately regulated by mesenchymal-epithelial interaction between dermal papilla and dermal sheath cells derived from the mesenchyme and the inner and outer root sheaths, matrix, and hair shaft cells [2]. Hair inductivity of dermal papilla cells is very necessary for induction and maintenance of hair follicular development and growth, and DPCs transport various growth factors and nutrients to facilitate hair follicular growth. Hence, that would be interesting research themes to finding out certain substances which can affect the viability of hair follicular cells and hair growth.

Treating hair loss by enhancing the growth of hair follicles (HFs) has steadily become the treatment strategy of interest to hair researchers. Many drugs with the potential to promote hair growth have been investigated; the mechanisms underlying their effects have been evaluated as well [3].

Phosphodiesterase is an enzyme that breaks phosphodiester bonds in molecules. Due to its action, the intracellular levels of cyclic nucleotide decrease. Commonly, PDE refers to cyclic nucleotide phosphodiesterase. Eleven mammalian PDE families have been discovered so far (type 1-11) [4]. Inhibition of PDE can enhance or prolong the effects of physiological processes mediated by cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate, as their degradation by PDE is blocked [4]. Despite the primary structural differences and diverse tissue expression patterns among the PDE subfamilies [4-7], intriguingly, the

PDE3B protein was detected in human HFs in a mass spectrometry-based human proteome study combined with Proteomics DB, which is a high-performance, in-memory database for real-time analysis of terabytes of big data [8]. However, PDE3A protein expression in human HFs remains to be elucidated.

One example of PDE3 inhibitors, cilostazol, that similarly suppresses both PDE3A and PDE3B and causes vasodilation by increasing intracellular cAMP levels in vascular smooth muscle cells. It also has an antiplatelet effect [9,10]. PDE3 inhibitors, for example cilostazol, are widely used for the supportive treatment of chronic peripheral vascular diseases such as intermittent claudication [11,12] and other peripheral arterial occlusive disorders [11-16]. Moreover, it is widely used for the prevention of thrombogenesis and restenosis after percutaneous coronary intervention [10,16]. Because of these well-established therapeutic indications of PDE3 inhibitors, their safety profile have been successfully studied [17-20]. However, there are only two dermatological reports on the effects of PDE3 inhibitors [21,22]. It has been shown that following topical application of cilostazol, a typical PDE3 inhibitor, enhanced cutaneous blood flow and wound healing in rabbit skin [21]. Moreover, cilostazol was shown to have anti-wrinkle property in ultraviolet-B-irradiated mice. Cilostazol decreased wrinkle formation and skin thickness in UVB-irradiated mice, as well as increased staining of collagen fibers and inhibition of reactive oxygen species (ROS) formation in the skin [22].

HFs and HF stem cells are important cellular contributions to wound healing and reepithelialization [23-28]. Moreover, during the wound-healing process, cutaneous blood flow increases due to vasodilatation and complex microvascular responses such as significant changes in the levels of vasodilation mediators [29,30]. Stimulating cutaneous blood flow in human balding scalps enhances microcirculation in the surrounding HFs and can eventually lead to the promotion of hair growth and hypertrichosis [31-33]. However, there is no report on the potential effects of PDE3 inhibitor on hair growth.

Therefore, in the present study, we investigated the effects of PDE3 inhibitor on

hair growth in human dermal papilla cells and human outer root sheath cells. We also investigated the effects of PDE3 inhibitor on hair shaft elongation in an *ex vivo* organ culture of human HFs. Lastly, we evaluated PDE3 inhibitor's effects on anagen induction in C57BL/6 mice.

Materials and Methods

1. Ethics statement

Study protocols were approved by the institutional research board of Seoul National University Hospital (IRB No. H-1612-028-812), and written informed consent was obtained from all subjects. All experimental procedures using human tissues were performed according to the principles described in the Declaration of Helsinki.

2. Isolation and culturing of human HFs

Scalp tissue samples (1.5 × 1.0 cm) from the occipital region were taken from healthy male volunteers without current or prior scalp diseases. The HFs were used in cell culture and organ culture studies. The follicles were isolated and cultured using a previously described method with minor modifications [34,35]. Briefly, a subcutaneous fat portion of scalp skin including the lower part of HFs was dissected from the epidermis and dermis. The follicles were isolated under a microscope (Olympus, Tokyo, Japan) with forceps and maintained in William's E medium (Sigma-Aldrich, St. Louis, MO, USA) at 37℃ in a humidified atmosphere of 95% O_2 and 5% CO_2.

3. Culture of DPCs and ORSCs

Human DPCs were isolated from the bulbs of dissected HFs and cultured as described previously [36]. Briefly, candlelight-shaped DPs were dissociated and incubated in Dulbecco's modified Eagle medium (DMEM; Welgene, Daegu, Republic of Korea) supplemented with 10% fetal bovine serum (FBS, Welgene) and

1 × antibiotic/antimycotic solution (Gibco BRL, Gaithersburg, MD, USA) containing penicillin and streptomycin at 37℃ in a 5% CO_2 atmosphere. For the isolation of ORSCs, the hair shaft and hair bulb regions of the HF were cut off to prevent contamination with other cells. Trimmed HFs were immersed in DMEM supplemented with 20% FBS. On the third day of culture, the medium was changed to KGM-Gold™ Keratinocyte Growth Medium (Lonza, Walkersville, MD, USA) supplemented with basal medium (bovine pituitary extract, human epidermal growth factor, bovine insulin, hydrocortisone, gentamicin, amphotericin B, epinephrine, and transferrin) at 37℃ in a 5% CO_2 atmosphere.

4. Preparation of the conditioned medium from hDPCs (DP-CM)

To prepare DP-CM, DPCs were grown on a 100-mm cell culture dish to 100% confluency in DMEM supplemented with 10% FBS. After washing the cells with phosphate-buffered saline three times, the DPCs were cultured with serum-free KGM-Gold™ Keratinocyte Growth Medium and treated with various concentrations of cilostazol (Sigma-Aldrich, St. Louis, MO, USA). After 72 hours, the conditioned medium was collected, centrifuged at 1800 rpm for 10 minutes, and filtered using a 0.22-μm syringe filter. The filtrate was then centrifuged in 3-kDa molecular weight cut-off Vivaspin (Sartorius Stedim Biotech GmbH, Goettingen, Germany) and concentrated.

5. Proliferation assays

Cell proliferation was measured by the bromodeoxyuridine (BrdU) and water-soluble tetrazolium salts-1 assays. DPCs (5 × 10^3 cells/well) and ORSCs (1 × 10^4 cells/well) were seeded into 96-well plates, serum-starved for 24 hours, and

then treated with cilostazol for 2days. For the BrdU assay, Cell Proliferation ELISA (BrdU chemiluminescent) (Roche, Mannheim, Germany) was used according to the manufacturer's instructions. After the addition of BrdU labeling solution, the cells were incubated for 4 hours at 37℃ in 5% CO_2. After the final washing step, 100 μL/well substrate solution was added to the cells, followed by analysis using a luminometer (Victor 3; PerkinElmer, Waltham, MA, USA). ORSCs were seeded at a density of 5×10^3 cells/well in 96-well plates. After 24 hours of incubation, the culture medium was replaced with a new medium containing various concentrations of cilostazol-treated DP-CM. After the required incubation with stimulants for 72 hours, cell proliferation was measured using EZ-Cytox Cell Viability Assaykit (Daeillab Service, Seoul, Korea). Briefly, 10 μL of EZ-Cytox solution was added to each well and the plate was incubated at 37℃ for 2 hours. Absorbance was then measured at 450nm using an ELISA reader (Tecan, Grödig, Austria).

6. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from DPCs and ORSCs using RNAiso Plus reagent (Takara Bio, Shiga, Japan). Approximately 2 μg of total RNA was used for cDNA synthesis reaction using First Strand cDNA Synthesis Kit (Fermentas, Sankt Leon-Rot, Germany) according to the manufacturer's instructions. Subsequently, 50-100 ng of the cDNA was used in PCR amplification and the primers used to amplify each target gene were as follows: PDE3A (forward, CGTCGGGGAGGATCACTTAC; reverse, TGAATGCCCCATGAGCTGTT), PDE3B (forward, AGGCACAGAGTGAACAG-CAA; reverse, ACAGGCAGCCATAACTCTCA), and glyceraldehyde-3-phosphate de-hydrogenase (GAPDH; forward, ATTGTTGCCATCAATGACCC; reverse, AGTAGAGG-CAGGGATGATGT). To normalize for differences in cDNA loading, primers designed to amplify GAPDH were used. The conditions for PCR were 3 minutes at 94℃, 30 seconds at 94℃, 30 seconds at 60℃, 45 seconds at 72℃, and 1 minute at 72℃

for 35 cycles.

7. Human growth factor antibody array

Growth factor array was carried out using RayBio® Human Growth Factor Antibody Array C1 (AAG-GF-1-8; RayBiotech, Norcross, GA, USA) according to the manufacturer's instructions. Membranes were first blocked with blocking buffer. Next, 1 mL of cilostazol-treated DP-CM was added and the membranes were incubated overnight at 4℃. Finally, immunoreactivity was assessed and quantified using Image J software (National Institutes of Health, Bethesda, MD, USA).

8. Human HF organ culture

Human scalp HFs were isolated and cultured as described previously [37,38]. Each dissected HF was cut from the bottom of the DP into pieces (approximately 3.5 mm in length) and cultured in William's E medium (Gibco BRL) supplemented with 10 ng/mL hydrocortisone (Sigma-Aldrich), 10 mg/mL insulin (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), and 1× antibiotic/antimycotic solution (Gibco BRL) containing penicillin and streptomycin. Follicles were maintained at 37℃ in a 5% CO_2 atmosphere. The human scalp HFs were obtained from three different individuals (> 60 HFs per subject) and treated with 1 and 10 μM cilostazol for 6 days. In all experiments, the tissue culture medium was changed every other day. HF elongation was measured directly at 2, 4, and 6 days of culture using a stereomicroscope (Olympus).

