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The effect of HNG, a Humanin analogue, on the enhancement of anagen phase

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모발 성장기의 향상에 있어서 휴매닌 유사체인 HNG의 효과

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김성민

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The effect of HNG, a Humanin analogue, on the enhancement of anagen phase

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This thesis has been examined and approved.

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List of Abbreviations

DP	Dermal Papilla
DPCs	Dermal Papilla cells
HNG	Humanin analogue
MS	Minoxidil Sulfate
gp130	glycoprotein130
Erk1/2	Extracellular signal-regulated kinase1/2
Stat3	Signal transducer and activator of transcription 3
VEGF	Vascular Endothelial Growth Factor

Abstract

Hair follicle goes on repetitive cycles; growth phase *(Anagen)*, apoptosis-driven regression phase *(Catagen)*, and quiescence phase *(Telogen)*. Dermal pailla cells (DPCs) residing in the basement of the hair follicle play an important role in regulating of hair cycle, maintaining of *Anagen* phase and signaling among keratinocytes of hair follicle.

Humanin consisted of 24-amino-acid was identified as a survival factor in brain cells of patients with Alzheimer's disease. HNG is a humanin analogue substituted 14-serine with glycine. HNG has 1000 time more potency than humanin. HNG affects cell growth, proliferation and cell cycle as well as protects cells from apoptosis. However, the effect of HNG against hair loss has not been yet discovered. This study was aimed to investigate HNG effect on hair growth and show potentiality of HNG as a new drug for hair loss. HNG significantly increased proliferation of DPCs. In hair follicle organ culture as an *ex vivo* experiment, HNG increased hair-shaft elongation. Anagen initiation inductivity and Anagen maintenance were tested by an *in vivo* experiment. Topical treatment with HNG did not facilitate anagen initiation faster, but exerted the anagen phase longer significantly compared to the control and prevented from apoptosis of hair follicle cells, which indicated that HNG inhibited the transition of anagen to catagen phase. In action of mechanisms, HNG activated Erk1/2, Akt, and Stat3 within minutes and up-regulated VEGF level known for prolongating anagen phase as one of the Stat3 target genes on DPCs. HNG could promote hair growth in various ways, but with one of mechanisms, HNG could maintain hair growth phase longer by preventing from apoptosis with up-regulation of VEGF levels via activation of Erk1/2, Akt, and Stat3.

Key words: HNG, Humanin, hair growth, Dermal papilla cell, VEGF, Erk1/2, Akt, Stat3

CONTENTS

LIST OF FIGURE

This study was published in the journal, *International Journal of Molecular Sciences*.

1. Introduction

The hair not only functions as physical protection, body temperature maintenance, sensory perception to mammals, but also for cosmetic effect in human.

Recently, a number of people have suffered from hair loss, in other words *alopecia*, and the number is still increasing. Nonetheless, so far, only two drugs, *finasteride* and *minoxidil*, have been approved by the FDA and are being used in clinics. However, the use of *finasteride* and *minoxidil* as a drug for alopecia is limited due to transient action and adverse effects.[1] Therefore, the development of new therapies is necessary for curing hair loss.

The hair follicle is embedded in all layers of the skin and is a dynamic mini-organ. To start with, the hair follicle consists of 20 types of epithelial cells and undergoes a repetitive cycle, interacting with one another: growth (anagen), apoptosis-driven regression (catagen), and quiescence (telogen).[2,3] Hair follicle stem cells (HFSCs) are located in two regions in hair follicle[4]: quiescent one located in the bulge (Bu-SCs) and another population located in the hair germ. During the transition from Telogen to Anagen phase, the hair follicle stem cells within hair germ proliferate first by receiving signals, such as NOG, FGF-7, FGF-10, and TGF-β2 from dermal papilla cells (DPCs). After that, it develops into matrix, a pool of transit amplifying cells (TACs). Then TACs construct hair shaft and their surrounding channel which is the inner root sheath while terminally differentiating. On the other hand, Bu-SCs give rise to the outer root sheath (ORS), engulfing dermal papilla (DP), and then the hair shaft starts to grow at the onset of Anagen. During Catagen, the matrix and IRS undergo apoptosis, but ORS cells remain and develop a new bulge right next to the original one. DPCs are placed below a new bulge in the state not engulfed from ORSs. DPCs, mesenchymal population located in the base of hair follicle, play a key role, not only in the regulation of hair cycle by epithelial-mesenchymal interaction,[4-8] but also in the regulation of

follicle morphogenesis and the diameter of the hair shaft.[3,9-11] Especially, the number of DPCs is crucial for the maintenance of the duration of Anagen, hair size, and hair shape.[3,12-14] Transitioning from Telogen to Anagen phase, the dermal papilla is recovered with the same number of cells in previous Anagen by cell division within the dermal papilla itself and influx of dermal sheath cells to dermal papilla.[15] Most of *alopecia* has destructive hair cycle because of the failure to maintain DP cell number by the increase in efflux from the dermal papilla and/or the decrease in influx from the dermal sheath.[3,16] Thus, either or both to maintain or to increase DP cell number is the way to cure alopecia.

Humanin is a poly-peptide made up of 24 amino-acids.[17-20] It was first discovered in the survival brain cells of patients with Alzheimer's disease. It has been revealed that Humanin protects apoptosis in living cells of the patients' brain.[17,21] HNG, a 14th-serine replaced with a glycine, is an analogue of Humanin, and it has 1000 times more enhanced potency than the original one.[17,21-23] It has been reported that treatment with HNG activates 57 proteins in the cell. These proteins that HNG activates can be subdivided into different molecular functional types: kinase, transcription regulator, translational regulator, transmembrane receptor, transporter, and so on. Furthermore, their proteins are significantly interrelated with cellular growth, proliferation, and cell cycle.[24] HNG protects neuroblastoma cells from apoptosis by activating extracellular signal-regulated kinase (ERK)1/2, Akt, and signal transducer and activator of transcription 3 (STAT3) signaling.[24]

However, the effect of HNG on the regulation of hair growth has not yet been revealed. Therefore, this study was carried out to investigate the promoting effect and the action mechanisms of HNG on hair growth. Here, it is demonstrated that HNG increases proliferation of DPCs, up-regulates expression of vascular endothelial growth factor (VEGF), activates Erk1/2, Akt, and Stat3, elongates hair shaft, and promotes hair growth.

2. Material and Methods

2.1 Reagents and Antibodies

HNG (a potent analogue of humanin, substituted with a glycine at 14th of 24 amino acids) was purchased from the Peptide Institute, Inc. (Ibaraki-Shi, Osaka, Japan). HNG was dissolved in and diluted with triple distilled water. Minoxidil sulfate (MS),[25,26] the active metabolite of minoxidil, was purchased from the Sigmal-aldrich to use it as positive control. The MINOXYL^{TM} (minoxidil 5%) was purchased from the Hyundai Pharm. Co. (Gangnam-gu, Seoul, Korea) for usage in *in vivo* experiment as positive control. Antibodies used in this study were noted subsequently: anti-phospho-p44/42 MAPK antibody (Cat. #9101), anti-MAPK antibody (Cat. #9102), anti-phospho-Akt antibody (Cat. #9271), anti-Akt antibody (Cat. #9272), anti-phospho-Stat3(Tyr^{705}) antibody (Cat. #9145) and anti-Stat3 antibody (Cat. #12640). These above-listed antibodies were purchased from the Cell Signaling Technology (Danvers,MA,USA). Mouse anti-beta actin (Cat. #A5316)(Sigma-aldrich,St.Louis,MO,USA) and mouse anti-GP130 antibody (Cat.#sc-376280)(Santa Cruz Biotechnology, Dallas, Texas, USA) were also purchased.

2.2 Cell Culture and Treatment

Rat vibrissa immortalized dermal papilla cell line[27] was donated by the Skin Research Institute, Amore Pacific Corporation R&D Center, South Korea. Rat vibrissa immortalized dermal papilla cells 2D-culture was conducted in high glucose Dulbecco's modified Eagle's medium (DMEM; GE Healthcare life Science, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Grand Island. NY, USA) and 1% antibiotic-antimycotic (Anti-Anti; Life Technologies, Carlsbad, CA, USA). In the experiments of MTT, western blot, and Real-time PCR assay, cells were seeded in DMEM medium containing 0.5% FBS and 1% Anti-Anti. Seeded after 24 hrs, the cells were

treated with the reagents.

2.3 Cell Proliferation Assay

Rat vibrissa immortalized DPCs were seeded in 96-well plates as each well contains 200 μL at 0.5×10^4 cell/ml. Seeded after 24 hrs, DPCs were treated with HNG or MS for 72 hrs. Thereafter, 50 μL of MTT solution (2mg/mL) was added into each well. 4 hrs later, supernatant of each well was discarded and 200 μL of DMSO was added to dissolve formazan crystals for 20 min by shaking the plates. Subsequently, the optical density was measured at 540 nm by the Versamax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.4 Western Blot Analysis

Rat vibrissa immortalized DPCs were lysed with protein extraction solution (iNtRON Biotechnology, Seongnam, Gyeonggi-do, Korea) for 30 min. The supernatant containing protein content was collected by centrifugation at 15,000×g for 15 min at 4℃. The quantity of protein content in the cellular lysates was assessed using the Protein assay dye reagent concentration (Bio-rad, Hercules, CA, USA). Quantified equivalent amounts of proteins were loaded on 4-20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis(SDS-PAGE) gels and separated according to protein size. Thereafter, the SDS-PAGE gels were transferred onto polyvinylidenefluoride(PVDF) membranes (GE healthcare, Little Chalfont, Buckinghamshire, UK). Blocked with 5% skimmed milk, the membranes were incubated with primary antibody at 4℃ overnight. Washed three times with Tris-buffered saline containing 0.1% Tween-20, the membranes were incubated with the proper HRP-conjugated secondary antibody at room temperature. Enhanced chemiluminescence was used for detecting the specific proteins. To assess the quantification

of specific proteins, ImageJ software was used.

