Hair loss (alopecia) is one of the most emotionally stressed conditions in current human life. The alopecia could be classified by causes; disease, mechanical stress, nutritional deficiency, hormone imbalance, and aging.\(^1\)\(^2\) Although diverse treatment strategies for hair loss have been suggested, the efficacy was limited. Only two drugs, minoxidil and finesteride, have been approved for alopecia treatment by the U.S. Food and Drug Administration (FDA).\(^3\) Minoxidil, originally developed for hypertension treatment, was discovered to promote hair growth and hair growth cycle.\(^1\) When compared with non-balding DPCs, balding DPCs had slower growth rate and tended to lose proliferative capacity.\(^5\) In addition, balding DPCs isolated from androgenic alopecia patients showed different gene transcription profiles that regulate hair follicle anagen stage and cell survival. On the contrary, the expression of p16 and p21 was down-regulated by HC extract. In addition, HC extract enhanced the secretion of platelet-derived growth factor (PDGF)-aa and vascular endothelial growth factor (VEGF) and induced phosphorylation of extracellular signal-regulated kinase (ERK) and AKT. Furthermore, HC extract prolonged anagen stage in organ cultured human hair follicles. Our data strongly suggest that HC extract could support hair growth by stimulating proliferation of DPCs and elongating anagen stage, resulted from enhanced cellular energy metabolism and modulation of gene expression related to cell cycle, apoptosis, and growth factors.

**Key words** Houttuynia cordata extract; human dermal papilla cell; hair growth; Bcl2

INTRODUCTION

Hair loss (alopecia) is one of the most emotionally stressed conditions in current human life. The alopecia could be classified by causes; disease, mechanical stress, nutritional deficiency, hormone imbalance, and aging.\(^1\)\(^2\) Although diverse treatment strategies for hair loss have been suggested, the efficacy was limited. Only two drugs, minoxidil and finesteride, have been approved for alopecia treatment by the U.S. Food and Drug Administration (FDA).\(^3\) Minoxidil, originally developed for hypertension treatment, was discovered to promote hair growth. Minoxidil was clarified as a potassium channel opener but the exact mechanism for hair growth is not fully understood.\(^5\) The effect of minoxidil is temporary and several side-effects, such as pruritus, dermatitis and irritation, have been reported.\(^5\) Finasteride works as a competitive inhibitor of type2 5-alpha reductase. It is administrated for treatment of androgenic alopecia and blocks conversion of testosterone to dihydrotestosterone and prevents androgen dependent hair follicle miniaturization.\(^6\)\(^7\) However, it has potential risks for malfunction of reproductive organs by hormonal imbalance. Because the efficacy of established drugs is limited and there could be unpredictable side effects, new therapeutic alternatives are needed to be suggested for hair loss prevention and/or hair growth promotion.

The hair follicle experiences three stages for its turnover; anagen (proliferation), categen (involution) and telogen (resting).\(^8\) Hair follicles are composed of several cell layers, outer root sheath (ORS), inner root sheath (IRS), germinal matrix and dermal papilla cells (DPCs).\(^9\) Dermal papilla cells, differentiated from mesenchyme cells and located in the base of hair follicle, are one of the major regulators of hair cycle by responding to external stimuli and signals delivered through cytokines and junctions.\(^9\)\(^10\) In particular, minoxidil has been reported to have a proliferative effect on DPCs.\(^11\) Therefore, enhancement of viability of dermal papilla cells might be a major target for activating hair cycle and hair growth. Balding DPCs isolated from androgenic alopecia patients showed different gene transcription profiles that regulate hair growth and hair growth cycle.\(^5\) When compared with non-balding DPCs, balding DPCs had slower growth rate in vitro\(^5\) and tended to lose proliferative capacity.\(^5\) In addition, balding DPCs underwent premature senescence in vitro, associated with expression of p16/pRb.\(^5\)