9. Immunofluorescence staining

Human DPCs and ORSCs were seeded at 1.0 × 10^4cells/500 μL per chamber into a chamber slide, serum-starved for 24 hours, and then treated with cilostazol for 24 hours. The cells were then treated with 4% paraformaldehyde for 10 minutes. Cultured DPCs and ORSCs were incubated with anti-PDE3A and anti-PDE3B antibodies at 4℃ overnight and then with Alexa Fluor 594-labeled goat anti-rabbit IgG (Invitrogen, Kyoto, Japan). A 4',6-diamidino-2-phenylindole (DAPI) mounting media kit was used to counterstain nuclei. Immunofluorescence staining for Ki67 and CD31 was performed on 5-μm frozen sections of human HFs and 15-μm frozen sections of mouse skin as previously described [39]. TUNEL labeling (Millipore, Billerica, MA, USA) was used as an indicator of cell apoptosis. The following antibodies were used: anti-PDE3A (Novus Biologicals, CA, USA), anti-PDE3B (Novus Biologicals), anti-Ki67 (DAKO, Carpinteria, CA, USA), anti-CD31 (BioLegend, San Diego, CA, USA), anti-hepatocyte growth factor (anti-HGF) (R&D systems, Minneapolis, MN, USA), and anti-vascular endothelial growth factor (anti-VEGF) (DAKO, Carpinteria, CA, USA) antibodies. Immunofluorescence images were acquired using a digital fluorescence microscope (Nikon, Tokyo, Japan). The number of Ki67-positive cells was counted and normalized to the number of DAPI-stained cells. Ki67-positive cells and CD31-stained vessel size were measured using Image J software.

10. Induction of anagen hair growth in C57BL/6 mice

We performed an anagen induction assay as previously described [40]. The back skin of 8-week-old C57BL/6 female mice in the telogen phase was shaved with a clipper. Vehicle (30% polyethylene glycol + 70% ethanol), cilostazol (0.2 and 1%), and minoxidil (2%) were then topically applied to the shaved skin every weekday for 3 weeks. At day 21, skin samples were obtained from the treated site for histological analysis by hematoxylin and eosin (H&E) staining. Skin thickness and

anagen induction score were assessed using a slightly modified version of previously described methods [41-43]. Anagen induction scores were calculated using assigned score values (telogen = 1, anagen I-VI = 2-7) [41-43]. The resulting values were added and divided by the number of HFs (n = 70 HFs/group). In the histological analysis by H&E staining, skin thickness was measured as the distance from the epidermis to the subcutaneous fat using the Image J software.

11. Western blot analysis

Mouse skin tissues were lysed in radioimmunoprecipitation assay buffer (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Equal amounts of total proteins were separated by electrophoresis using 10% sodium dodecyl sulfate-polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane (Amersham, Buckinghamshire, UK) using semi-dry Trans-Blot® Turbo™ Transfer System (Bio-Rad, CA, USA). The blotted membranes were incubated with the respective primary antibodies at 4℃. The following antibodies were used: anti-phospho extracellular signal-regulated kinase (ERK, #9101), anti-total ERK (#9102), anti-phospho c-Jun N-terminal kinase (JNK, #9251), anti-total JNK (#9252), anti-phospho P38 (#9211), anti-total P38 (#9212), anti-Bcl-2 (#2872), anti-BAX (#5023) (Cell Signaling Technology, Beverly, MA, USA), anti-hHGF (#AF-294-NA), anti-p-HGFR (#AF-2480; R&D systems, Minneapolis, MN, USA), anti-VEGF (#ab150766), anti-platelet-derived growth factor (anti-PDGF) (#ab34074; Abcam, Cambridge, UK), anti-α tubulin (SC-8035), and anti-β-actin (SC-1616; Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies. Membranes were probed with anti-rabbit and anti-goat-IgG-horseradish peroxidase conjugates (Santa Cruz Biotechnology) for an hour at 25℃. Antibody-antigen complexes were detected using an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Little Chalfont, UK).

12. Statistical analysis

Statistical significance was determined by Student's *t*-test. *P*-values were two-tailed, and statistical significance was considered at $P < 0.05$. Repeated-measures ANOVA analysis of variance was used for statistical analysis of data from the organ culture studies.

Results

1. PDE3A and PDE3B expression in human DPCs, ORSCs, and HFs

We first investigated the expression of PDE3A and PDE3B in cultured DPCs and ORSCs isolated from human HFs (n = 4) by RT-PCR. PDE3A and PDE3B were highly expressed in hDPCs; however, the expression levels of PDE3A and PDE3B were not detectable in hORSCs (Fig. 1A). We also evaluated PDE3A and PDE3B expression patterns in cultured human DPCs, ORSCs, and HFs by immunofluorescence staining. The expression patterns of PDE3A and PDE3B proteins were similar to the respective mRNA expression patterns in hDPCs and hORSCs. PDE3A and PDE3B were highly expressed in both the nucleus and cytoplasm of hDPCs but not in those of hORSCs (Fig. 1B). Furthermore, PDE3A was markedly expressed in DPs and the lower part of the dermal sheaths of the HFs. PDE3B was also detected at the same site; however, its expression level was lower than that of PDE3A. We used human colon tissue as a positive control (Fig. 1C).

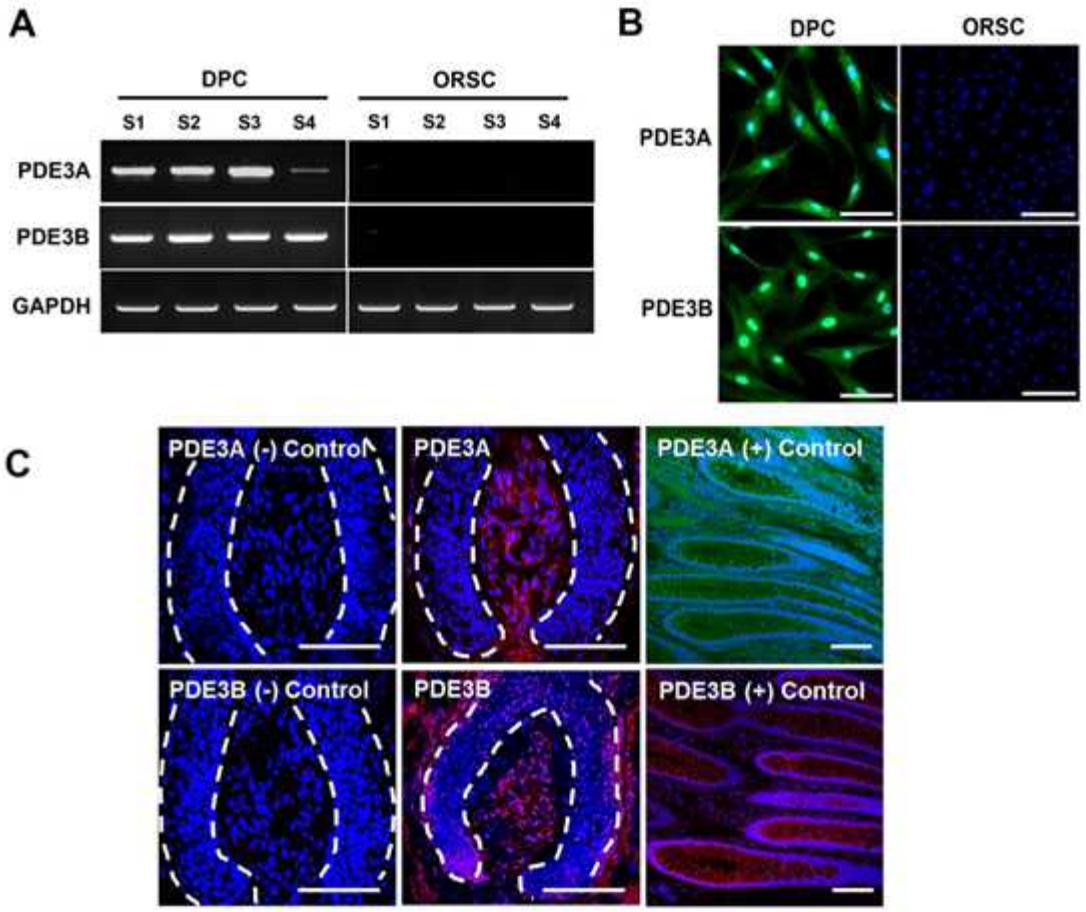

Fig. 1. Expression of phosphodiesterase (PDE) 3A and PDE3B in human dermal papilla cells (DPCs), outer root sheath cells (ORSCs), and hair follicles (HFs). (A) mRNA levels of PDE3A and PDE3B genes in DPCs and ORSCs, as determined by RT-PCR (S, subject; n = 4). Immunofluorescent staining of PDE3A and PDE3B was performed in (B) cultured human DPCs, ORSCs (green fluorescence), and (C) HFs (red fluorescence). Human colon tissue was selected as the positive control (C) (green fluorescence for PDE3A, red fluorescence for PDE3B), and the negative control was human HF without attached primary antibody. Nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI, blue fluorescence). Scale bar = 100 μm. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

2. PDE3 inhibitor enhances the proliferation of human DPCs and ORSCs

To investigate the effect of PDE3 inhibitor on HFs, we treated human DPCs and ORSCs for 48 hours with various concentrations of cilostazol (0.1-10 μM), a PDE3 inhibitor, and evaluated changes in cell proliferation by the BrdU incorporation assay. The results showed that cilostazol significantly enhanced the proliferation of DPCs more than the vehicle did (Fig. 2A); however, it did not enhance the proliferation of ORSCs (Fig. 2B). In addition, since DPCs secrete diverse paracrine factors, which stimulate the growth of keratinocytes and other DPCs, we specifically evaluated the conditioned medium derived from cilostazol-treated DPCs (DP-CM). Furthermore, we treated human ORSCs with DP-CM for 3 days to investigate whether cilostazol alters paracrine factors and growth factors secreted by DPCs and whether the proliferation of ORSCs is regulated by these factors. The results showed that at concentrations of 1-10 μM, cilostazol-treated DP-CM noticeably enhanced ORSC proliferation more than the vehicle-treated DP-CM. In addition, the most effective concentration of cilostazol-treated DP-CM was 1 μM (Fig. 2C).