2.5 Real-time quantitative Polymerase Chain Reaction (Real-Time PCR)

Total RNA was extracted with the TRIzol™Reagent (Thermofisher Scientific, Carlsbad, CA, USA). RNA yield and purity were calculated at 260/280 nm on Jenway 7315 spectrophotometer (Bibby Scientific, Stone, Staffordshire, UK). An equivalent amount of RNA (2 μg per sample) was used to synthesize cDNA using the MG cDNA synthesis kit (MGmed, Geumcheom-gu, Seoul, Korea). Reverse transcription was conducted using Random octamer at 65℃ for 5 min and MMLV reverse transcriptase at 42℃ for 30 min. The VEGF-specific forward and reverse primers were 5'-AAC GAA AGC GCA AGA AAT CC-3' and 5'-GCT CAC AGT GAA CGC TCC AG-3', respectively. In addition, the b-actin (reference gene) -specific forward and reverse primers were 5'TCC TGG CCT CAC TGT CCA C-3' and 5'-GGG CCG GAC TCA TCG TAC T-3', respectively. It was shown briefly in Table 1.

Table 1. Primers for rat β-actin and vascular endothelial growth factor (VEGF)

VEGF *forward*	*5′-AAC GAA AGC GCA AGA AAT CC-3′*
VEGF *reverse*	*5′-GCT CAC AGT GAA CGC TCC AG-3′*
β-actin *forward*	*5′-TCC TGG CCT CAC TGT CCA C-3′*
β-actin *reverse*	*5′-GGG CCG GAC TCA TCG TAC T-3′*

Real-time PCR, followed by the thermal cycling protocol of iQ™SYBR®Green Supermix, was performed using iQ™SYBR®Green Supermix. The thermal cycling protocol condition was the initial polymerase activation, DNA denaturation step at 95℃ for 3 min, and the amplification step consisted of denaturation at 95℃ for 10 sec, annealing and extension at 55℃ for 30 sec, and 40 cycles, which were conducted subsequently. After the amplification step, the melt curve analysis was conducted in order to check the specificity of

the generated products. The relative expression level of VEGF mRNA was normalized to β-actin mRNA and calculated by the $2^{-\Delta\Delta CT}$method. The results were presented as the expression in a time-dependent manner and the relative expression to the control at the same time.

2.6 Animal

All animals were cared for using protocols (20180030) approved by the Institutional Animal Care and Use Committee (IACUC) of Jeju National University. Female 6-week-old C57BL/6 mice and male 3-week-old Wistar rats were purchased from Orient Bio (Seongnam, Gyeonggi, Korea). A standard laboratory diet and water *ad libitum* were given.

2.7 Isolation and Culture of Rat Vibrissa Follicles

The rat vibrissa follicles in Anagen phase (17 day-old) were separated and cultured using the method explained previously.[28] In short, mystacial pads of wistar rat were obtained and placed in a 1:1 (vol/vol) solution of Earle's balanced salts solution and phosphate-buffered saline(PBS) supplemented with 100 unit penicillin per mL and 100 mg streptomycin per mL (E/P buffer). Each vibrissa follicle was isolated by microdissection and shifted to a petri dish containing E/P buffer. Each vibrissa follicle was distributed into 5 groups (n = 7) as considered the size of obtained follicles among groups and cultured in each well of 24-well plates in 500 μL of Williams E medium supplemented with 2 mM L-glutamine, 10 μg insulin per mL, 10 ng hydrocortisone per mL, 100 unit penicillin per mL, and 100 μg streptomycin per mL in the atmosphere of 5% CO_2/95% air at 37℃. The medium was changed every 3 days with HNG or MS. The lengths of the hair follicles were assessed using DP controller software (Olympus, Tokyo, Japan).

2.8 Hair-growth *in vivo* experiment

It has been reported that 7-week-old mice are in Telogen phase in accordance with time-scale for the murine hair cycle, and Anagen phase from Telogen phase is induced by hair depilation or shaving.[29] Therefore, 7-week-old female C57/BL6 mice were purchased. At the beginning of the mice experiment, the mice were randomly divided into 5 groups (n = 6) and shaved to induce Anagen phase. Topical treatments with HNG (50, 100 and 200 nM) or MINOXYL™ (minoxidil 5%) were applied once a day for 43 days. The back skin of the mice was observed and photographed in the 1st, 12th, 19th, 26th and 34th days after depilation. Results were assessed for quantification using dot matrix planimetry.[30]

2.9 TUNEL(Tdt-mediated dUTP-Dig nick and labeling) assay and Haematoxylin and eosin (H&E) staining

The dorsal skin of mice was dissected, fixed in 4% paraformaldehyde (Biosesang, Seongnam, Gyeonggi-do, Korea), embedded in paraffin, and cut into 5μm-thick sections for TUNEL(Tdt-mediated dUTP-Dig nick and labeling) and haematoxylin and eosin (H&E) staining. For TUNEL to evaluate apoptotic cells in hair follicle, DeadEND™ Colorimetric TUNEL System kit (G7130 and G7360) was purchased from the Promega (Madison, WI, USA) and used. After deparaffinization, soaked with xylene for 10 min, 3 min, and 3 min subsequently, tissue sections were applied with 20 μg/mL Proteinase K solution for 30 min. Then, tissue sections were washed with distilled water and soaked in 0.3% hydrogen peroxide in methanol to inhibit the reaction of endogenous peroxidases for 20 min. After washing with distilled water and PBS one after another, tissue sections were equilibrated with equilibration buffer for 10 min. Then, the tissue sections were incubated in a rTdT reaction mix for 60 min inside a humidified chamber. The reaction was terminated in 2X SSC for 15 min. After washing with PBS, the tissue sections were activated by the

Streptavidin HRP solution for 30 min. Colour was developed using the diaminobenzidine(DAB) solution, and tissue sections were counterstained with hematoxylin. For H&E staining, 5μm-thick sections were deparaffinized through the same procedure in TUNEL assay. After then, to rehydrate, those sections were soaked with alcohol along downward concentration gradients; 100%, 95%, 90%, 80%, and 70% for 3 min respectively and subsequently, and finally washed with tap water for 5 min. To stain, those sections were soaked with Mayer's Hematoxylin for 1 min. After then, those sections were washed in tap water with 10-to-20-time dipping. Subsequently, those sections were soaked with eosin for 1 min. To dehydrate, those sections were dipped with alcohol along upward concentration gradients; 80%, 90%, and 95%, and soaked with 100% alcohol three times for 2 min respectively and xylene in the end. As a result, nucleus was stained with deep blue, and cytoplasm and connective tissue were stained with shades of pink.

2.10 Statistical analysis

Data are shown as mean ± standard deviation (S.D) or mean ± standard error (S.E) of at least triplicate experiments. The results were subjected to student's t-test using the SigmaStat software ver. 3.5 (San Jose, CA, USA) to assess significant differences. In all cases, a $p<0.05$ was considered statistically significant.

3. Results

3.1 HNG increases proliferation of dermal papilla cells.

To analyze whether HNG increases proliferation of DPCs, the proliferation assay using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide(MTT) solution was performed. HNG at 100 nM, 200 nM, and 500 nM increased the proliferation of DPCs significantly by 109.6±3.9%, 112.2±6.4%, and 108.1±3.8%, respectively (Figure 1). 1 μM Minoxidil sulfate(MS), an active metabolite of minoxidil as positive control, also increased the proliferation of DPCs significantly by 113.2±2.9% as reported (Figure 1).[31] HNG could stimulate hair growth by DPC proliferation.

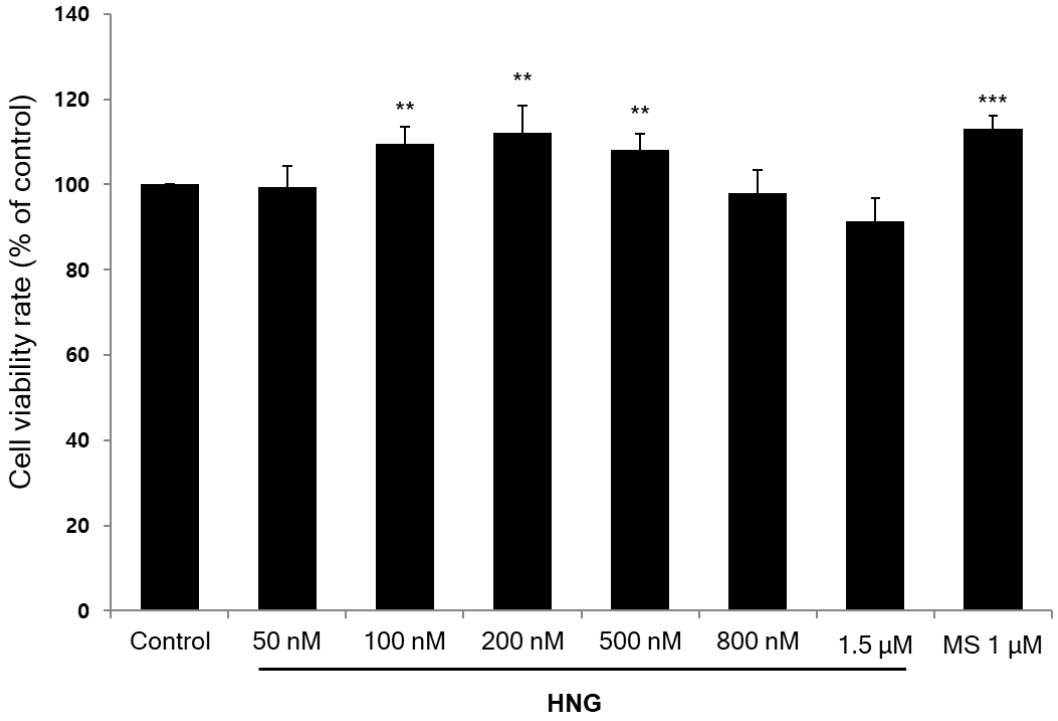
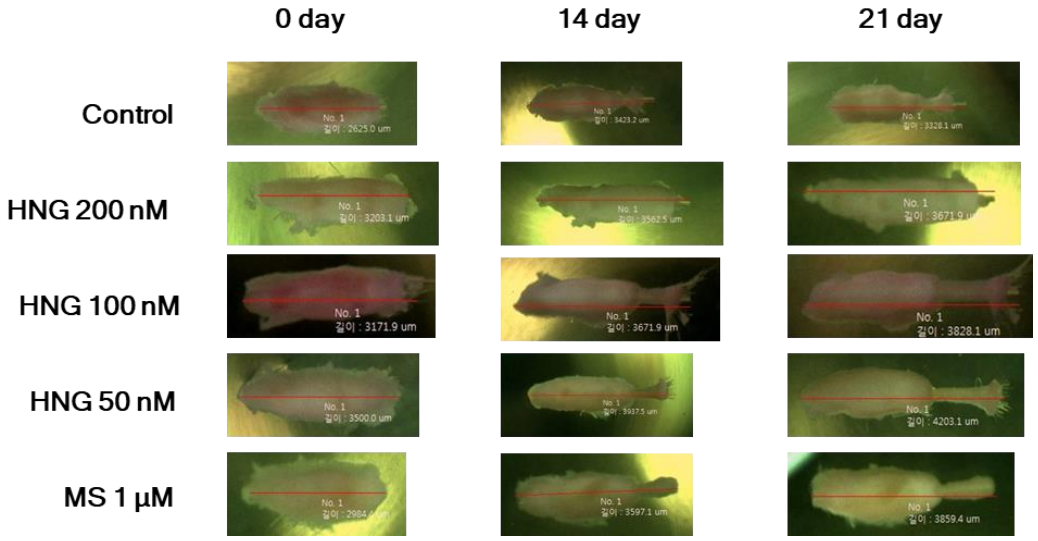