_Houttuynia cordata_ Thunberg (HC, Saururaceae), ‘Eu-Sung-Ch’ in Korean, is a perennial herbal plant for traditional medicine widely distributed throughout Southeast Asia. In recent years, HC was reported as an important source of natural polysaccharides and flavonoids,\(^16\) It has been used in traditional medicine for immune stimulation and anti-cancer treatment. HC was also known to have a variety of pharmacological effects; anti-viral,\(^17\) anti-leukemic,\(^18\) anti-oxidative,\(^19\) anti-inflammatory\(^20\) and anti-bacterial\(^21\) activities. Furthermore, Chung _et al._ reported the effect of herbal complex containing _Houttuynia cordata_ Thunb, _Perilla frutescens_ and _Camellia sinensis_ on hair growth promotion in mice.\(^22\) However,
the effects and molecular mechanisms of HC on human cell system concerning hair growth were not well documented.

In this study, the physiological effects of *Houttuynia cordata* extract (HC extract) on cultured human dermal papilla cells were investigated. HC extract enhanced the viability of cultured human DPCs by stimulating energy metabolism and induced G1 phase progression which resulted in enhanced proliferative capacity. Furthermore, we have found that these possible hair promoting effects were mediated by regulating the gene expressions related to cell cycle (CDK4, CCNA2, and CCNB1), anti-apoptosis (Bcl2), growth factors (platelet-derived growth factor (PDGF)-aa and vascular endothelial growth factor (VEGF)) and signal transduction (extracellular signal-regulated kinase (ERK) and AKT). In addition, HC extract prolonged the anagen period in human hair follicle (hHF) organ culture model.

**MATERIALS AND METHODS**

**Plant Materials and Preparation of the HC Extract**

The dried aerial parts of *Houttuynia cordata* were purchased from Humanherb (Lot.A5616071, Daegu, Korea) in July 2016 and identified by Prof. Seok-Seon Roh in the College of Korean Medicine, Daejeon University. The dried aerial parts of *H. cordata* (40 g) were extracted with 50% aqueous ethanol for 2 d at room temperature, filtered through Whatman No. 4 filter paper. The filtrate was concentrated by rotary evaporator under reduced pressure to give 50% aqueous ethanol extract (8.24 g).

**Cell Culture**

Human DPCs were purchased from Promocell (Heidelberg, Germany). DPCs were cultured in basal medium supplemented with 4% fetal calf serum, 0.4% bovine pituitary extract, 1 ng/mL basic fibroblast growth factor and 5 µg/mL insulin (Supplement Mix, Promocell). Cells were maintained in humidified incubator at 37°C with 5% CO2. Before HC treatment, serum limitation was done by replacing the medium with Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, MA, U.S.A.) supplemented with 1% fetal bovine serum (FBS) (Gibco) and 1 ng/mL basic fibroblast growth factor (bFGF) (Merck, Darmstadt, Germany) and culturing for 24h to minimize the effects of serum and growth supplements.

**Cell Proliferation Assay**

The effect of the HC extract on the proliferation of DPCs was examined using bromo-deoxy-uridine (BrdU) incorporation assay kit (Roche, Mannheim, Germany) following the manufacturer’s protocol. Briefly, DPCs (1.5 × 10⁴ cells/well) were seeded in 24-well plates. Cells were treated with various concentrations of HC extract for 24h and fixed with 4% paraformaldehyde. Mitochondria were stained with 100 nM mitotracker. Mitochondrial network was visualized by taking fluorescence images with EVOS™ FL Auto2 Imaging System (Thermofisher Scientific, MA, U.S.A.) at 600 nm (580 nm excitation).

**Staining of Mitochondria in Cultured Cell**

The effect of HC extract on mitochondria was examined using Mitotracker (Invitrogen, Paisley, U.K.) following the manufacturer’s protocol. Briefly, DPCs (1.5 × 10⁴ cells/well) were treated for 24h plates. Cells were treated with various concentrations of HC extract for 24h and fixed with 4% paraformaldehyde. Mitochondria were stained with 100 nM mitotracker. Mitochondrial network was visualized by taking fluorescence images with EVOS™ FL Auto2 Imaging System (Thermofisher Scientific, MA, U.S.A.) at 600 nm (580 nm excitation).