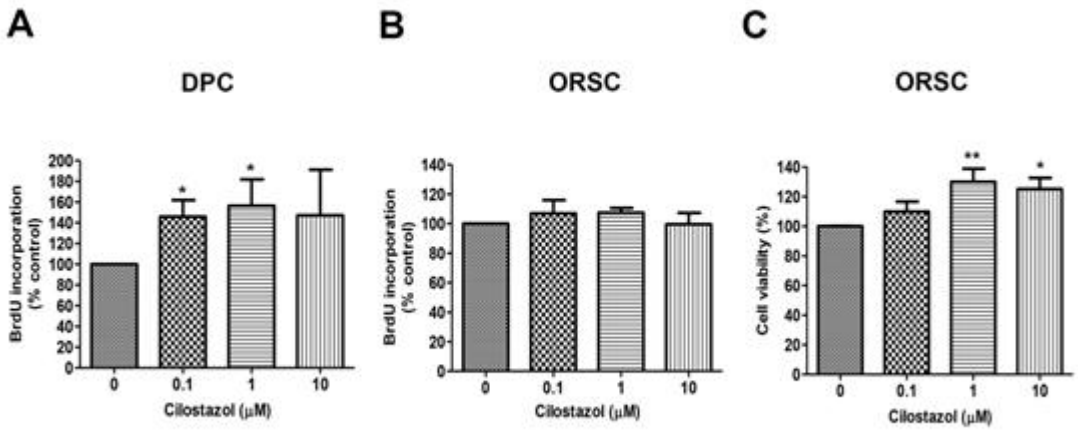

Fig. 2. Effect of PDE3 inhibitor on the proliferation of human dermal papilla cells (DPCs) and outer root sheath cells (ORSCs). The proliferation of (A) DPCs and (B) ORSCs was measured by the BrdU incorporation assay after treatment of the cells with cilostazol (0, 0.1, 1, or 10 μM), a PDE3 inhibitor, for 48 hours. (C) ORSCs were incubated with cilostazol-treated dermal papilla conditioned medium (DP-CM) for 72 hours and cell proliferation was measured by the WST-1 assay. The results are shown as mean ± standard error values (n = 4). ** indicates $P < 0.01$ and * indicates $P < 0.05$ when compared to the control group.

3. PDE3 inhibitor regulates growth factor secretion by DPCs

We hypothesized that PDE3 inhibitor can regulate the growth factors secreted by DPCs to enhance ORSC proliferation. Therefore, we treated DPCs with 1 μM cilostazol, a PDE3 inhibitor, and used human growth factor array to investigate the levels of growth factors secreted. Spot density was measured using a densitometer. The signal intensities of insulin-like growth factor binding protein (IGFBP)-1, IGFBP-3, insulin-like growth factor-2 (IGF-2), macrophage colony-stimulating factor (M-CSF), M-CSF receptor (M-CSFR), PDGF receptor β (PDGF-Rβ), PDGF-AB, and PDGF-BB significantly increased in DP-CM (Fig. 3B).

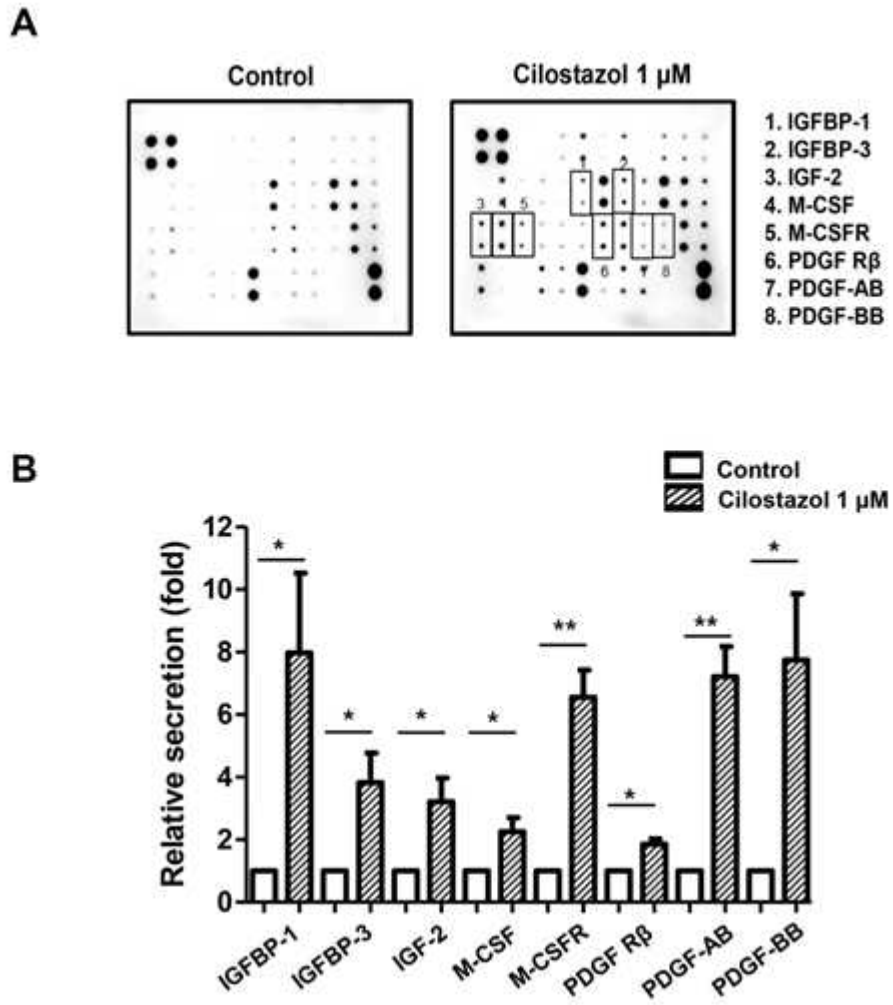

Fig. 3. Effect of PDE3 inhibitor on the secretion of growth factors in human dermal papilla cells (DPCs). (A) Growth factor secretion in the conditioned medium derived from DPCs (DP-CM) was measured using a human growth factor antibody array. The antibody array measured 41 growth factors. (B) The levels of IGFBP-1, IGFBP-3, IGF-2, M-CSF, M-CSFR, PDGF-Rβ, PDGF-AB, and PDGF-BB in vehicle-treated DP-CM were compared to the respective levels in cilostazol-treated DP-CM. The results are shown as mean ± standard error values (n = 3). ** indicates $P < 0.01$ and * indicates $P < 0.05$ when compared to the control group.

4. PDE3 inhibitor enhances hair shaft elongation in *ex vivo* hair organ culture

To determine whether PDE3 inhibitor stimulates hair shaft elongation, we examined the effects of cilostazol, a PDE3 inhibitor, using an *ex vivo* organ culture of human HFs. Human HFs were cultured in 1 and 10 μM cilostazol for 6 days, and elongation of the hair shaft was measured every other day. The results showed that cilostazol promoted hair growth in the organ cultures in a dose-dependent manner. In addition, 1 μM cilostazol significantly enhanced hair shaft elongation after 6 days (Fig. 4A). Furthermore, we performed immuno-fluorescence staining of Ki-67, which is a proliferation marker, when HFs were treated with cilostazol for 2 days.

We also analyzed the number of Ki-67 positive cells (green fluorescence) and normalized it to the number of DAPI-stained cells (blue fluorescence) in follicular matrix keratinocytes of the hair bulb. We counted only the cells below the line of Auber. As shown in Fig. 4B, cilostazol treatment significantly increased the number of Ki-67-positive keratinocytes, which accounted for 20.8% of the vehicle-treated hair matrix, 34.9% of the 1 μM cilostazol-treated hair matrix, and 33.5% of the 10 μM cilostazol-treated hair matrix. This is an indication that cilostazol significantly increases the proliferation of keratinocytes (Fig. 4C). However, cilostazol did not likely affect the apoptosis of hDPCs and ORSCs (Fig. 5).

