



Udenafil Induces the Hair Growth Effect of Adipose-Derived Stem Cells

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Abstract

Udenafil, which is a PDE₅ inhibitor, is used to treat erectile dysfunction. However, it is unclear whether udenafil induces hair growth via the stimulation of adipose-derived stem cells (ASCs). In this study, we investigated whether udenafil stimulates ASCs and whether increased growth factor secretion from ASCs to facilitate hair growth. We found that subcutaneous injection of udenafil-treated ASCs accelerated telogen-to-anagen transition *in vivo*. We also observed that udenafil induced proliferation, migration and tube formation of ASCs. It also increased the secretion of growth factors from ASCs, such as interleukin-4 (IL-4) and IL12B, and the phosphorylation of ERK1/2 and NFκB. Furthermore, concomitant upregulation of IL-4 and IL12B mRNA levels was attenuated by ERK inhibitor or NFκB knockdown. Application of IL-4 or IL12B enhanced anagen induction in mice and increased hair follicle length in organ culture. The results indicated that udenafil stimulates ASC motility and increases paracrine growth factor, including cytokine signaling. Udenafil-stimulated secretion of cytokine from ASCs may promote hair growth via the ERK and NFκB pathways. Therefore, udenafil can be used as an ASC-preconditioning agent for hair growth.

Key Words: Udenafil, Adipose-derived stem cells, IL-4, IL12B, Hair growth

INTRODUCTION

Adipose-derived stem cells (ASCs) are multipotent cells that not only differentiate into mesenchymal lineage cells but also secrete various growth factors thereby promoting hair growth (Bunnell *et al.*, 2008; Won *et al.*, 2010; Festa *et al.*, 2011; Jeong *et al.*, 2013b; Kim *et al.*, 2014a, 2014b, 2015; Jin and Sung, 2016). ASCs release multiple growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factors (b-FGF) which induce the proliferation of dermal papilla cells (DP cells), thereby promoting hair growth (Won *et al.*, 2010). The lineage experiment revealed that premature adipocyte drives hair cycling as skin stem cell niche releasing platelet-derived growth factor-A (PDGF-A) to regulate follicular stem cell activity (Festa *et al.*, 2011). ASC transplantation promotes hair growth *in vivo*, and a conditioned medium of ASCs increased the proliferation of hair-compositing cells *in vitro* (Jin and Sung, 2016). Therefore, transplantation of ASC-enriched adipose tissue has shown promise as an alternative approach to treating hair loss in men and women (Perez-Meza

et al., 2017). However, our many trials have found that only ASC or conditioned medium of ASCs is not enough to promote hair growth significantly and effectively. Therefore, ASC preconditioning with identified stimulators is one of the best strategy that can be used to enhance the efficacy of ASCs.

We have identified stimulators that enhance the hair growth potential of ASCs *in vivo*. For instance, vitamin C and low-dose UVB increased secretion of hair growth-promoting factors from ASCs and induced the rate of anagen to telogen in animal models (Jeong *et al.*, 2013b; Kim *et al.*, 2014a). Platelet-derived growth factor-D (PDGF-D) revealed strong effects on ASCs and increased secretion of growth factors via mitogen-activated protein kinase (MAPK) pathways and ROS generation in mitochondria (Kim *et al.*, 2015). LL-37 increased the secretion of growth factors and the hair growth efficacy of ASCs via early growth response 1 (EGR1) protein and the MAPK pathway (Yang *et al.*, 2016). The vasodilator, such as minoxidil, are known to stimulate hair growth (Buhl *et al.*, 1989; Headington, 1987; Michelet *et al.*, 1997; Li *et al.*, 2001; Kwack *et al.*, 2011; Otomo, 2002; Han *et al.*, 2004). However, the

Open Access <https://doi.org/10.4062/biomolther.2018.195>

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Received Oct 8, 2018 Revised Jan 1, 2019 Accepted Feb 25, 2019

Published Online Apr 10, 2019

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exact mechanism for hair growth of vasodilators has not yet to be understood. The general hypothesis is that the increase in blood flow causes dormant follicles to reconnect with the dermal papillae, and this structures located under the follicle provide a blood supply to the hair bulb. There is strong evidence that minoxidil directly promotes hair growth via stimulation of DP cells and epithelial cells (Han *et al.*, 2004). In addition, we found that preconditioned ASCs with minoxidil promoted hair growth releasing growth factors including PDGF-C, PD-ECGF and CXC1 via ERK pathway (Choi *et al.*, 2018). Therefore, we next wanted to examine hair promoting effect of other vasodilators such udenafil.