Fig. 1. The effect of HNG on the increase of proliferation of dermal papilla cells (DPCs). Immortalized rat DPCs(0.5×10^4 cell/ml) were seeded in 96-well plates. DPCs were treated with HNG in a concentration-dependent manner; 50 nM to 1.5 μM. DPC proliferation was measured using the MTT assay after 72 hrs after treatment. All experiments were performed in triplicate. Data are shown as the mean ± the S.D. **p<0.05, **p<0.01, ***p<0.001 vs. control*.

3.2 HNG increases hair shaft elongation.

To examine whether HNG increases hair shaft elongation, rat vibrissa follicles in Anagen phase were cultured in treatment with vehicle as the control, HNG (50, 100, and 200 nM) and MS (1 and 10 μM**)** as positive controls, in groups (n=7) for 21 days. HNG (50 nM) increased hair shaft elongation by 163.9±22.7% on the 21st day. MS (1 μM), as a positive control, also significantly increased hair shaft elongation by 166.0±30.7% on the 21st day as reported (Figure 2 and A1).[31,32] Morphologically, vibrissa follicles treated with HNG or MS resulted in early Catagen shape, while those of untreated controls resulted in late Catagen shape on the 21st day. Therefore, we suggest that HNG increase hair shaft elongation by delaying Catagen progression *ex vivo*.



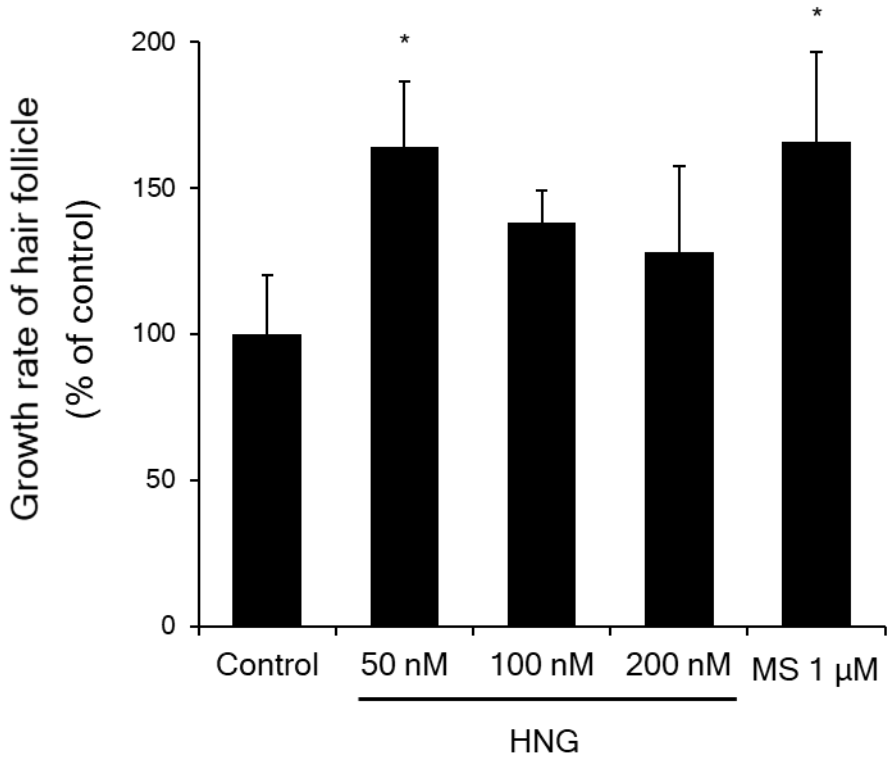


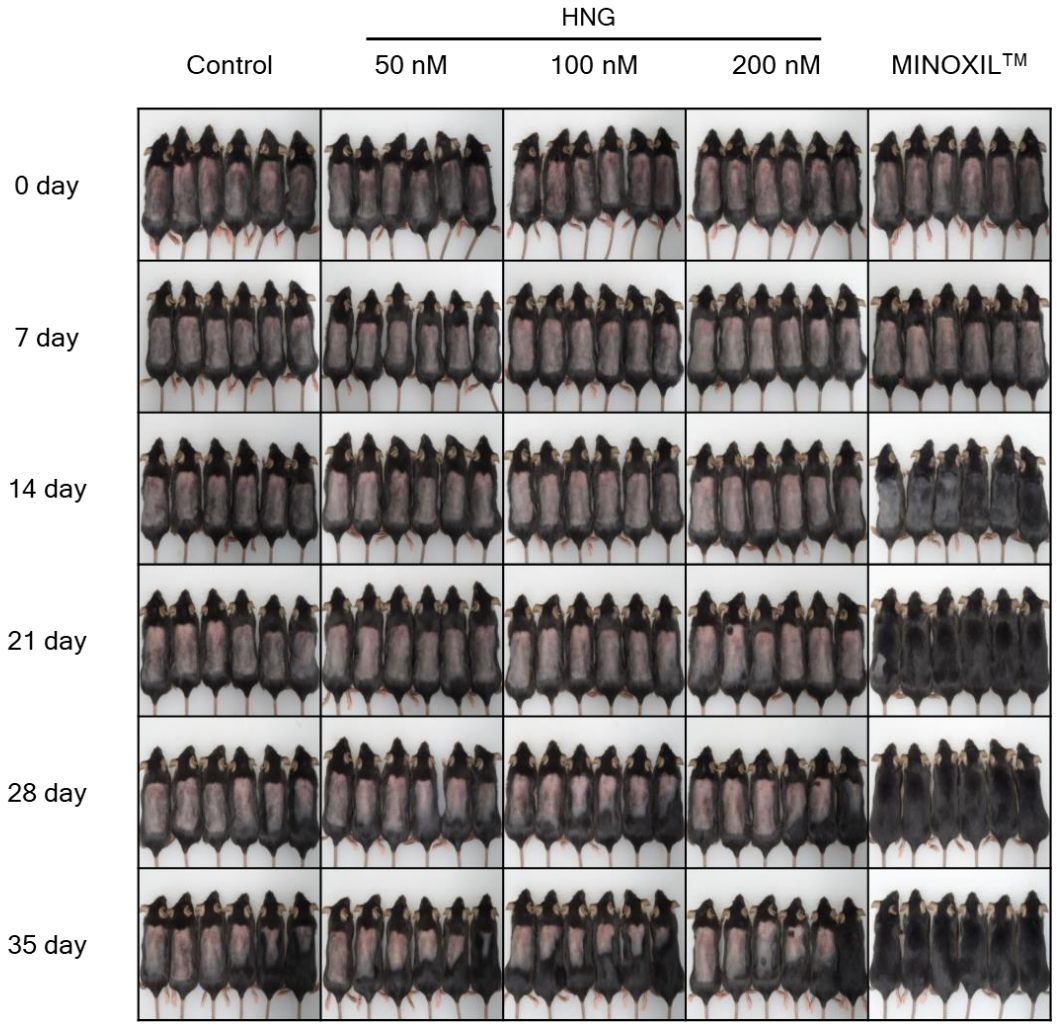
Fig. 2. The effect of HNG for hair follicle elongation.

Vibrissa follicles in Anagen phase were treated with HNG in the following concentration; 50, 100, and 200 nM for 21 days. The medium was refreshed every three days. The mean of the growth rate of the vibrissa follicle of the control group on 21st day was considered to 100%. Data are shown as the mean ± S.E.M (N=7). *$p<0.05$ *vs. control*.

3.3 HNG promotes hair growth.

To assess whether HNG promotes hair growth, the shaved C57/BL6 mice on the dorsal back were treated with vehicle as control (PBS and ethanol in 1:1 (vol/vol)), HNG (50, 100, and 200 nM), and MINOXIL™ (minoxidil 5%) as positive control (Figure 3A). As expected, treatment with MINOXIL™ (minoxidil 5%) promoted hair growth and showed statistical significance within 14 days (Figure 3A, B). Interestingly, treatment with HNG (100 nM) promoted hair growth and showed statistical significance within 35 days (Figure 3A, B). However, there was no significant difference in the initiation of the Anagen phase between the groups treated with HNG and the control group treated with vehicle. It has been reported that Catagen-associated changes such as the skin colour, thickness, and apoptosis of the cells in hair follicle are first seen on the 17th day when the hair follicle transits to Catagen from Anagen phase.[29,33] As reported, we investigated the apoptosis of the cells in hair follicle on the 21st day. Surprisingly, the number of the apoptotic cells in HNG treatment (100 nM) was prominently lesser than in vehicle treatment as control (Figure 3D). The entire dermis and subcutis of the dorsal skin in HNG treatment (100 nM) or MINOXIL™ (minoxidil 5%) group were thicker than the control on the 17th and 21st days, which mean that the duration of Anagen phase was extended. In addition, we examined quantitative histomorphometry based on the shape and size of DP (Figure 3F).[29,34] The number of Catagen hair follicle was significantly decreased by HNG (100 nM) and MINOXIL™ (minoxidil 5%) treatment (control, 86±10.7%; HNG 100 nM, 44±6.2%; MINOXIL™, 2.0±9.1%; Figure3F).