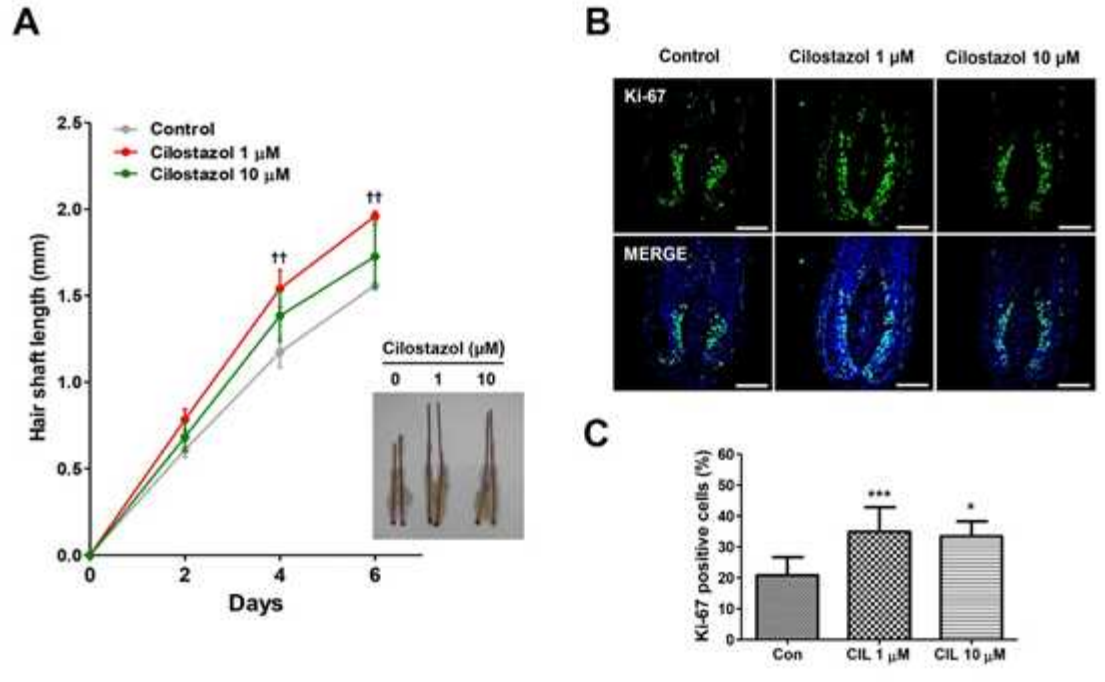

Fig. 4. Effect of PDE3 inhibitor on hair shaft elongation in an *ex vivo* hair organ culture. (A) Human HFs were treated with vehicle or cilostazol (1 or 10 μM), a PDE3 inhibitor, for 6 days. HF elongation was measured at 2, 4, and 6 days after culturing the cells using a stereomicroscope (n = 3; n ≥ 60 follicles for each subject). †† indicates the statistically significant difference ($P < 0.01$) between different groups using repeated-measures ANOVA analysis. (B) Human HFs were cultured with vehicle or cilostazol (1 or 10 μM) for 2 days and analyzed for proliferation (Ki67, green fluorescence) by immunofluorescence staining. DAPI was used to counterstain nuclei (blue fluorescence). Scale bar = 100 μm. (C) The number of Ki67-positive cells was counted and normalized to the number of DAPI-stained cells. The results are shown as mean ± standard error values (n = 3). *** indicates $P < 0.001$ and * indicates $P < 0.05$ when compared to the control group using Student's *t*-test.

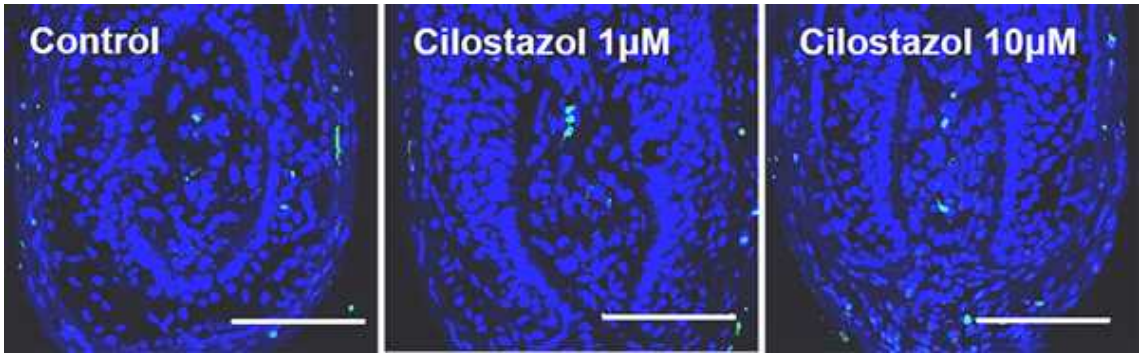

Fig. 5. Immunofluorescent staining of TUNEL assay for analysis of apoptosis. Human HFs were cultured *in vitro* with vehicle, cilostazol 1 or 10 μM for 3 days and analyzed for apoptosis in the hair matrix keratinocytes by using immunofluorescence staining (TUNEL, green fluorescence). For quantitative analysis, TUNEL positive cells were counted and normalized to DAPI-stained cells by Meta Morgh V.7.8.10 Premier software for offline analysis and processing. Nuclei were stained by 4',6-diamidino-2-phenylindole (DAPI, blue fluorescence). Scale bar = 100 μm.

5. PDE3 inhibitor stimulates hair growth in C57BL/6 mice

We investigated whether cilostazol (0.2 and 1%), would accelerate spontaneous anagen entry after shaving gently with clipper in 8-week-old C57BL/6 female mice. We found that cilostazol-treated mice exhibited gross skin color change from pink to light gray at day 7 after hair growth induction, indicating transition of the hair growth phase from the telogen phase to the anagen phase. In addition, their hair shafts were visible at day 14. At day 21, the area of black skin was significantly larger in the cilostazol-treated group than in the control group. In addition, the hair coat was poorly regenerated in the vehicle-treated group (Fig. 6A). Histological studies showed more anagen HFs and thicker back skin in the cilostazol-treated group than in the control group (Fig. 6B). The thickness of interfollicular whole skin and HF size significantly increased after treatment with cilostazol (Fig. 6C). When anagen HFs were scored according to the anagen phase of each HF, the score was significantly higher in the cilostazol-treated group than in the control group (Fig. 6D). These results suggest that PDE3 inhibitor significantly accelerates the growth of HFs and induces a fast entry into the anagen phase. Furthermore, cilostazol treatment-induced increase in the levels of potent anagen inducers such as HGF, VEGF, and PDGF-A could enhance hair growth (Fig. 7).

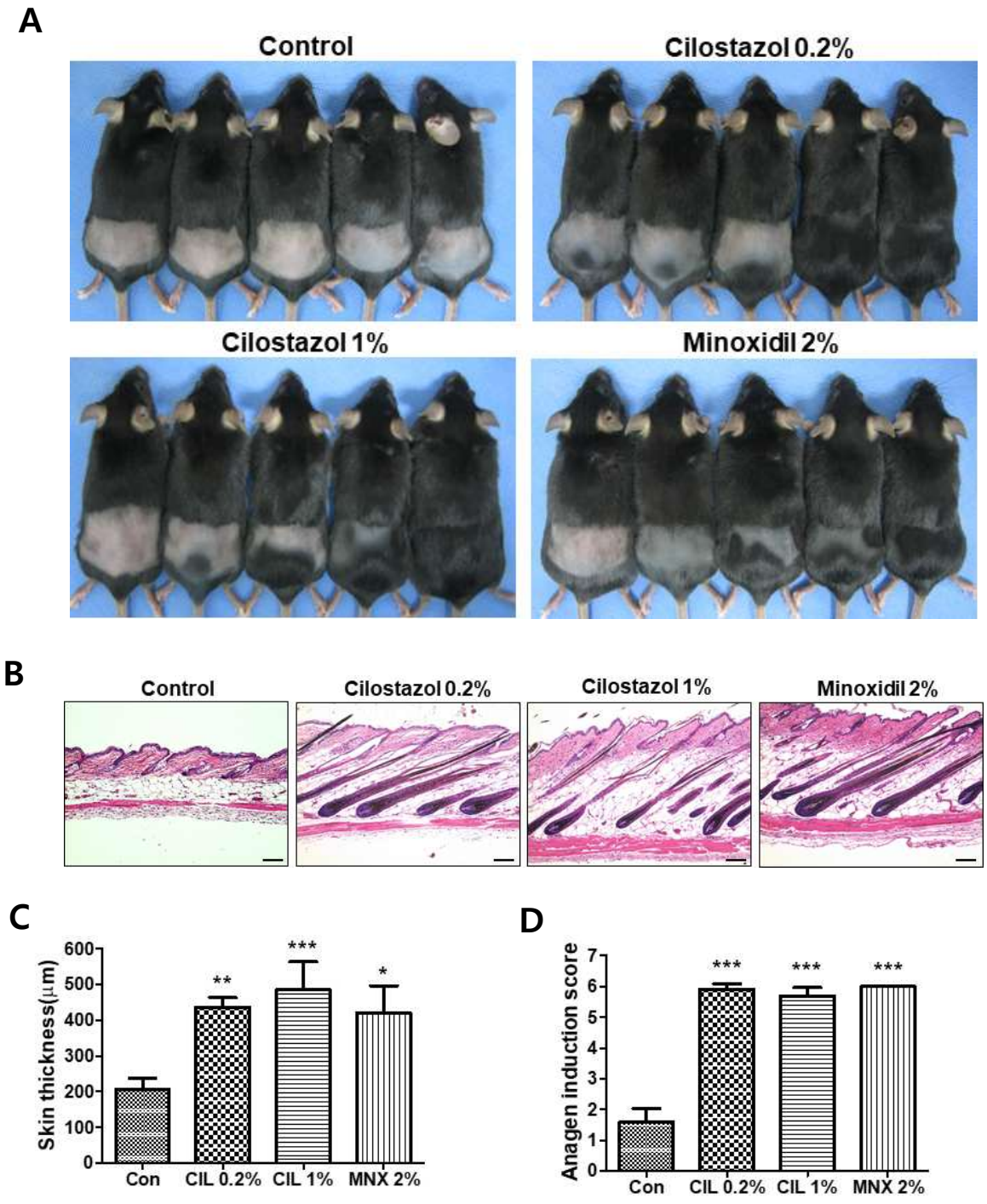

Fig. 6. Effect of PDE3 inhibitor on anagen induction in 8-week-old female C57BL/6 mice. (A) The backs of the mice (n = 5 in each group) were shaved, after which they were treated with vehicle, 0.2% cilostazol, 1% cilostazol, or 2% minoxidil for 3 weeks. (B) Histological analysis of paraffin-embedded skin samples

by hematoxylin and eosin staining. Scale bar = 100 μm. (C) Skin thickness was measured as the distance from the epidermis to subcutaneous fat. Numbers on the bars are relative percentages compared with the control. (D) Anagen induction scores (telogen = 1, anagen I-VI = 2-7, n = 70 HFs/group). The results are shown as mean ± standard error values. *** indicates $P < 0.001$, ** indicates $P < 0.01$, and * indicates $P < 0.05$ when compared to the control group.