**Quantitative Real-Time PCR**

Real-time PCR was used to investigate the effects of HC extract on the expression of viability related genes of DPCs. Cells were seeded in 6-well plates (1.5 × 10⁴ cells/well) and incubated for 24h. The cells were cultured in DMEM medium supplemented with 1% FBS and 1 ng/mL bFGF for 24h and then HC extract was treated at concentrations of 10, 20 and 50 µg/mL for 24h, with non-treated cells served as control. Total RNA was extracted using Rneasy RNA extraction kits (Qiagen Inc., CA, U.S.A.). cDNA synthesis was performed using cDNA synthesis kit (Philekorea, Seoul, Korea) with ThermoCycler (R&D Systems, MN, U.S.A.), according to the manufacturer’s protocol. cDNA samples obtained from control and treated cells were subjected to real-time RT PCR analysis.

**Western Blotting Analysis**

DPCs (1 × 10⁶ cells/dish) were seeded in 100 mm dishes and cultured for 24h. HC extract were treated at concentrations of 10, 20 and 50 µg/mL for 24h. Then cells were washed with ice-cold phosphate buffered saline (PBS) and lysed on ice in M-PER buffer (Thermofisher Scientific) supplemented with Complete™ protease inhibitor cocktail and phosphatase inhibitor (Roche, Indianapolis, IN, U.S.A.). 40 µg of protein was analyzed by Western blotting with corresponding antibodies; Bcl2 (1:1000, Abcam), p16 adenine dinucleotide (NADH) and ATP generation were measured. The NADH generation was determined by CCK-8 assay. The absorbance was read at 450 nm using micro-plate reader. For ATP measurement, cells were lysed and ATP synthesis was blocked by detergent solution. Luminescence from ATP/luciferin reaction was measured. The mitochondrial membrane potential was measured by JC-1 staining. HC extract treated DPCs were stained with 1 µM JC-1 Solution. Fluorescence intensities from JC-1 aggregate and monomer forms were measured at 590 nm (535 nm excitation) and 530 nm (475 nm excitation), respectively.

**Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) assay**

Early suppl. id Hs01034249_m1; MDM2 assay id Hs00540450_s1; CASP1 assay id 4352934E; MKI67 assay id Hs04260396_g1; TP53 assay id Hs00962278_m1; BAD assay id Hs00188930_m1; p16 assay id Hs00231713_m1. TaqMan One-Step RT-PCR Master MixReactions included 1 µl cDNA, 5 µl master mix, and 0.5 µl of a 1:10000 dilution of each primer. TaqMan probes for RT-PCR used in this study were as follows: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) assay id 4352934E; MKI67 assay id Hs04260396_g1; TP53 assay id Hs01034249_m1; MDM2 assay id Hs00540450_s1; CASP1 assay id Hs00354836_m1; CASP3 assay id Hs00234387_m1; CASP9 assay id Hs00962278_m1; BAD assay id Hs00188930_m1; Bax assay id Hs00180269_m1; Bcl2 assay id Hs00608023_m1; CDKN1A assay id Hs00355782_m1; CDK2 assay id Hs00923894_m1; CDK1 assay id Hs00938777_m1; CDK2 assay id Hs01548894_m1; CDK4 assay id Hs00364847_m1; CCND1 assay id Hs00765553_m1; CCNA2 assay id Hs00153138_m1; CCNB1 assay id Hs01030099_m1; CREBI assay id Hs00231713_m1. TaqMan One-Step RT-PCR Master MixReagents (Life Technologies, CA, U.S.A.) was used. The PCR reactions were performed on ABI7500 Real Time PCR system following the manufacturer’s protocol. The resulting data were analyzed with ABI software.