A



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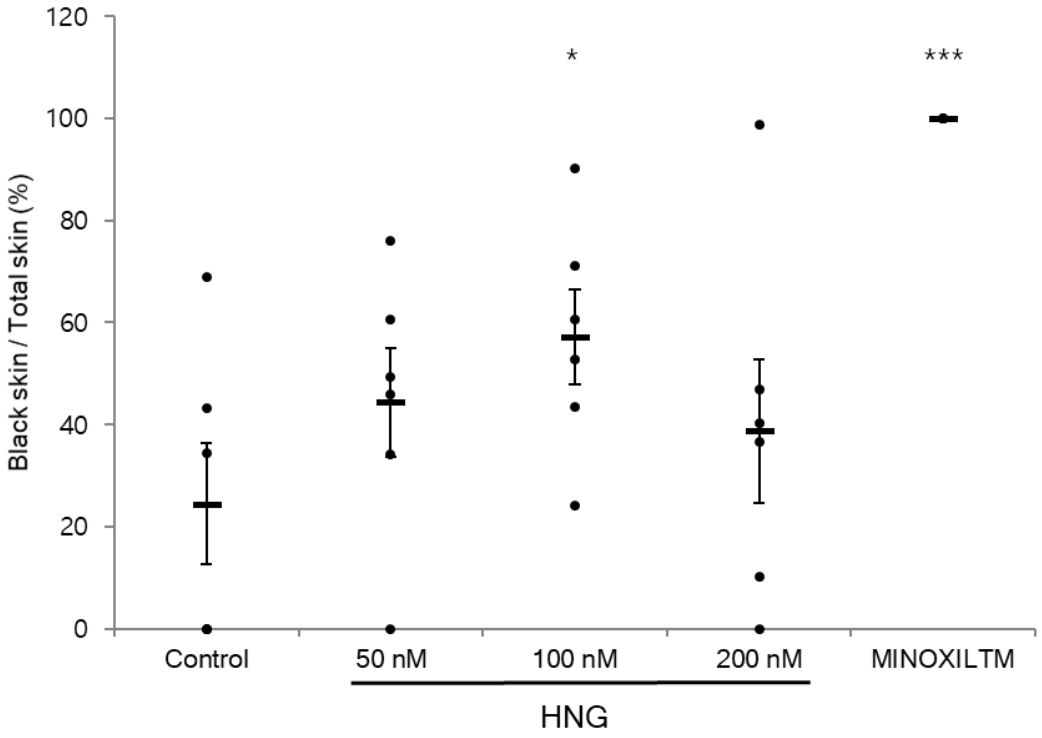

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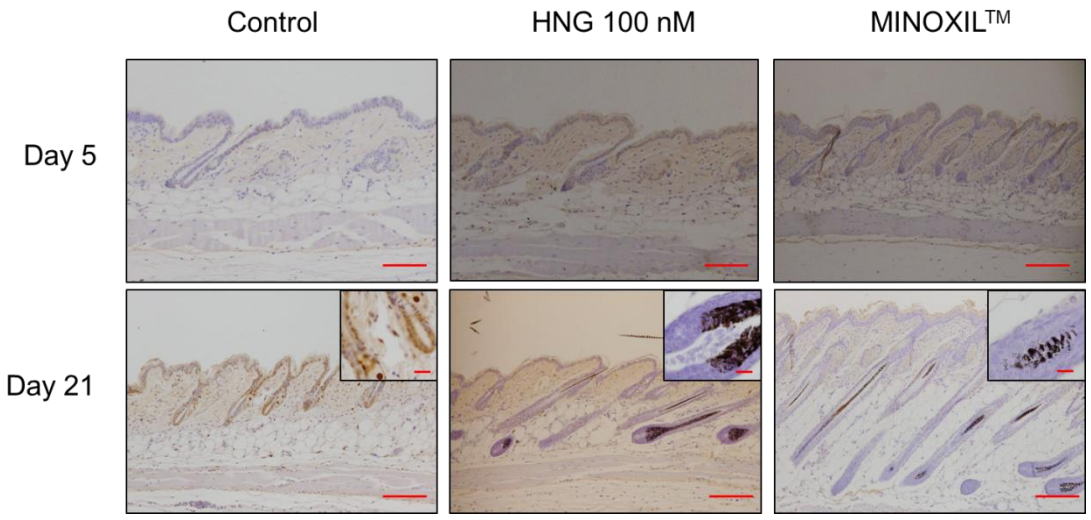

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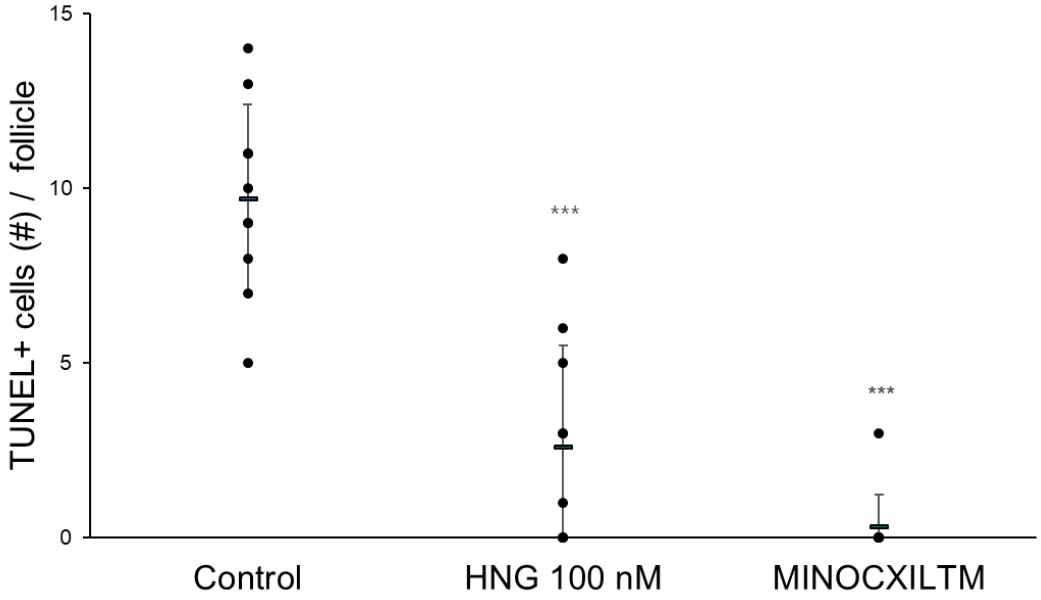

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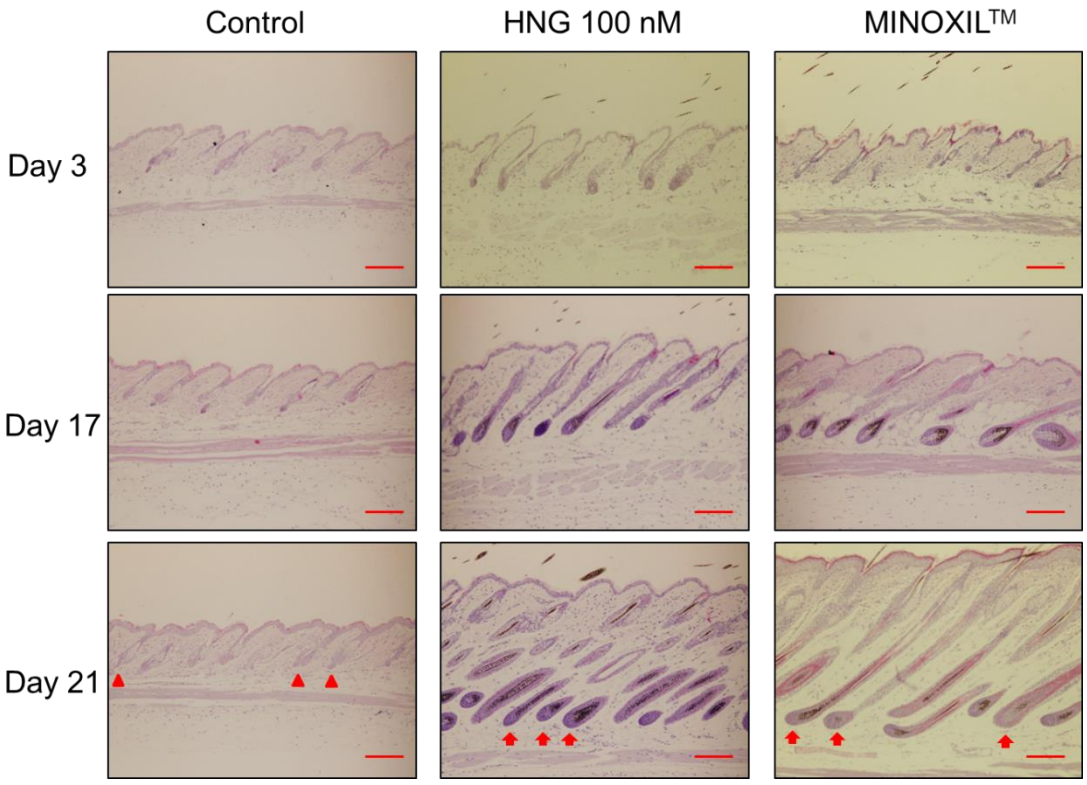