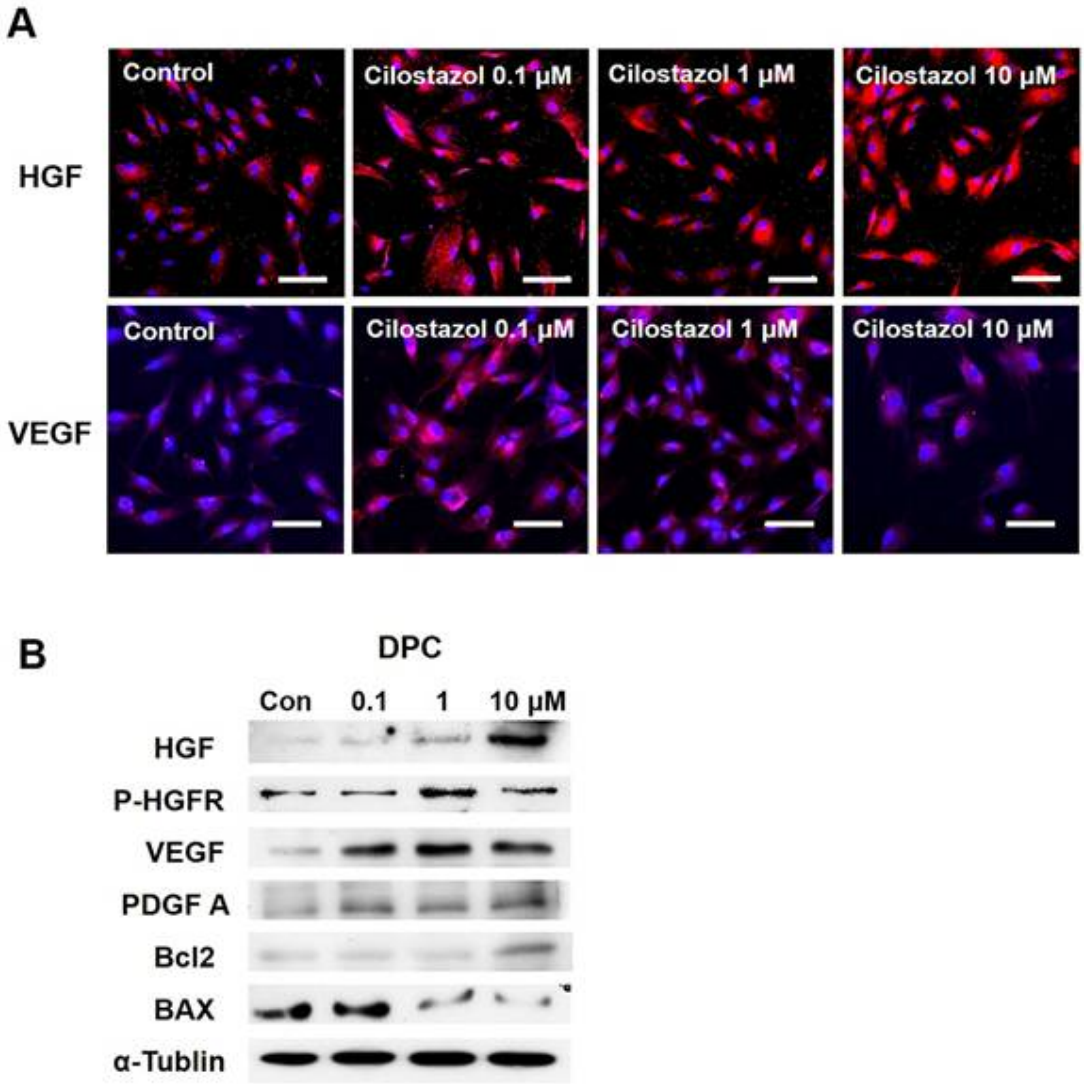

Fig. 7. Effect of PDE3 inhibitor on multiple anagen inducers: Immunocytochemistry for hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) and Western blotting assay on HGF, VEGF, platelet-derived growth factorA (PDGF-A), B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (BAX) in hDPCs. (A) hDPCs were cultured with vehicle or cilostazol (0.1, 1 or 10 μM) for 24hrs and analyzed for protein expression of HGF and VEGF (red fluorescence) by Immunocytochemistry. (B) hDPCs treated with cilostazol and were lysed and analyzed by Western blotting using primary antibodies against HGF, P-HGF, VEGF, PDGF-A, Bcl-2 and BAX. hDPCs were cultured with cilostazol 0.1, 1, or 10 μM for 24 hrs. Scale bar = 100 μm.

6. PDE3 inhibitor increases vessel formation and activates the mitogen-activated protein kinase (MAPK) signaling pathway

To investigate whether PDE3 inhibitor promotes angiogenesis, we performed immunofluorescence staining of the blood vessel marker CD31. In order to ensure proper selection of the control groups, we used mice that had naturally entered the anagen phase as controls, and excluded mice in the telogen phase on day 21. As shown in Fig. 8A, large vessels were observed surrounding the elongated HFs and bulb after cilostazol treatment. In addition, perifollicular vessel size was more than a 2-fold greater in the cilostazol-treated group (average size of 1900 μm^2) than in the vehicle-treated group (average size of 900 μm^2) (Fig. 8B). To further determine the mechanisms by which cilostazol stimulates hair growth and vessel formation, we analyzed the expression of various signaling molecules involved in cell proliferation and angiogenesis, such as ERK, JNK, and P38, in cilostazol-treated mouse skin by western blotting. We found that PDE3 inhibitor significantly enhanced the expression levels of phosphorylated ERK, JNK, and P38 in mouse skin (Fig. 8C). We also found that PDE3 inhibitor increased p-ERK, p-JNK, and p-P38 protein expression to a relatively greater degree in hDPCs than in ORSCs (Fig. 9).

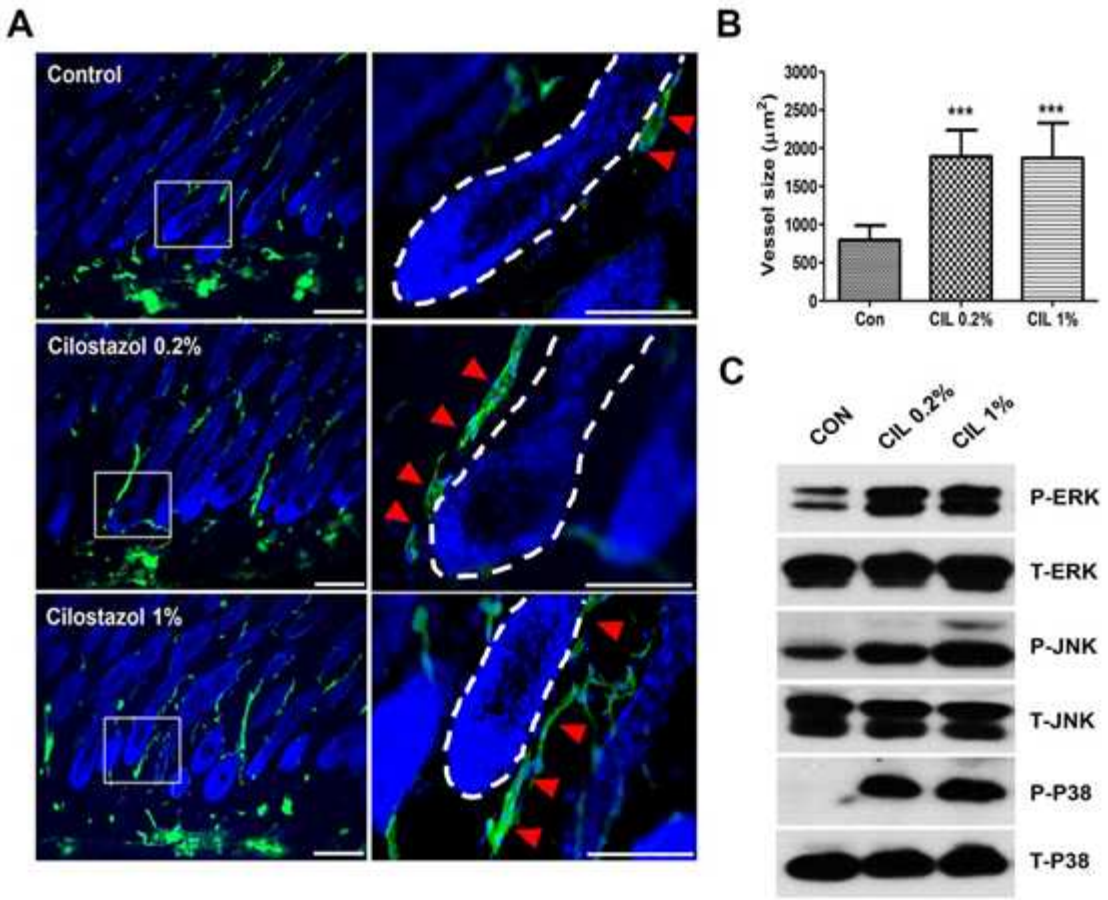

Fig. 8. Effect of PDE3 inhibitor on vessel formation and mitogen-activated protein kinase expression. (A) Immunofluorescent staining of CD31 (green fluorescence). DAPI was used to counterstain nuclei (blue fluorescence). Red arrowheads mark the outlines of the vessels surrounding HFs. Scale bars = 100 μm (left) and 50 μm (right). (B) Quantification of vessel size in vehicle- or cilostazol-treated mice (n = 5 random fields, n = 4 mice/group). Data are presented as mean ± standard error values. *** indicates $P < 0.001$ when compared to the control group. (C) Mouse skin was lysed and analyzed by western blotting using primary antibodies against total-p42/44 ERK, phospho-p42/44 ERK, total-JNK, phospho-JNK, total-P38, phospho-P38, and β-actin. The results are shown as a representative figure of three individual experiments.