**Western Blotting Analysis**

DPCs (1 × 10⁶ cells/dish) were seeded in 100 mm dishes and cultured for 24h. HC extract were treated at concentrations of 10, 20 and 50 µg/mL for 24h. Then cells were washed with ice-cold phosphate buffered saline (PBS) and lysed on ice in M-PER buffer (Thermofisher Scientific) supplemented with Complete™ protease inhibitor cocktail and phosphatase inhibitor (Roche, Indianapolis, IN, U.S.A.). 40 µg of protein was analyzed by Western blotting with corresponding antibodies; Bcl2 (1:1000, Abcam), p16
(1:1000, Abcam, Inc. Cambridge, MA, U.S.A.), AKT (1:1000, Santa Cruz, CA, U.S.A.), Erk (p44/42) (1:1000, Santa Cruz), GAPDH (1:2000, Santa Cruz), phospho(Ser473)-Akt (1:1000, Cell Signaling Technology, MA, U.S.A.) and phospho(Thr202/Tyr204)-Erk (p44/42), (1:1000, Cell Signaling Technology). Western blot was analyzed by chemiluminescence detector (Vilber Lourmat, Collégnue, France).

**Dot Blot Analysis of Growth Factors** Human growth factor antibody kit was used (Abcam) to elucidate the changes in growth factor profiles of DPCs. Total of 41 human growth factors could be analyzed at once. Briefly DPCs (1 × 10^5 cells/well) were seeded in 24-well plates and cultured for 24 h. The cells were treated with 20 µg/mL of HC extract for 24 h and then culture supernatant were collected for growth factor analysis. Fresh medium and culture supernatant from non-treated cells were used as blank and control, respectively. Conventional immunohistochemical was performed following the manufacturer’s instruction. Biotin-conjugated anti-cytokine antibodies were used as detection antibodies and HRP-conjugated streptavidin was used for chemiluminescence detection. The resulting blots were analyzed under identical condition using chemiluminescence detector Fusion FX5 (Vilber Lourmat, Collégnue, France).

**DPC Spheroid Construction** DPCs (2 × 10^5 cells/well) were seeded in 24-well Hydrocell plate (Nunc, Roskilde, Denmark), which had chemically coated surface to prevent attachment of cells to plate surface, resulting in spheroid formation. The cells were incubated for 72 h to generate spheroids with/without HC extract. Then the size of spheroids was measured by microscopic photography (Leica, Wetzlar, Germany) and sorted by diameter.

**DPC Spheroid Immunocytochemistry** DP spheroids were transferred to 96-well Hydrocell plate (Nunc, Roskilde, Denmark) and cultured overnight. After PBS wash, plates were fixed with 4% paraformaldehyde at room temperature for 10 min. The cells were then permeabilized with PBS containing 0.1% triton X-100 and blocked with PBS containing 5% FBS and 1% BSA. After consecutive incubation with Bcl2 antibody (1:200) at 4°C for 12 h and secondary antibody (1:1000, abcam) at room temperature for 1 h, nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (1:1000, Thermostar Scientific) in the dark for 10 min. High resolution fluorescence images were taken using EVOS™ FL Auto2 Imaging System (Thermofisher Scientific).

**Human Hair Follicle Organ Culture and Hair Cycle Scoring** Human scalp skin specimens were obtained from patients undergoing reconstructive plastic surgery after obtaining informed consent, following Declaration of Helsinki principles. The study was approved by the Institutional Review Board of the CHA Bundang Medical Center (IRB No. 2018-09-009).

Anagen human hair follicles were isolated by microdissection and maintained in William’s E medium (WelGENE, Kyungsan, Korea) supplemented with 10 µg/mL insulin (Sigma-Aldrich, MO, U.S.A.), 10 ng/mL hydrocortisone (Sigma-Aldrich), 20 mM N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid (HEPES) (Invitrogen-Gibco-BRL, NY, U.S.A.) and 1x antibiotic-antimycotic (Invitrogen-Gibco-BRL) for 1 d.