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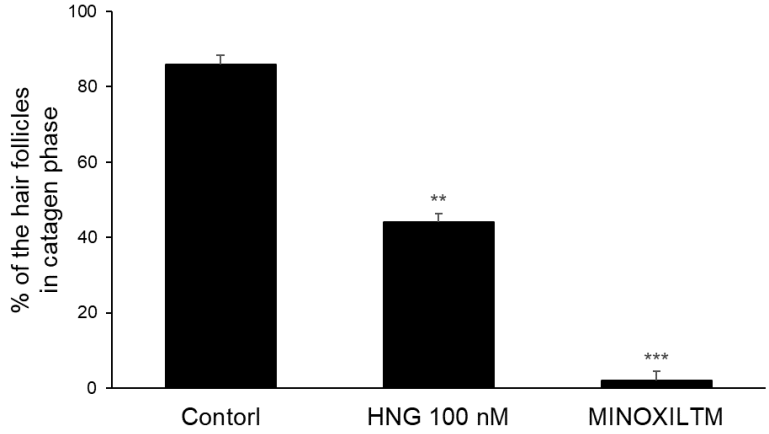
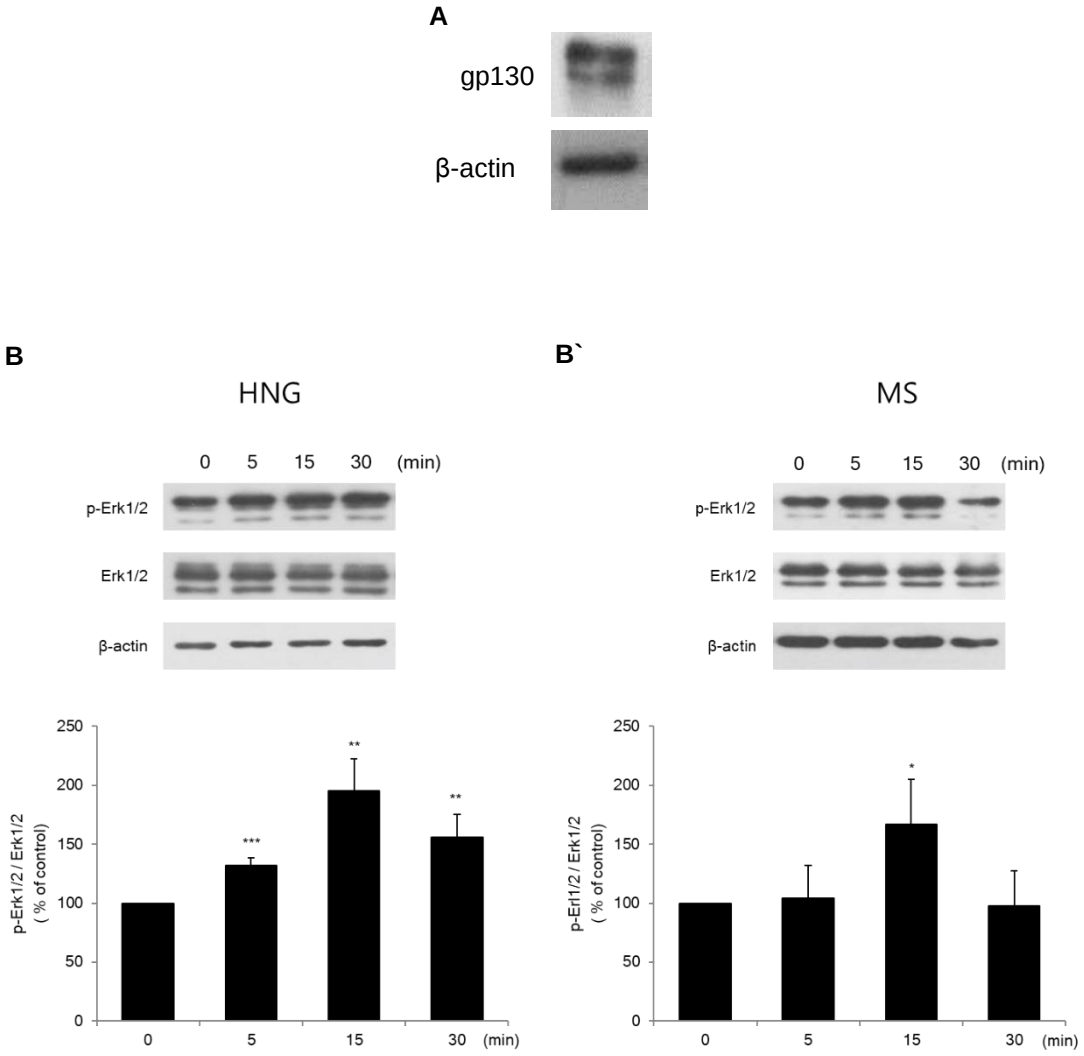


Fig. 3. HNG stimulates Anagen phase in C57/BL6 mice.

After having been shaved, the dorsal skin of the mice was topically treated with vehicle, HNG, and MINOXIL^{TM} (minoxidil 5%) once a day for 43 days. (A) Photos of dorsal skin of C57/BL6 mice on the 0, 7th, 14th, 21st, 28th, and 35th day after depilation. (B) Quantified hair growth of control, HNG 50, 100, 200 nM, and MINOXIL^{TM} (minoxidil 5%) groups on the 35th day using dotmatrix planimetry. Data are shown as the mean ± S.E.M (N=6). *$p<0.05$, **$p<0.01$, ***$p<0.001$ *vs. control.* (C) Coloricmetric Tdt-mediated dUTP-Dig nick and labeling (TUNEL) assay on 21st day. Scale bars represent 100 μm, whereas 200 μm in sub-figures. (D) Quantitative analysis of TUNEL-positive cells on the 21st day in (C). (E) Haematoxylin and eosin staining. Scale bars represent: 100 μm; Anagen hair follicle pointed by *arrow*; Catagen hair follicle pointed by *arrowhead*. (F) Quantification (%) of catagen hair follicle in (E) according to histomorphometry (hair follicles (n≥100) on the 21st day examined). Mean values ± S.D. **$p<0.01$ *vs. control,* ***$p<0.001$ *vs. control.*

3.4 HNG activates Erk1/2, Akt, and Stat3 through a gp130 receptor in DPCs.

It has been reported that treatment with HNG activates Erk1/2, Akt, and Stat3 via a glycoprotein 130 (gp130) receptor.[24] Therefore, we identified the existence of the gp130 receptor and activation of Erk1/2, Akt, and Stat3 in DPC with treatment with HNG. We found the existence of gp130 receptor in DPC (Figure 4A). Further, treatment with HNG (200 nM) activated Erk1/2, Akt, and Stat3 within 5 min (Figure 4B, C, D). Treatment with MS (1 μM) as positive control also activated Erk1/2 and Akt, but not Stat3 (Figure 4B`, C`, D`).



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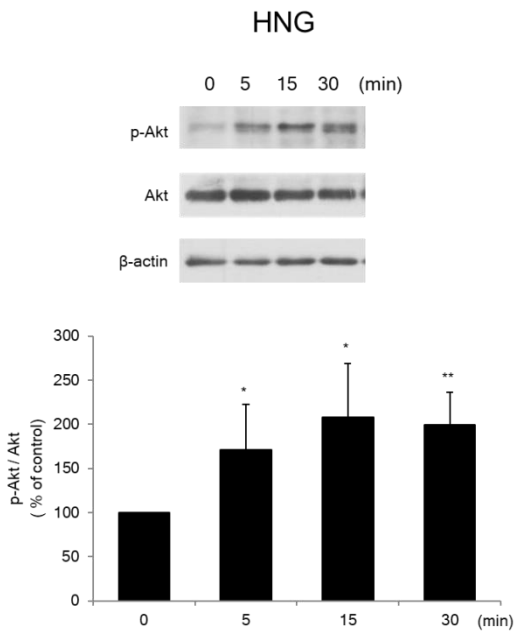

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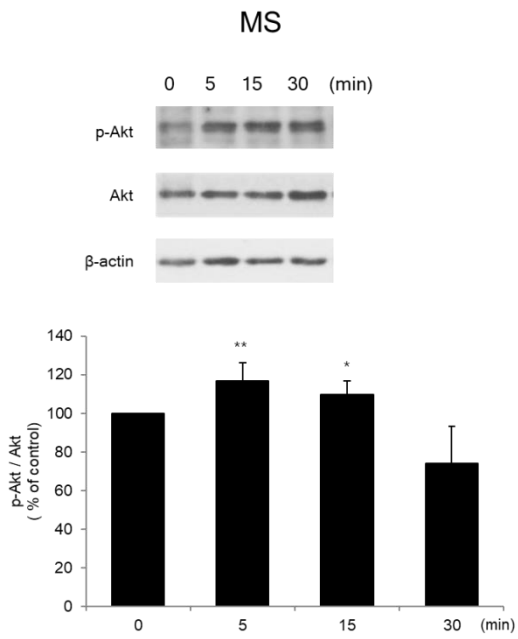

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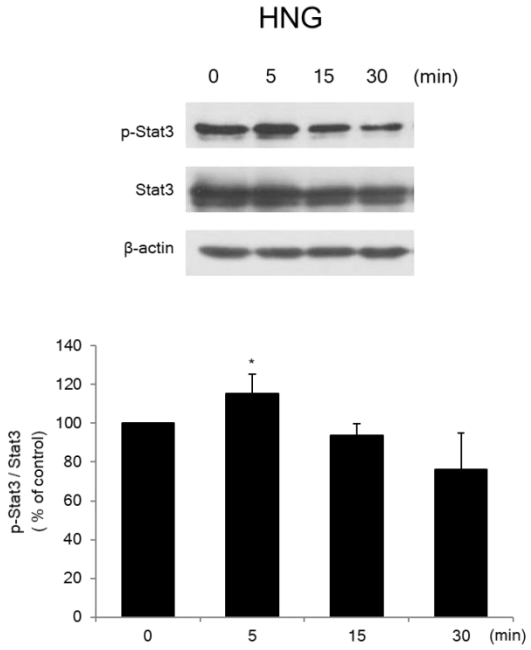