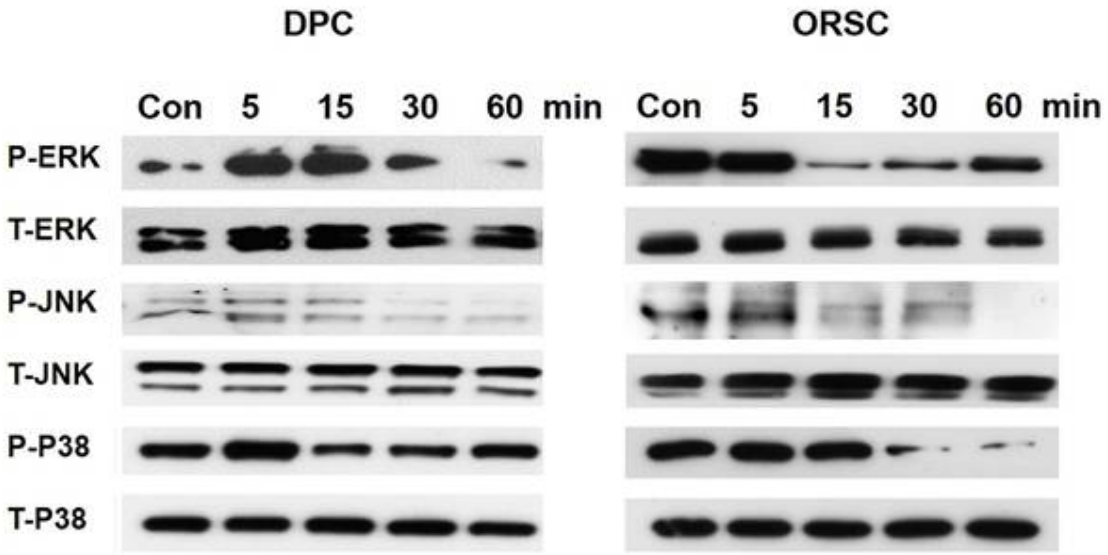

Fig. 9. Effect of PDE3 inhibitor on mitogen-activated protein kinase expression. hDPCs treated with cilostazol 1 μM (for 5, 15, 30, 60 min) were lysed and analyzed by Western blotting using primary antibodies against total-p42/44 ERK, phospho-p42/44 ERK, total-JNK, phospho-JNK, total-P38, and phospho-P38.

Discussion

In this study, cilostazol, a typical PDE3 inhibitor, was investigated for its potential to enhance hair growth. As previously mentioned, increasing cutaneous blood flow in the balding scalp enhances microcirculation, which may accelerate the growth of HFs [31-33]. PDE3 inhibitor is a vasodilator used to treat and prevent peripheral vascular diseases [11-16]; therefore, we believed that it could promote hair growth.

First, we found that PDE3A and PDE3B genes were highly expressed in hDPCs but not in hORSCs. A similar expression pattern was observed through immuno-fluorescent staining for PDE3A and PDE3B detection in individual human HFs, as well as through the immunocytochemical analysis of hDPCs and hORSCs. Furthermore, we demonstrated that PDE3 inhibitor significantly increased the viability and proliferation of hDPCs; however, it did not enhance the proliferation of hORSCs directly. Taken together, the results indicate that PDE3 inhibitor could have a direct effect on hDPCs but not on hORSCs. Nevertheless, because DPCs and ORSCs closely interact during the entire hair growth cycle [44], we assumed that the effects of PDE3 inhibitor on DPCs could indirectly influence the physiology of ORSCs and result in hair growth. Subsequently, when we cultured hORSCs with cilostazol-treated DP-CM, we found that cilostazol-treated DP-CM effectively increased the proliferation of hORSCs compared to the control cells. We consequently assumed that some small molecules such as growth factors and cytokines secreted by DPCs might affect the growth of ORSCs. Therefore, we investigated the paracrine effects of PDE3 inhibitor on human HFs using cilostazol-treated DP-CM. The secretion levels of the following three groups of growth factors significantly increased in cilostazol-treated DP-CM: IGFBP-1, IGFBP-3, and IGF-2, which are IGF-associated growth factors; M-CSF and M-CSF R; and PDGF-Rβ, PDGF-AB, and PDGF-BB, which were all well-known secreted molecules associated with HF growth and the hair cycle. In particular, IGFs are

potent hair growth stimulators that enhance keratinocyte proliferation and prevent HFs from entering into a catagen-like phase [34,45]. IGFBPs also affect the growth of HFs because they can modulate the action of IGF subfamilies [46,47]. In addition, increased secretion of M-CSF and M-CSFR promotes hair growth and accelerates skin wound healing [48,49]. Moreover, previous studies have demonstrated that PDGF-AA, PDGF-BB, and PDGF-Rβ signaling can induce and maintain the anagen phase of HFs. Additionally, the important ability of these three PDGF molecules to preserve hair inductive activity by modulating HF dermal stem cells has been confirmed [49-52]. PDGF-Rβ is a well-known component secreted from adipose-derived stem cells and promotes hair growth. It can be stimulated by PDGF-AB, a hybrid form that has a high affinity for PDGF-Rβ and PDGF-BB [49,53]. These growth factors are well-known components of human HF conditioned medium, and can interact and promote the growth of HFs. The effects of conditioned medium containing growth factors for clinical hair growth have also been reported [48]. In the present study, we hypothesized that these growth factors may affect the surrounding conditions of HFs, especially ORSCs, and act as important factors in enhancing final hair growth.

In addition, we investigated the effects of PDE3 inhibitor on hair growth in an *ex vivo* organ culture of human HFs and found that PDE3 inhibitor promotes hair shaft elongation. It was also found that PDE3 inhibitor increased the proliferation of human hair matrix keratinocytes, which was confirmed by immunofluorescence staining for Ki-67 detection. These results suggested that PDE3 inhibitor stimulates hair growth by increasing the proliferation of hair matrix cells.

Furthermore, an anagen induction test revealed that topically applied PDE3 inhibitor markedly accelerated anagen induction in C57BL/6 mice, due to its promoting effect on the hair growth cycle. We assumed that the cilostazol treatment-induced increase in the levels of potent anagen inducers such as HGF, VEGF, and PDGF-A could enhance hair growth. Moreover, cilostazol and 2% minoxidil, a hair growth inducer used as a positive control, similarly thickened the

back skins of the mice. These findings suggest that topical PDE3 inhibitor enhances hair growth effectively. The concentration of cilostazol was relatively higher *in vivo* testing than *in vitro* or *ex vivo* studies. The different concentration was selected separately because of the consideration of the penetration of cilostazol through the mouse skin. In other words, direct delivery of treated substance would be more efficient *in vitro* or *ex vivo* conditions than in animal assay. Nevertheless, cilostazol is suitable for topical use because it is expected to be absorbed through the skin without difficulty due to its low molecular weight (369.46 g/mol). Moreover, the long clinical history of its oral use guarantees its systemic safety following topical use, particularly because its serum level is much lower when it is applied topically [22]. Additionally, in the present study, cilostazol-treated mice did not show any evident adverse effects.

We also performed western blotting to investigate the signaling pathway through which PDE3 inhibitor enhances hair growth and increases cell proliferation. The results showed that PDE3 inhibitor increased the phosphorylation of ERK and P38, which are known to promote cell survival and prevent cell death [39,54-57]. Since this MAPK-associated signaling pathway is closely related to survival promotion and apoptosis prevention [39,54-57], we concluded that the MAPK signaling pathway is specifically involved in hair growth enhancement by PDE3 inhibitor.

Although there are no reports on the effect of PDE3 inhibitor on HFs, PDE3 inhibitor is known to potentiate angiogenesis [10,58,59]; therefore, we investigated whether PDE3 inhibitor affects cutaneous vessel formation around HFs. We performed immunofluorescence staining of CD31 and found that PDE3 inhibitor promotes HF vessel formation. An increment in the number of new vessels was also detected in adjacent DPs, which suggests that molecules secreted by DP, such as PDGF, may affect angiogenesis [60]. Taken together, these results show that PDE3 inhibitor increased neovascularization near HFs, which allowed for the effective transport of the various growth factors secreted by DP to adjacent matrix keratinocytes and ORSCs. This resulted in the stimulation of hair growth,

probably through the MAPK signaling pathway.

In summary, our results demonstrate that PDE3 inhibitor significantly stimulates hair growth both *in vitro* and *in vivo*. In addition, it accelerates *in vitro* proliferation of DPCs, enhances *ex vivo* hair shaft elongation, and induces *in vivo* anagen hair induction through the promotion of vessel formation. Furthermore, the possible mechanisms underlying the effects of PDE3 inhibitor, which make it a potential treatment for alopecia, include direct stimulation of DPC proliferation through the MAPK signaling pathway and facilitation of neovascularization around HFs. Our findings show that PDE3 inhibitor effectively induces the growth of human HFs; therefore, it may be a new therapeutic agent for hair loss.