Each group of 20 isolated hHFs was cultured in medium containing HC extract at concentrations of 2, 20 and 50 µg/mL. On days 3, 6, and 9, medium was replaced and hHFs were photo-documented. Hair cycle stage of cultured human hair follicles was determined on days 0 and 9, according to hair cycle guideline.21,24

**Statistical Analysis** All experimental data were presented as the mean ± standard deviation (S.D.) of at least three independent experiments. Experimental results were analyzed using the SigmaPlot (Systat Software Inc., IL, U.S.A.). The statistical significance of the difference was determined using Student’s t-test. The value of p < 0.05 considered statistically significant.

**RESULTS**

**HC Extract Increased Cell Viability and Proliferation of Cultured Human DPCs** The cell viability and proliferation of DPCs were evaluated by CCK-8 assay and BrdU incorporation assay, respectively. As shown in Fig. 1, treatment of HC extract increased both cell viability and proliferation in dose dependent manners. Cell viability was enhanced by 13, 51 and 71% by HC extract treatment at concentrations of 1, 10 and 50 µg/mL, respectively. The increment was comparable with that induced by minoxidil, the most well-known hair growth stimulating agent (Fig. 1A). The proliferation of human DPCs treated with HC extract was also stimulated, revealed by BrdU incorporation into newly synthesized DNA. The treatment of HC extract at concentration of 1 and 20 µg/mL stimulated the growth of cultured human DPCs by 8% and 22%, respectively, comparable result of minoxidil. Treatment over this concentration range, the growth stimulating effect tended to reach a plateau (Fig. 1B).

**HC Extract Enhanced Energy Metabolism of Cultured Human DPCs** Because NADH/nicotinamide adenine dinucleotide phosphate (NADPH) generation was dramatically enhanced by HC extract treatment, another parameters related to cellular energy metabolism were evaluated. The cellular ATP level, a biological energy source and mitochondrial membrane potential (ΔΨ), a marker of mitochondrial energy production, were investigated. As shown in Fig. 2 the amount of cellular ATP and membrane potential were increased in dose dependent manners. The ATP level was increased by 25% and 30% in the presence of 10 µg/mL and 20 µg/mL of HC extract, respectively (Fig. 2A). On the other hand, HC extract at concentration of 20 µg/mL and 50 µg/mL increased mitochondrial membrane potential by 31% and 39%, respectively (Figs. 2B, C). Minoxidil exerted comparable results (Figs. 2A, B). Our data showed that treatment of HC extract increased the indicators of cells’ energy metabolic potential, NADH, ATP, and ΔΨ in cultured human DPCs.

**HC Extract Increased the Expression Level of Bcl2 mRNA** The mRNA expression levels of cell viability and proliferation related genes were determined in cultured human DPCs using RT-PCR analysis. Total of 56 genes were examined and normalized to expression level of GAPDH. As a result, the expression of 12 genes was significantly changed by HC extract. As proliferative/apoptotic markers in mitochondria, Bad gene expression level was not changed but Bcl2 level was increased by HC extract in a concentration dependent manner. In addition, Ki67 and CREB level was also increased in HC extract treated samples. Caspase 9, an apoptotic marker, was also decreased by 0.79- and 0.78-fold at HC
The extract concentration of 10 and 20 µg/mL, although caspase 3 level was not changed (Fig. 3). Because HC extract treatment resulted in proliferative and anti-apoptotic effect, the expression of cell cycle related genes was additionally investigated. Cyclin A (CCNA2) and B (CCNB1) mRNA levels were decreased but, on the contrary, Cyclin D (CCND1) level was not changed. Among cyclin-dependent kinases (CDKs), the expression level of CDK1 and CDK2 was not changed, but CDK4, which participates in the G1 phase progression, was significantly increased by 1.40- and 1.29-fold with 20 and 50 µg/mL of HC extract treatment, respectively. As check point regulators, mouse double minute 2 homolog (MDM2) level was increased by 2.15-fold and p21 (CDKN1A) level was decreased by 0.83-fold in HC extract (Fig. 3). These results suggest that HC extract could facilitate cell cycle progression at G1/S phase.
HC Extract Increased the Protein Level of Bcl2 in Cultured DPCs and DPC Spheroids