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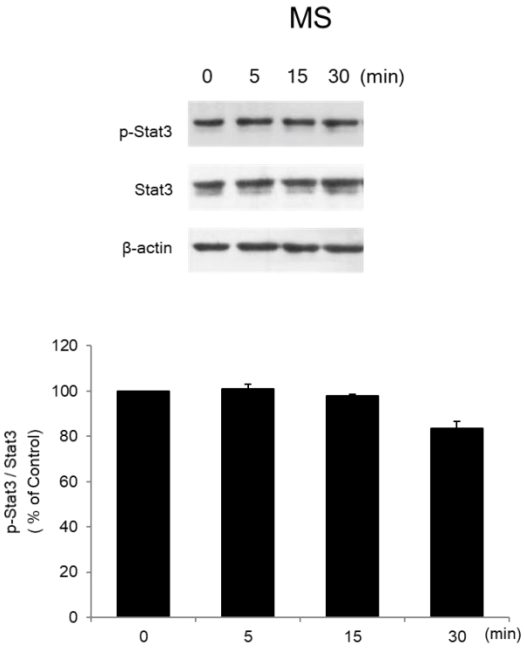


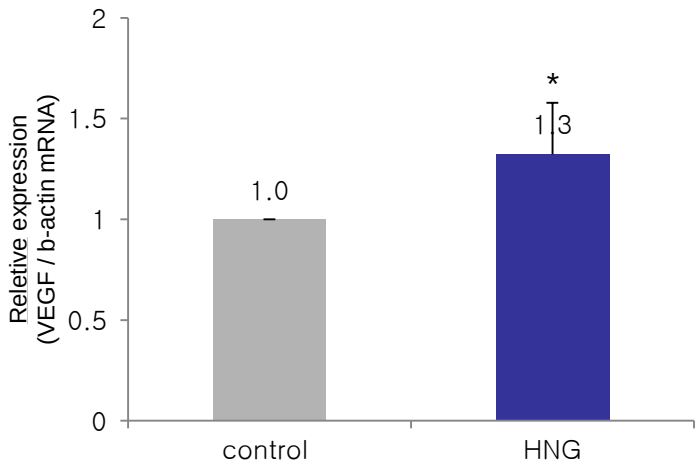
Fig. 4. HNG activates Erk1/2, Akt, and Stat3 in DPCs.

DPCs(1×10^5 cells/ml) were seeded in DMEM, supplemented in 0.5% FBS. After 24 hrs, the cells were treated with HNG 200 nM for the indicated time periods. Total cell lysates were immnoblotted using anti-phospho-Erk(Thr^{202}/Tyr^{204}), -Akt(Ser^{473}), and -Stat3(Tyr^{705}). All experiments were performed in triplicate. (A) Western blot of the gp130 receptor known as which HNG binds. Quantification and representative western blot of (B and B`) Erk1/2 activation, (C and C`) Akt activation and (D and D`) Stat3 activation. Data are shown as the mean ± the S.D. *$p<0.05$, **$p<0.01$, ***$p<0.001$ *vs. control*.

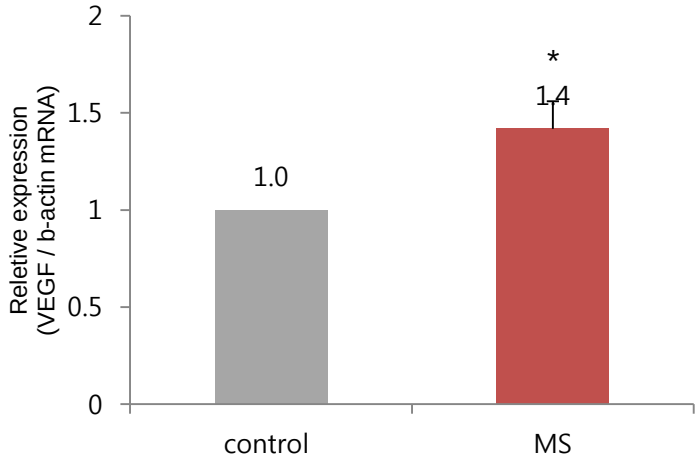
3.5 HNG up-regulates the expression of VEGF mRNA in DPCs.

It has been reported that VEGF, known as one of the anagen prolongation factors, is one of the Stat3 target genes and DPCs express VEGF.[2,6,7,35,36] As Stat3 was activated by HNG treatment, we further investigated whether the expression level of VEGF mRNA is up-regulated in DPCs. After 2 hrs from the treatment with HNG, the expression level of VEGF mRNA was up-regulated in DPCs, where the up-regulation of the expression level of VEGF mRNA was maintained for 24 hrs (Figure 5A, B, C, D). The peak of up-regulation level of VEGF mRNA in DPC treated with HNG was 1.3-fold relative to control (Figure 5A). The level of VEGF mRNA in DPCs treated with MS used as positive control was also up-regulated, which was also maintained for 24 hrs (Figure 5A`, B`, C`, D`).