References

[1] M.R. Schneider, R. Schmidt-Ullrich, R. Paus, The hair follicle as a dynamic miniorgan, Curr. Biol. 19 (3) (2009) 132-142.

[2] S.E. Millar, Molecular mechanisms regulating hair follicle development, J. Invest. Dermatol, 118 (2002) 216-225.

[3] Y. Kelly, A. Blanco, A. Tosti, Androgenetic Alopecia: An Update of Treatment Options, Drugs. 76 (14) (2016) 1349-1364.

[4] D.H. Maurice, H. Ke, F. Ahmad, Y. Wang, J. Chung, V.C. Manganiello, Advances in targeting cyclic nucleotide phosphodiesterases, Nat. Rev. Drug. Discov. 13 (4) (2014) 290-314.

[5] T. Keravis, C. Lugnier, Cyclic nucleotide phosphodiesterase (PDE) isozymes as targets of the intracellular signalling network: benefits of PDE inhibitors in various diseases and perspectives for future therapeutic developments, Brit. J. Pharmacol. 165 (5) (2012) 1288-1305.

[6] S.H. Francis, M.A. Blount, J.D. Corbin, Mammalian cyclic nucleotide phosphodiesterases: molecular mechanisms and physiological functions, Physiol. Rev. 91 (2) (2011) 651-690.

[7] M. Conti, J. Beavo, Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signaling, Annu. Rev. Biochem. 76 (2007) 481-511.

[8] M. Wilhelm, J. Schlegl, H. Hahne, A.M. Gholami, M. Lieberenz, M.M. Savitski, E. Ziegler, L. Butzmann, S. Gessulat, H. Marx, T. Mathieson, S. Lemeer, K. Schnatbaum, U. Reimer, H. Wenschuh, M. Mollenhauer, J. Slotta-Huspenina, J.H. Boese, M. Bantscheff, A. Gerstmair, F. Faerber, B. Kuster, Mass-spectrometry-based draft of the human proteome, Nature. 509 (7502) (2014) 582-587.

[9] A. Hashimoto, M. Tanaka, S. Takeda, H. Ito, K. Nagano, Cilostazol Induces PGI2 Production via Activation of the Downstream Epac-1/Rap1 Signaling Cascade to

Increase Intracellular Calcium by PLC ε and to Activate p44/42 MAPK in Human Aortic Endothelial Cells, PLoS. One. 10 (7) (2015) e0132835.

[10] Y. Liu, Y. Shakur, M. Yoshitake, J. Kambayashi Ji, Cilostazol (pletal): a dual inhibitor of cyclic nucleotide phosphodiesterase type 3 and adenosine uptake, Cardiovasc. Drug. Rev. 19 (4) (2001) 369-386.

[11] J.W. Stevens, E. Simpson, S Harnan, H. Squires, Y. Meng, S. Thomas, J. Michaels, G. Stansby, Systematic review of the efficacy of cilostazol, naftidrofuryl oxalate and pentoxifylline for the treatment of intermittent claudication, Br. J. Surg. 99 (12) (2012) 1630-1638.

[12] W.R. Hiatt, Current and future drug therapies for claudication, Vasc. Med. 2 (3) (1997) 257-262.

[13] G. de Donato, F. Setacci, M. Mele, G. Giannace, G. Galzerano, C. Setacci, Restenosis after Coronary and Peripheral Intervention: Efficacy and Clinical Impact of Cilostazol, Ann. Vasc. Surg. 41 (2017) 300-307.

[14] S. Dindyal, C. Kyriakides, A review of cilostazol, a phosphodiesterase inhibitor, and its role in preventing both coronary and peripheral arterial restenosis following endovascular therapy, Recent. Pat. Cardiovasc. Drug. Discov. 4 (1) (2009) 6-14.

[15] P. Robless, D.P. Mikhailidis, G.P. Stansby, Cilostazol for peripheral arterial disease, Cochrane. Database. Syst. Rev. (1) (2008) Cd003748.

[16] G.G. Biondi-Zoccai, M. Lotrionte, M. Anselmino, C. Moretti, P. Agostoni, L. Testa, A. Abbate, J. Cosgrave, A. Laudito, G.P. Trevi, I. Sheiban, Systematic review and meta-analysis of randomized clinical trials appraising the impact of cilostazol after percutaneous coronary intervention, Am. Heart. J. 155 (6) (2008) 1081-1089.

[17] S.R. Money, J.A. Herd, J.L. Isaacsohn, M. Davidson, B. Cutler, J. Heckman, W.P. Forbes, Effect of cilostazol on walking distances in patients with intermittent claudication caused by peripheral vascular disease, J. Vasc. Surg. 27 (2) (1998) 267-274.

[18] M.B. Elam, J. Heckman, J.R. Crouse, D.B. Hunninghake, J.A. Herd, M.

Davidson, I.L. Gordon, E.B. Bortey, W.P. Forbes, Effect of the novel antiplatelet agent cilostazol on plasma lipoproteins in patients with intermittent claudication, Arterioscler. Thromb. Vasc. Biol. 18 (12) (1998) 1942-1947.

[19] D.L. Dawson, B.S. Cutler, W.R. Hiatt, R.W. Hobson, 2nd, J.D. Martin, E.B. Bortey, W.P. Forbes, D.E. Strandness, Jr., A comparison of cilostazol and pentoxifylline for treating intermittent claudication, Am. J. Med. 109 (7) (2000) 523-530.

[20] H.G. Beebe, D.L. Dawson, B.S. Cutler, J.A. Herd, D.E. Strandness, Jr., E.B. Bortey, W.P. Forbes, A new pharmacological treatment for intermittent claudication: results of a randomized, multicenter trial, Arch. Intern. Med. 159 (17) (1999) 2041-2050.

[21] I. Ono, H. Gunji, K. Suda, F. Kaneko, Effects of cilostazol lotion on blood flow in rabbit skin, J. Dermatol. Sci. 7 (2) (1994) 79-83.

[22] H.N. Kim, C.H. Gil, Y.R. Kim, H.K. Shin, B.T. Choi, Anti-photoaging properties of the phosphodiesterase 3 inhibitor cilostazol in ultraviolet B-irradiated hairless mice, Sci. Rep. 6 (2016) 31169.

[23] A.N. Vagnozzi, J.F. Reiter, S.Y. Wong, Hair follicle and interfollicular epidermal stem cells make varying contributions to wound regeneration, Cell cycle. 14 (21) (2015) 3408-3417.

[24] M. Ito, Y. Liu, Z. Yang, J. Nguyen, F. Liang, R.J. Morris, G. Cotsarelis, Stem cells in the hair follicle bulge contribute to wound repair but not to homeostasis of the epidermis, Nat. med. 11 (12) (2005) 1351-1354.

[25] J.B. Brown, F. McDowell, Epithelial healing and the transplantation of skin, Ann. surg. 115(6) (1942) 1166-1181.

[26] G. Cotsarelis, Epithelial stem cells: a folliculocentric view, J. Invest. Dermatol. 126 (7) (2006) 1459-1468.

[27] T.S. Argyris, B.F. Argyris, Factors affecting the stimulation of hair growth during wound healing, Anat. Rec. 142 (1962) 139-145.

[28] D.M. Ansell, J.E. Kloepper, H.A. Thomason, R. Paus, M.J. Hardman, Exploring the “hair growth-wound healing connection“: anagen phase promotes wound

re-epithelialization, J. Invest. Dermatol. 131 (2) (2011) 518-528.

[29] M.S. Rendell, M.L. Johnson, D. Smith, D. Finney, C. Capp, R. Lammers, S. Lancaster, Skin blood flow response in the rat model of wound healing: expression of vasoactive factors, J. Surg. Res. 107 (1) (2002) 18-26.

[30] M.S. Rendell, B.K. Milliken, M.F. Finnegan, D.A. Finney, J.C. Healy, The skin blood flow response in wound healing, Microvasc. Res. 53 (3) (1997) 222-234.

[31] V.C. Weiss, D.P. West, T.S. Fu, L.A. Robinson, B. Cook, R.L. Cohen, D.A. Chambers, Alopecia areata treated with topical minoxidil, Arch. Dermatol. 120 (4) (1984) 457-463.

[32] E.E. Vanderveen, C.N. Ellis, S. Kang, P. Case, J.T. Headington, J.J. Voorhees, N.A. Swanson, Topical minoxidil for hair regrowth, J. Am. Acad. Dermatol. 11 (3) (1984) 416-421.

[33] R.C. Wester, H.I. Maibach, R.H. Guy, E. Novak, Minoxidil stimulates cutaneous blood flow in human balding scalps: pharmacodynamics measured by laser Doppler velocimetry and photopulse plethysmography, J. Invest. Dermatol. 82 (5) (1984) 515-517.

[34] M.P. Philpott, D.A. Sanders, T. Kealey, Effects of insulin and insulin-like growth factors on cultured human hair follicles: IGF-I at physiologic concentrations is an important regulator of hair follicle growth in vitro, J. Invest. Dermatol. 102 (6) (1994) 857-861.

[35] M. Magerl, S. Kauser, R. Paus, D.J. Tobin, Simple and rapid method to isolate and culture follicular papillae from human scalp hair follicles, Exp. Dermatol.11 (4) (2002) 381-385.

[36] A.G. Messenger, The culture of dermal papilla cells from human hair follicles, Br. J. Dermatol. 110 (6) (1984) 685-689.