To examine the change of Bcl2 level by HC extract, cultured DPCs were treated with 10, 20, and 50 µg/mL of HC extract for 24h, and the amount of Bcl2 protein was measured by Western blotting. HC extract significantly increased the Bcl2 level by 104.0% at 20 µg/mL treatment (Figs. 4A, B).

The effect of HC extract on the process of spheroid formation was also investigated. DP spheroids with diameter above 400 µm, which could mimic the size of actual dermal papilla in hair follicle, were used for the experiment. Autonomously constructed spheroids on Hydro-cell plate with/without HC extract (10, 20, 50, and 100 µg/mL). The constructed DPC spheroids were cryosectioned and immunostained with Bcl2(red) antibody and DAPI(blue). (D) The intensity of Bcl2(red) in cultured hDPC spheroids was measured. N.T, non-treated control. Significantly different compared with N.T (*p<0.05, **p<0.01, ***p<0.001). (Color figure can be accessed in the online version.)

Fig. 4. Effect of HC Extract on Bcl2 Expression in Cultured hDPC and hDPC Spheroids

DPCs (1×10^6 cells/dish in 100 mm dishes) were seeded and cultured for 24h. The cells were treated with HC extract at concentrations of 10, 20, 50 µg/mL for 24h. (A) Whole cell lysates (40 µg protein) from DPCs were analyzed by immunoblotting to determine the levels of Bcl2 and (B) the band intensity was quantitated. DPCs (2×10^5 cells/well in 24well Hydrocell plate) were seeded and incubated for 72h to generate spheroids with/without HC extract (10, 20, 50, and 100 µg/mL). (C) The constructed DPC spheroids were cryosectioned and immunostained with Bcl2(red) antibody and DAPI(blue). (D) The intensity of Bcl2(red) in cultured hDPC spheroids was measured. N.T, non-treated control. Significantly different compared with N.T (*p<0.05, **p<0.01, ***p<0.001). (Color figure can be accessed in the online version.)
The proportion of hHFs in anagen stage tended to increase retaining the characteristic morphology of anagen stage, so group. As shown in Fig. 8, HC extract rendered cultured hHFs gen stage (Fig. 8) comparable with minoxidil treated control (Fig. 5).

Proliferation of DPCs,25) ined, PDGF-aa and VEGF, which were reported to activate the culture media were examined. Among growth factors examined, p16 showed a marked change. As shown in Fig. 5, HC extract significantly down-regulated the expression of p16, a CDK4 inhibitor playing a critical role in cell cycle regulation by prohibiting cell progression from G1 phase to S phase. The p16 level in 20 and 50 µg/mL of HC extract treated DPCs decreased to 30.7 and 24.0% of non-treated control (Fig. 5B). Our data suggest that HC could induce G1/S phase progression by suppressing a CDK4 inhibitor, p16.

**HC Extract Decreased the p16 Protein** To confirm the mechanism underlying the enhanced cell viability exerted by HC extract, the expression levels of cell cycle regulatory proteins and proliferative/apoptotic signaling proteins were evaluated. Cultured DPCs were treated with various concentrations of HC extract (10, 20, and 50 µg/mL) for 24h, and then the amount of protein was measured by Western blotting.

Among proteins analyzed, p16 showed a marked change. As shown in Fig. 5, HC extract significantly down-regulated the expression of p16, a CDK4 inhibitor playing a critical role in cell cycle regulation by prohibiting cell progression from G1 phase to S phase. The p16 level in 20 and 50 µg/mL of HC extract treated DPCs decreased to 30.7 and 24.0% of non-treated control (Fig. 5B). Our data suggest that HC could induce G1/S phase progression by suppressing a CDK4 inhibitor, p16.