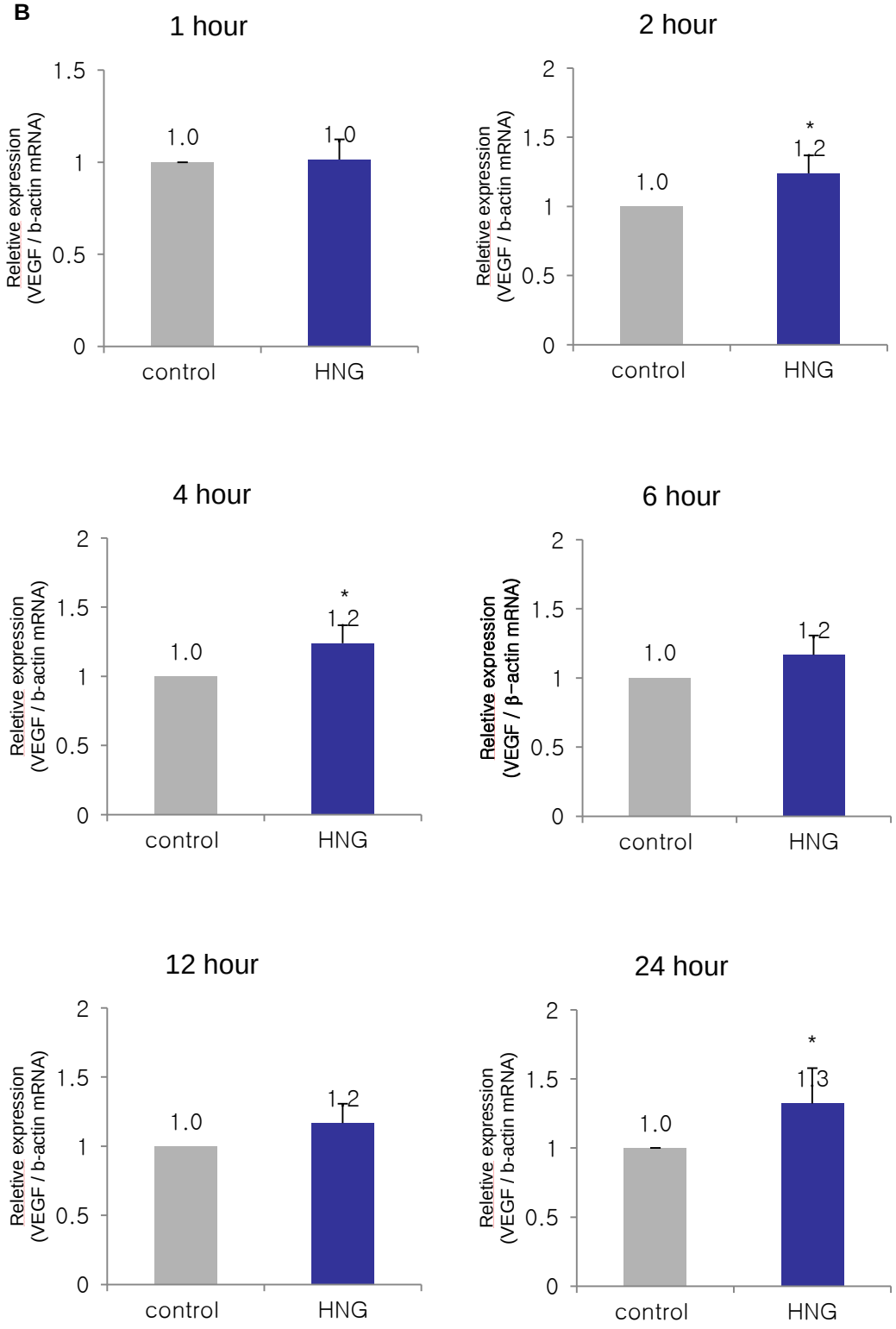
A



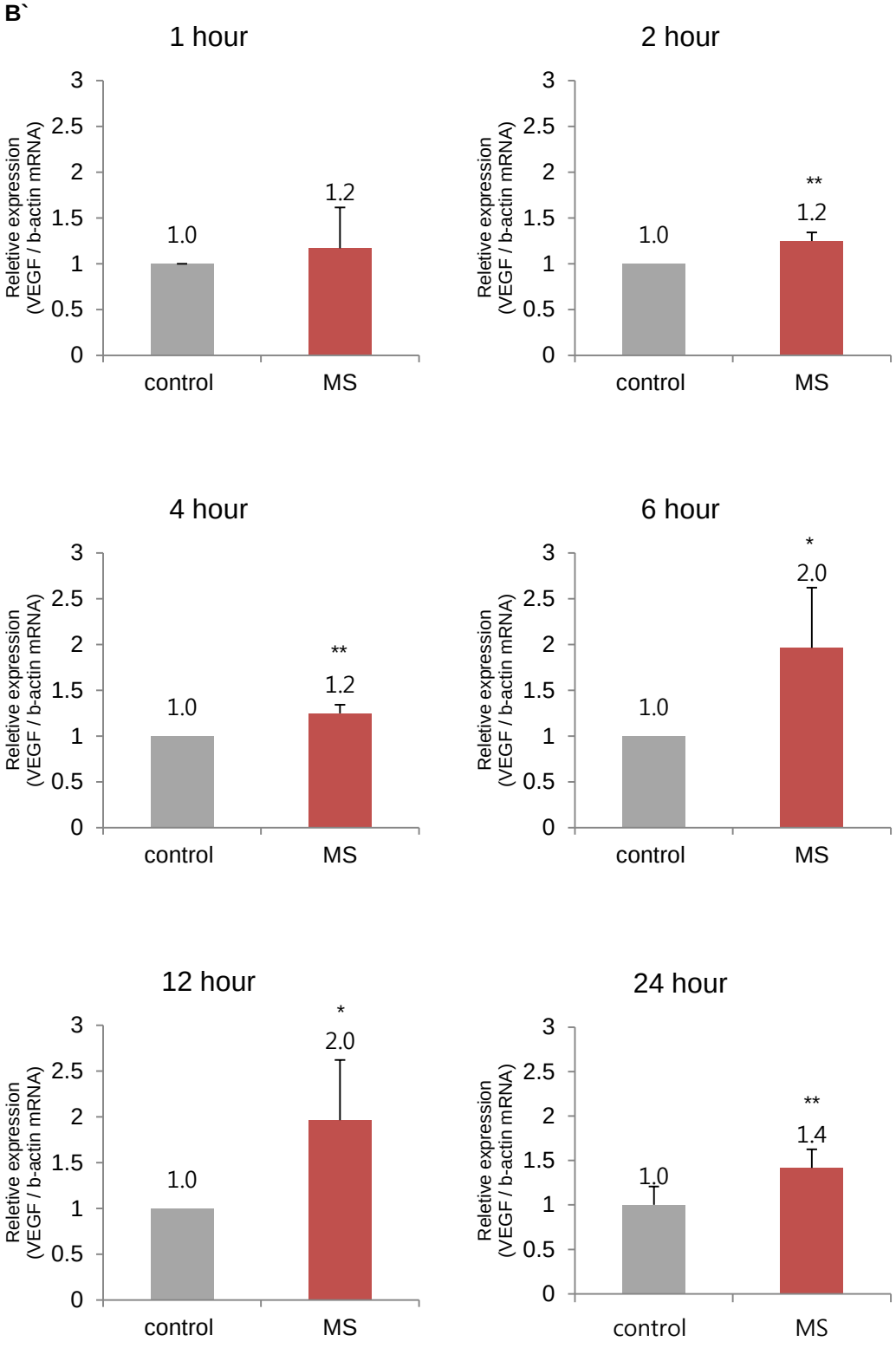
A`



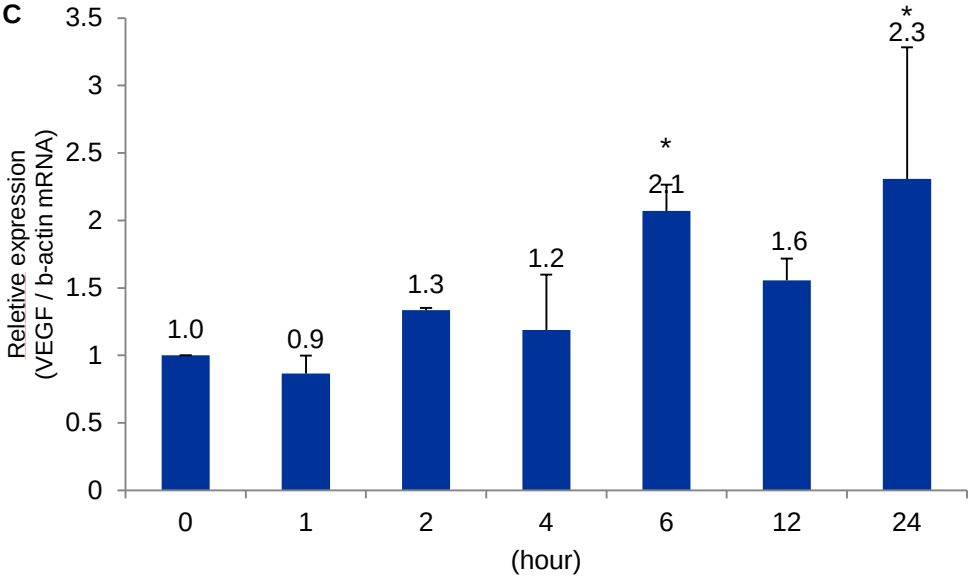
B



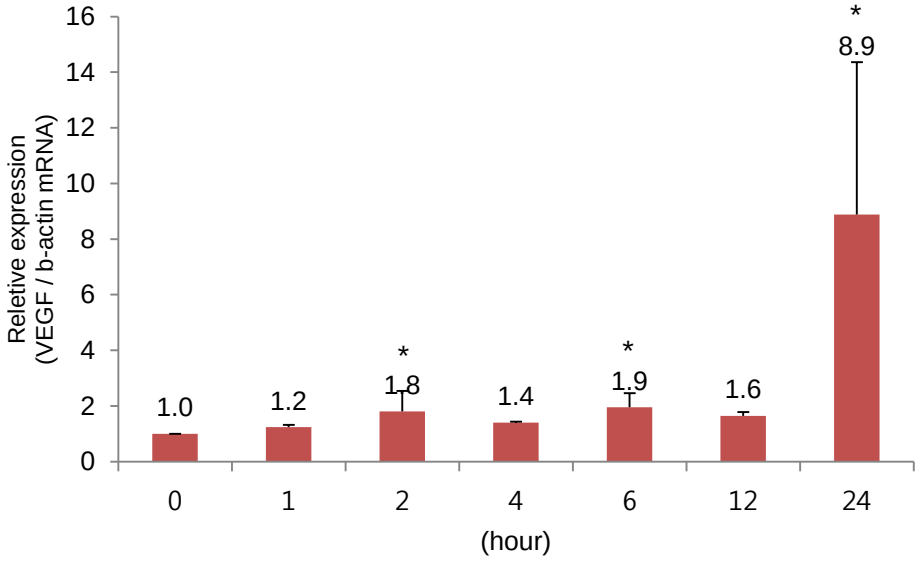
B`



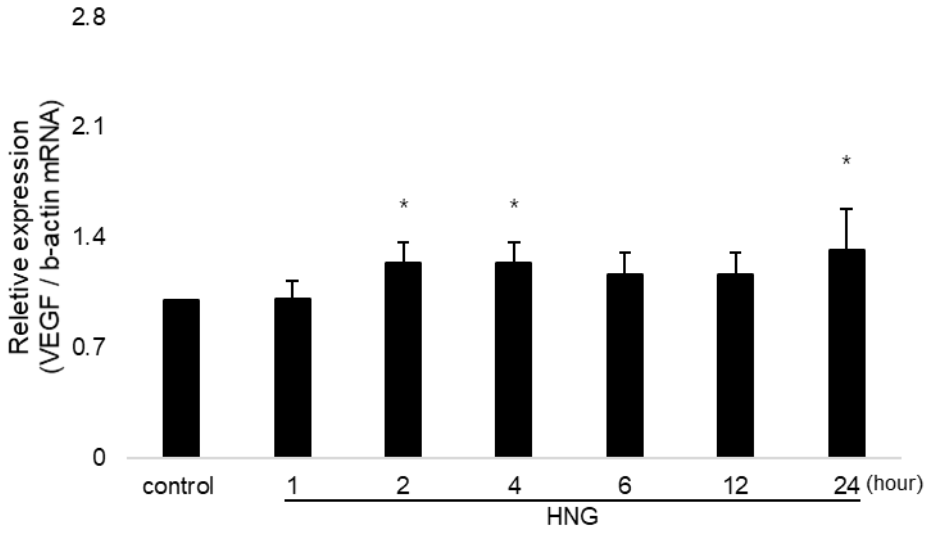
C



C`



D



D`

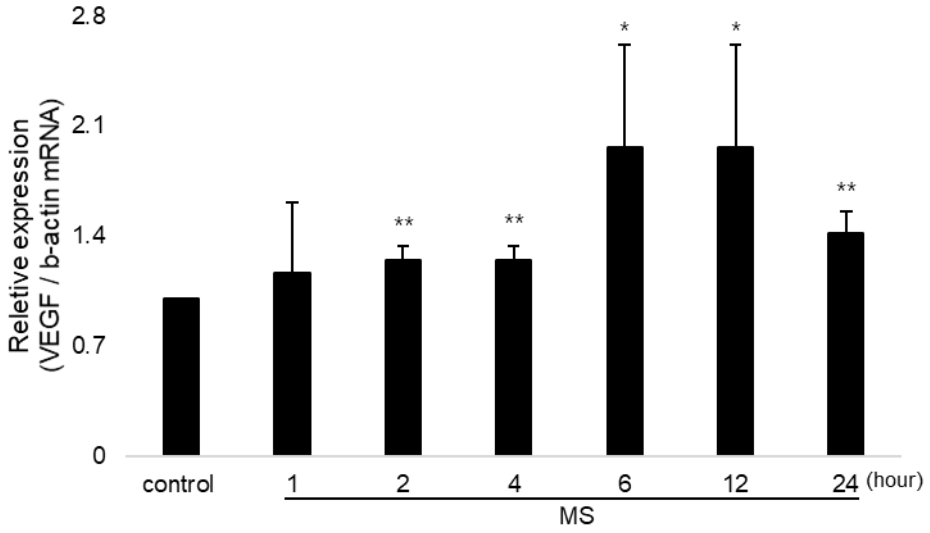


Figure 5. The expression level of VEGF is up-regulated in DPCs treated with HNG.