[37] M.P. Philpott, M.R. Green, T. Kealey, Human hair growth in vitro, J. Cell. Sci. 97 (Pt 3) (1990) 463-471.

[38] S.Y. Yoon, K.T. Kim, S.J. Jo, A.R. Cho, S.I. Jeon, H.D. Choi, K.H. Kim, G.S. Park, J.K. Pack, O.S. Kwon, W.Y. Park, Induction of hair growth by insulin-like growth factor-1 in 1,763 MHz radiofrequency-irradiated hair follicle cells, PloS

one 6 (12) (2011) e28474.

[39] S.Y. Yoon, J.S. Yoon, S.J. Jo, C.Y. Shin, J.Y. Shin, J.I. Kim, O. Kwon, K.H. Kim, A role of placental growth factor in hair growth, J. Dermatol. Sci. 74 (2) (2014) 125-134.

[40] R. Paus, K.S. Stenn, R.E. Link, The induction of anagen hair growth in telogen mouse skin by cyclosporine A administration, Lab. Invest. 60 (3) (1989) 365-369.

[41] M. Maurer, E.M. Peters, V.A. Botchkarev, R. Paus, Intact hair follicle innervation is not essential for anagen induction and development, Arch. Dermatol. Res. 290 (10) (1998) 574-578.

[42] S. Muller-Rover, B. Handjiski, C. van der Veen, S. Eichmuller, K. Foitzik, I.A. McKay, K.S. Stenn, R. Paus, A comprehensive guide for the accurate classification of murine hair follicles in distinct hair cycle stages, J. Invest. Dermatol. 117 (1) (2001) 3-15.

[43] P.C. Arck, B. Handjiski, E.M. Peters, E. Hagen, B.F. Klapp, R. Paus, Topical minoxidil counteracts stress-induced hair growth inhibition in mice, Exp. Dermatol. 12 (5) (2003) 580-590.

[44] C.C. Yang, G. Cotsarelis, Review of hair follicle dermal cells, J. Dermatol. Sci. 57 (1) (2010) 2-11.

[45] E.K. Neely, V.B. Morhenn, R.L. Hintz, D.M. Wilson, R.G. Rosenfeld, Insulin-like growth factors are mitogenic for human keratinocytes and a squamous cell carcinoma, J. Invest. Dermatol. 96 (1) (1991) 104-110.

[46] M.M. Rechler, A.L. Brown, Insulin-like growth factor binding proteins: gene structure and expression, Growth Regul. 2 (2) (1992) 55-68.

[47] J.A. Batch, F.A. Mercuri, G.A. Werther, Identification and localization of insulin-like growth factor-binding protein (IGFBP) messenger RNAs in human hair follicle dermal papilla, J. Invest. Dermatol. 106 (3) (1996) 471-475.

[48] Y. Li, R.B. Jalili, A. Ghahary, Accelerating skin wound healing by M-CSF through generating SSEA-1 and -3 stem cells in the injured sites, Sci. Rep. 6 (2016) 28979.

[49] B.S. Park, W.S. Kim, J.S. Choi, H.K. Kim, J.H. Won, F. Ohkubo, H. Fukuoka, Hair growth stimulated by conditioned medium of adipose-derived stem cells is enhanced by hypoxia: evidence of increased growth factor secretion, Biomed. Res. 31 (1) (2010) 27-34.

[50] R. González, G. Moffatt, A. Hagner, S. Sinha, W. Shin, W. Rahmani, A. Chojnacki, J. Biernaskie, Platelet-derived growth factor signaling modulates adult hair follicle dermal stem cell maintenance and self-renewal, NPJ Regen. Med. 2 (1) (2017) 11.

[51] Y. Tomita, M. Akiyama, H. Shimizu, PDGF isoforms induce and maintain anagen phase of murine hair follicles, J. Dermatol. Sci. 43 (2) (2006) 105-115.

[52] M. Kiso, T.S. Hamazaki, M. Itoh, S. Kikuchi, H. Nakagawa, H. Okochi, Synergistic effect of PDGF and FGF2 for cell proliferation and hair inductive activity in murine vibrissal dermal papilla in vitro, J. Dermatol. Sci. 79 (2) (2015) 110-118.

[53] M.A. Heidaran, J.H. Pierce, J.C. Yu, D. Lombardi, J.E. Artrip, T.P. Fleming, A. Thomason, S.A. Aaronson, Role of alpha beta receptor heterodimer formation in beta platelet-derived growth factor (PDGF) receptor activation by PDGF-AB, J. Biol. Chem. 266 (30) (1991) 20232-202327.

[54] S.Y. Ahn, L.Q. Pi, S.T. Hwang, W.S. Lee, Effect of IGF-I on Hair Growth Is Related to the Anti-Apoptotic Effect of IGF-I and Up-Regulation of PDGF-A and PDGF-B, Ann. Dermatol. 24 (1) (2012) 26-31.

[55] M. Iino, R. Ehama, Y. Nakazawa, T. Iwabuchi, M. Ogo, M. Tajima, S. Arase, Adenosine stimulates fibroblast growth factor-7 gene expression via adenosine A2b receptor signaling in dermal papilla cells, J. Invest. Dermatol. 127 (6) (2007) 1318-1325.

[56] A. Cuadrado, A.R. Nebreda, Mechanisms and functions of p38 MAPK signalling, Biochem. J. 429 (3) (2010) 403-417.

[57] W. Zhang, H.T. Liu, MAPK signal pathways in the regulation of cell proliferation in mammalian cells, Cell Res. 12 (1) (2002) 9-18.

[58] T. Tanaka, T. Ishikawa, M. Hagiwara, K. Onoda, H. Itoh, H. Hidaka, Effects of

cilostazol, a selective cAMP phosphodiesterase inhibitor on the contraction of vascular smooth muscle, Pharmacology 36 (5) (1988) 313-320.

[59] J. Cone, S. Wang, N. Tandon, M. Fong, B. Sun, K. Sakurai, M. Yoshitake, J. Kambayashi, Y. Liu, Comparison of the effects of cilostazol and milrinone on intracellular cAMP levels and cellular function in platelets and cardiac cells, J. Cardiovasc. Pharmacol. 34 (4) (1999) 497-504.

[60] N. Sato, J.G. Beitz, J. Kato, M. Yamamoto, J.W. Clark, P. Calabresi, A. Raymond, A.R. Frackelton, Jr., Platelet-derived growth factor indirectly stimulates angiogenesis in vitro, Am. J. Pathol.142 (4) (1993) 1119-1130.

국문 초록

포스포디에스테라아제 3 (PDE3) 억제제는 세포내의 고리형 아데노신 일인산 (cyclic adenosine monophosphate, cAMP)의 수치를 올려서 혈관확장을 유도한다. 포스포디에스테라아제 3 억제제의 하나인 Cilostazol을 피부에 국소 도포하였을 때, 국소적인 혈류의 흐름을 증가시켜서 상처 회복을 촉진한다는 기존의 연구가 있었다. 하지만 포스포디에스테라아제 3 억제제의 사람 모낭에 대한 영향은 아직까지 밝혀진 바가 없었다.

이 연구는 포스포디에스테라아제 3 억제제의 모낭 성장에 대한 효과를 알아보기 위한 목적으로 진행되었다. 이번 연구에서 사람의 모유두세포와 겉뿌리싸개세포, 사람 모낭에서의 포스포디에스테라아제 3의 발현 정도를 먼저 확인하였다.

포스포디에스테라아제 3 억제제가 모낭의 모유두세포와 겉뿌리싸개세포의 증식에 미치는 영향을 BrdU와 WST-1 분석을 통하여 확인해 보았다. 또한 성장인자 항체 분석법을 통하여 모유두세포 내에서의 다양한 모발 관련 성장인자들의 발현을 확인할 수 있었다. 이에 더불어, 모낭 기관 배양을 통해서 모간의 신장을 측정하였고, C57BL/6 마우스에서의 모발 생장기 유도 실험도 함께 진행하였다.

나아가, 포스포디에스테라아제 3 억제제의 혈관 형성에 미치는 영향과 미토겐 활성화단백질 키나아제 (Mitogen-Activated Protein Kinase, MAPK) 신호전달체계의 활성에 대한 연관성도 함께 연구하였다.

결과적으로 사람의 모유두세포에서 포스포디에스테라아제 3의 mRNA와 단백질이 모두 높게 발현하는 것을 확인하였다. 포스포디에스테라아제 3 억제제는 사람 모발의 모유두세포의 증식을 촉진시킬 뿐만 아니라, 모발 성장에 도움이 되는 다양한 성장 인자의 발현 역시도 조절함을 알 수 있었다. 뿐만 아니라, 포스포디에스테라아제 3 억제제는 체외 실험에서 모낭기질의 각질형성세포의 증식을 유도함으로써 모간 신장을 촉진시키는 것을 확인하였다. 또한 포스포디에스테라아제 3 억제제를 C57BL/6 마우스에 국소적으로 도포하였을 때 가인산 세포 외 신호 조절 키나아제 (phosphorylated extracellular signal-regulated kinase), c-Jun N-말단 키나아제 (c-Jun N-terminal kinase), P38을 상승시키고 혈관 생성을 촉진함으로써 모발의 생장기 유도를 가속화시키는 역할을 한다는 것을 알

수 있었다.

결론적으로, 이 연구를 통하여 포스토디에스테라아제 3 억제제가 모발 성장을 촉진한다는 것을 확인하였고, 추가적인 연구를 통하여 추후 탈모 치료의 새로운 치료법을 제시할 수 있을 것이라 기대한다.

.....

주요어 : 모발, 모발성장, 모낭세포생존, 포스포디에스테라아제,
포스포디에스테라아제 억제제

학 번 : 2014-21987