**HC Extract Increased PDGF-aa and VEGF Expression** The DPCs secrete diverse growth factors for regulation of hair follicles. The changes of secreted growth factors in DPC culture media were examined. Among growth factors examined, PDGF-aa and VEGF, which were reported to activate the proliferation of DPCs,25) were significantly increased by HC extract (Fig. 6).

**HC Extract Induced Phosphorylation of ERK and AKT** In addition, changes in pivotal cellular signaling molecules, Erk and Akt, were also investigated. The protein levels of total Erk and Akt were not changed by HC extract. The phosphorylation of Erk and Akt, however, was significantly increased by HC extract in dose dependent manners (Fig. 7A). HC extract at concentrations of 20 and 50 µg/mL increased the ratios of pErk/Akt by 54.6 and 96.3%, respectively. On the other hand, pAkt/Akt ratios were increased by 47.1 and 102.1% in 20 and 50 µg/mL of HC extract treated DPCs, respectively (Fig. 7B). Our data suggest that the enhanced proliferative potential of DPCs by HC extract is exerted through the phosphorylation of Erk and Akt.

**HC Extract Elongated Anagen Stage in Organ Cultured Human Hair Follicles** The effect of HC extract on hair follicle stage was investigated in hHFs organ culture model. At the end of 9d incubation, 52.6% of hHFs stayed in anagen stage in non-treated control group. On the contrary, 73.7% of hHFs treated with HC extract (20 µg/mL) remained in anagen stage (Fig. 8) comparable with minoxidil treated control group. As shown in Fig. 8, HC extract rendered cultured hHFs retaining the characteristic morphology of anagen stage, so the proportion of hHFs in anagen stage tended to increase with the concentrations of HC extract.

**DISCUSSION**

Hair growth is a highly energy-consuming process.26) Measurement of hair follicle metabolism using radiolabeled metabolic substrate made it confirmed that glycolysis is a major energy metabolic pathway,27) but mitochondrial contribution was also strongly demonstrated in hair follicle cells, especially in DPCs.28,29) For example, l-carnitine promotes mitochondrial beta-oxidation and affects hair growth in vitro and in vivo.30,31) In addition, hair cycle is strongly conjugated with mitochondrial function. For example, the disruption of TFAM expression, a marker for mitochondria biogenesis, causes malfunction of hair follicle morphogenesis.32)

Mitochondria process carbon sources to generate NADH and ATP which are critical for multiple biosynthetic processes, such as amino-acid metabolic pathways, cell redox pathways and cell proliferation. We have shown that treatment of HC extract to cultured human DPCs resulted in enhancement of NADH and ATP production, strongly suggesting that HC extract stimulated mitochondrial activities. In addition, mitochondrial membrane potential (ΔΨ) was increased and mitochondrial network was also improved by HC extract (Fig. 9). Mitochondrial membrane potential is critical for positive steady state NAD/NADH coupling and ATP production by oxidative phosphorylation.33) Although further studies concerning mitochondrial biogenesis and other markers are needed, our data demonstrated that HC extract improved cellular en-
ergy metabolism of DPCs, resulted from enhanced mitochon-
drial function. In addition to enhancement of viability related
to energy metabolism, BrdU assay further confirmed that HC
extract promoted DNA synthesis which is crucial for cell pro-
liferation. The prevention of hair follicle miniaturization by
supporting the proliferation of DPCs could be expected by HC
extract, based on the fact that the reduction of dermal papilla
volume is closely related to it.