DPCs(1×10^5 cells/ml) were seeded in DMEM supplemented in 0.5% FBS (A-D`). After 24 hrs, the cells were treated with HNG 200 nM and MS 1 μM. (A and A`) The expression level of VEGF evaluated by real-time PCR and normalized to β-actin mRNA at 24 hrs after treatment. (B and B`) The expression level of VEGF at indicated time point compared to

control. (C and C`) Relative expression level of VEGF at 0 time as control. (D and D`) Relative expression level of VEGF in HNG and MS groups, and control at each time point. Data are shown as the mean ± S.D. *$p<0.05$, **$p<0.01$ *vs. control.*

4. Discussion

The repetitive hair cycle consisted of Anagen (growth), Catagen (regression), and Telogen (resting) phases is regulated by signaling while interactions among follicular cells. DPCs, mesenchymal derived fibroblasts among follicular cells, play as a key regulator in hair cycle.[13] In addition, the population of DP is decreased in most of the onset points of alopecia because DP is not recovered with the same number of cells in previous Anagen by cell division within the dermal papilla itself, and by the decrease of influx of dermal sheath cells to dermal papilla.[15] Accordingly, HNG was treated in DPCs and the effect of HNG for the proliferation of DPCs was investigated. The proliferation of DPCs was significantly increased in 100, 200, and 500nM HNG (Figure 1). In accord with the significant concentration range of HNG, all experiments were conducted thereafter. Nonetheless, further studies are needed to determine whether HNG treatment increase influx of dermal sheath cells to dermal papilla and the level of inductivity marker such as ALP and versican.[37] *Ex vivo*, HNG treatment increased the length of hair shaft of the hair follicle in the Anagen phase (Figure 2). The increase of the length of hair shaft will be due to the Anagen phase prolonged by the inhibition of transition of Anagen to Catagen phase because hair follicles in Anagen phase were isolated and cultured with treatment. In an i*n vivo* experiment, the initiation of Anagen from Telogen phase by HNG treatment was not faster than the others (vehicle and MINOXIL^{TM}); rather, the duration of Anagen would be considered as expanded and impeding the transition of Anagen to Catagen phase (Fig. 3A, 3B). On the 21st day, the number of apoptotic cells of mice hair follicle topically treated with HNG was significantly lesser than the vehicle (Figure 3C-E). The apoptotic cells are found in the hair follicle as the hair follicle transits from Anagen to Catagen phase. Because TUNEL+ cell was not found on four of the ten follicles of HNG 100 nM treatment group, Anagen and Catagen hair follicles were estimated respectively at 40% and 60% from figure 3D. It also implies that the duration of Anagen was considered as expanded by impeding the transition from Anagen to Catagen

phase. In addition, the quantitative histomorphometry result demonstrates that HNG prolongs the duration of Anagen by impeding transition to Catagen from Anagen phase (Figure 3F). The effective concentration of HNG in *ex vivo* assay is lower than other assays including in vitro and in vivo. According to previous research,[38,39] a possible explanation for this was as follows: In *ex vivo* experiments using the culture of vibrissa follicles, the culture medium containing HNG was refreshed every 3 days until the end of *ex vivo* assay. Therefore, the effective concentration was lower than expected, because HNG and its metabolites could be accumulated in hair follicle. In our previous studies, *ex vivo* experiments showed efficacy of the reagent at lower concentration compared to other *in vitro* experiments.[38,39] Then, western blotting was performed to investigate the signaling molecule and transcription factor activated by HNG treatment. HNG activates Erk1/2, Akt, Stat3 signaling via a gp130 receptor in SH-SY5Y neuroblastoma cells. This prevents from the apoptosis of the cells.[24,40] In the study, expression of the gp130 receptor was detected, and activation of Erk1/2, Akt, and Stat3 were examined thereafter in DPCs by HNG treatment (Figure 4). In the study, we have not yet determined whether HNG activates Erk1/2, Akt and Stat3 via the gp130 receptor with use of siRNA or blocker of the gp130 receptor. Nonetheless, HNG seems to activate Erk1/2, Akt, and Stat3 via the gp130 known as a specific receptor for HNG as reported.[24,40-42] Erk1/2 and Akt called signaling molecules are known to be associated with cell proliferation by up-regulating genes important for cell cycle progression, for example, Cdks, cyclins and growth factors, and apoptosis prevention, for example, Bcl-2 and cytokines.[43-48] As Erk1/2 and Akt was activated with HNG treatment in DPCs, the proliferation of the cells would be increased by the up-regulation of genes important for cell cycle progression and apoptosis prevention. Minoxidil and norgalanthamine significantly increase the proliferation of DPCs, activating Erk1/2 and Akt.[31,49] Sinapic acid also showed hair growth-promoting effects on DPCs in human hair follicle, activating AKT.[50] Vascular endothelial growth factor (VEGF) and hepatic growth

factor (HGF) which are known to prolong the Anagen phase by inhibiting the transition of Catagen phase, are of Stat3 target genes.[7,51-53] It has already been reported that DPCs express VEGF and HGF. As Stat3 was activated with HNG treatment, DPCs would up-regulate VEGF and HGF, and it would be signaling the inhibition of transition of Anagen to Catagen phase. Therefore, we investigated that the expression level of VEGF mRNA in DPCs treated with HNG and found that its expression level was up-regulated (Figure 5). In addition, up-regulated VEGF mRNA from treatment with HNG by activating Stat3 would also increase proliferation of DPCs in *in vitro* because it has been reported that VEGF induces proliferation of DPCs.[54] VEGF has been reported to increase the cell proliferation of outer root sheath (ORS) and DPC via VEGF-R2.[38,53] Furthermore, the gp130 receptor known to be bound by HNG is expressed and distributed in follicular keratinocytes, including matrix cells in hair follicle.[55] The expression of genes known to prolong the Anagen phase may also be up-regulated in many types of hair follicle cells by HNG treatment.[53] In other words, HNG stimulates many types of hair follicle cells, activating Erk1/2, Akt, and Stat3. Then, HNG induces the up-regulation of expression of its target genes. Alternatively, the Anagen phase could have been prolonged as the apoptosis of the hair follicle cells was directly protected from HNG, which is known to inhibit cell apoptosis.

In conclusion, our study provides efficacy of HNG as a treatment for alopecia. Further studies will be needed to test its potential for clinical use.

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6. 국문초록 (Abstarct in Korean)

모낭은 모발이 자라나는 성장기 (Anagen), 모발의 성장이 멈추고 모낭의 세포들이 사멸하는 퇴행기 (Catagen), 퇴행이 멈추고 성장기로 들어가기 전 단계인 휴지기 (Telogen)를 거쳐 다시 성장기로 들어가는 계속되는 주기를 갖는다. 모발이 자라나는 성장기에는 모낭의 세포들이 증식하고 분화하여 모낭의 외모근초 (outer root sheat), 내모근초(inner root sheat), 모발 (hair shaft) 등 모낭의 구조물을 형성하며, 모유두(Dermal papilla; DP)는 타원형 모양으로 커지며 모낭의 세포들에 의하여 둘러싸이게 된다. 모발의 성장이 멈추는 퇴행기에는 모낭의 세포들이 사멸하고 모낭의 크기가 작아지며 모유두의 크기는 줄어들고, 모유두는 모낭의 바닥으로부터 떨어지게 된다. 휴지기는 다음 성장기로 들어가기 전에 휴지하는 상태이다. 모유두세포 (Dermal papilla cells)의 수는 성장기에서 다음 성장기에 이르는 동안 세포의 증식과 모낭 세포의 이동을 통하여 유지가 되게 된다. 모발 주기의 조절과 성장기의 유지는 모낭의 모유두세포의 수 및 모낭과 모유두세포 사이의 주고 받는 신호들에 의하여 결정된다. 탈모는 이러한 모발의 주기가 잘 조절되지 않아서 휴지기에서 성장기로의 이행이 늦거나 성장기에서 퇴행기로의 이행이 빨라지게 되면 일어나게 된다. 또한 모유두세포의 수가 감소하게 되면 모발의 굵기가 가늘어지고 약해지며 탈모와 연관이 있다고 알려져 있다. 따라서

휴지기에서 성장기로의 이행을 빠르게 유도하든지 성장기에서 퇴행기로의 이행을 늦추는 것이 모발의 성장을 촉진하는 방법이 될 것이고, 또한 모유두세포의 수를 유지시키거나 증식시키는 것도 모발이 잘 성장하는 성장기를 오래도록 유지하는 방법이 된다.

휴매닌(Humanin)은 알츠하이머 질환(Alzheimer's disease)을 가지고 있는 환자의 살아남은 뇌세포에서 세포사멸을 막는 생존 인자로서 발견되어 이름 붙었다. 본래 휴매닌(HN)의 14번째 아미노산 염기가 serine에서 glycine으로 대체된 HNG은 본래 휴매닌(HN)의 활성보다 1000배를 초과한 강력한 효과를 갖는다. 이러한 HNG을 세포에 처리하면 57개의 단백질이 인산화 되는데, HNG에 의하여 인산화 되는 57개의 단백질을 기능에 따라 분류하면 kinase, transcription regulator, translational regulator, transmembrane receptor, transporter 등이다.

탈모 질환에 있어서 HNG의 효과는 아직 밝혀진 바가 없다. 본 연구는 모발 성장에 있어서 HNG의 효과를 밝혀낸 것이고, 탈모에 있어서 새로운 약물로써 HNG의 가능성을 보인 것이다. HNG는 모유두세포의 성장을 증가시켰다. HNG는 또한 모낭 배양에서 모간의 길이 성장을 대조군에 비하여 유의하게 증가시켰다. 마우스 동물 실험에서 HNG의 피부 도포는 모발 성장기를 효과적으로 연장하였고, 모낭세포의 사멸을 저해하였다. 작용기전으로 HNG은 모유두세포에

서 Erk1/2, Akt, Stat3의 활성을 증가시켰다. 또한 HNG은 모유두세포에서 Stat3의 표적 유전자라고 알려졌고, 모발 성장기를 연장시킨다고 알려진 Vascular Endothelial Growth Factor (VEGF)의 양도 증가시켰다. 연구결과를 종합하면, HNG는 모유두세포에서 Erk1/2, Akt, Stat3의 활성화, VEGF 발현 증가 및 모낭의 세포들의 사멸을 저해함으로써 모발의 성장기가 더 길게 유지되게 하여 모발 성장을 유도한다.

본 연구는 HNG의 탈모의 치료제로서 가능성을 제시하였다는 것에 의의가 있다. 본 연구를 토대로 실제 임상적으로 탈모환자에서 효과가 있는지, 효과가 있다면 약물의 효과적인 전달을 위한 제형에 대한 후속 연구가 필요하다.

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