In hair follicle, the anti-apoptotic Bcl-2 protein family
members were shown to facilitate the resistance of senescence
committing cells to apoptosis.34) For hair cycle, Bcl2 level
is essential for maintaining specific cell type in each stage;
anagen, telogen and catagen. Interestingly, dermal papilla
cells were Bcl2-positive in all stages, and during anagen stage,
Bcl2 was significantly in higher level compared with other

Fig. 7. Effect of HC Extract on ERK and AKT Phosphorylation in Cultured hDPCs
The DPCs (1 × 10^6 cells/dish in 100 mm dishes) were seeded and cultured for 24 h. HC extract were treated at concentrations of 10, 20, 50 µg/mL for 24 h. (A) Whole cell lysates (40 µg protein) from DPCs were analyzed by immunoblotting to determine the levels of Erk, phospho-Erk, Akt, and phospho-Akt. (B) The Ratio of pAkt/Akt and pErk/Erk was calculated. N.T, non-treated control. Significantly different compared with N.T (* p < 0.05, ** p < 0.01, *** p < 0.001).

Fig. 8. Effect of HC Extract on Bulb Stage in Human Hair Follicle Organ Culture
The anagen hair follicle were prepared and incubated for 9 d. HC extract were treated at concentrations of 2, 20, 50 µg/mL on every 3 d. (A) At day 9, the structure of hair follicle bulb was photo-documented. (B) Percentage of HF in anagen, early or late catagen state was determined. N.T, non-treated control; MNX, minoxidil.

Fig. 9. Effect of HC Extract on Mitochondrial Network in Cultured hDPCs
The DPCs (1.5 × 10^4 cells/well in 24-well plates) were seeded and treated with HC extract (50 µg/mL) for 24 h and fixed with 4% paraformaldehyde. Mitochondria were stained with mitotracker.
In addition, the balding DPCs tend to undergo premature senescence markers. Increased expression of p16 is associated with higher expression of p16 and are more sensitive to environmental stresses. In addition, the balding DPCs tend to undergo premature senescence with higher expression of p16 and are more sensitive to environmental stresses. HC extract decreased the expression of p16 and p21 proteins and, on the contrary, significantly increased the mRNA expression of ki67 and MDM2. Our data suggest that the HC extract could support the growth of dermal papilla cells by preventing DPCs from cellular senescence. We also investigated the underlying mechanism of growth stimulatory effect of HC extract. As previously demonstrated, dot blot analysis for growth factors revealed that PDGF-aa and VEGF expression was increased by HC extract, which were known to be key growth factors. We have also found that the increase in growth factors resulted in stimulation of ERK and AKT pathways via phosphorylation of ERK and AKT proteins (Fig. 7).

The natural plant extract composed of diverse chemicals. Therefore, the changes in specific phenotypes (e.g. ATP level or CDKN1A mRNA level) were not always exerted by NPE in total but rather by specific chemical(s) in NPE. In that sense, it might be possible the most effective concentration of each phenotypical parameter could be different. Above this concentration the effects could be diminished because of opposing or even cytotoxic effect of NPEs. Determining which concentration should be better for hair growth is a very complicated process with so many variables which might be agonistic, antagonistic and/or synergistic and should be done empirically. Since the effects of NPE resulted from orchestrated, overall actions of thousands chemicals with varying molecular signatures, it should be assessed by macroscopic features such as grown hair length or hair cycle stages, not by each molecular phenotypes. In this context, human hair follicle organ culture model is suitable for evaluating the overall effect of NPEs.

The HC has long been used in complex prescriptions for hair loss treatment but the effect of HC extract itself and the exact underlying mechanisms, as far as we know, were not clearly described yet. In this report, we have found that HC extract stimulated the proliferation and activated the energy metabolism of cultured human dermal papilla cells. We also found that these stimulation and activation were accompanied by up-regulation of Bcl2 and down-regulation of p16 and p21. In addition, our data suggest that HC extract exerted its proliferative effects by enhanced PDGF-aa and VEGF production and through the phosphorylation of ERK and AKT signal transduction pathways.

In conclusion, our data strongly demonstrate that HC extract could promote hair growth by stimulating proliferation of DPCs and elongating anagen stage, resulted from enhanced cellular energy metabolism and modulation of gene expression related to cell cycle, apoptosis, and growth factors.

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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