Udenafil (Dong-A, Seoul, Korea), a newly introduced drug in urology, is used to treat erectile dysfunction. It belongs to a class of drugs called phosphodiesterase type 5 (PDE₅) inhibitors, which also include avanafil, sildenafil, tadalafil, and vardenafil. Udenafil acts by inhibiting the degradation of cyclic guanosine monophosphate (cGMP) to guanosine monophosphate (GMP), which relaxes the smooth muscle and allows increased blood flow for penile tumescence (Gopal *et al.*, 2001). Previous studies have proven that increased cGMP levels due to an active PDE₅ inhibitor induces angiogenesis by forming capillary-like tubes in the corpus cavernosum and increasing VEGF synthesis (Pyriochou *et al.*, 2007; Sahara *et al.*, 2010). Preconditioning of PDE₅ inhibitors with ASCs improves the therapeutic efficacy of ASCs in myocardial infarction *in vivo*, releasing growth factors, including VEGF, b-FGF, and insulin-like growth factor (IGF) (Hoke *et al.*, 2012). In a previous study, chronic administration of udenafil promoted erectile function in a BCNI rat model and suppressed ameliorating fibrosis and apoptosis induced by cavernous nerve crush injury (Lee *et al.*, 2011). Udenafil improves penile reaction in diabetic animals, animals with spinal cord injury and hypertensive patients without increasing the frequency or severity of adverse events (Kang *et al.*, 2004; Paick *et al.*, 2009). In addition, in combination with covering ASCs, udenafil can improve erectile dysfunction (Jeong *et al.*, 2013a).

However, there is little evidence that preconditioning of udenafil on ASCs can affect hair growth. Therefore, in this study, we examined the preconditioning effect of udenafil in ASCs and growth factor secretion enhancing hair growth.

MATERIALS AND METHODS

Cell culture

Human ASCs were isolated via liposuction of subcutaneous fat, as described in a previous study (Kim *et al.*, 2007; Yi *et al.*, 2014): Briefly, the fat was washed with phosphate-buffered saline (PBS), 0.075% collagenase was added and the mixture was incubated for 45 min. at 37°C with gentle shaking. After centrifugation, the pellet was filtered through a 100 µm nylon mesh. Then, the cells were cultured with an essential medium including alpha Minimum Essential Medium (α-MEM) (Hyclone, Logan, UT, USA), 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA) and 1% antibiotics (Gibco) for three passages. Then, the medium was changed to α-MEM, 10% FBS and 1% penicillin/streptomycin (Gibco) for four passages. ASCs were used at passages 5-7 for all experiments. Characterization of ASCs was performed using flow cytometry. ASCs were positive for CD44, CD73, CD90, CD105, human leukocyte antigen (HLA)-I, and podocalyxin-like protein (PODXL)

but were negative for hematopoietic markers such as CD34 and CD45 (Kim *et al.*, 2007). The multipotent differentiation potential was examined, as described in a previous study (Yi *et al.*, 2014), and ASCs could be differentiated into adipocytes, osteocytes, and chondrocytes. ASCs were maintained at 37°C in a humidified 5% CO₂ incubator.

Cell growth assay

For cell growth assay, ASCs were seeded in 12-well plates, with 5×10³ cells/well, treated with udenafil (0.1, 0.5 or 1 µM) and incubated for 7 days. ASCs were also seeded in 12-well plates, with 1×10⁴ cells/well, treated with synthetic peptides, IL-4 or IL12B (100 ng/mL) and incubated for 7 days. The cells were then trypsinized, stained with trypan blue (Sigma-Aldrich, MO, USA) and counted each day using a hemocytometer.

Scratch wound-healing assay

ASCs were seeded into 6-well plates and cultured to confluence. A sterile 1 mL pipette tip was used to scratch the cell monolayer. The cultures were then washed with PBS to remove de-plated cells, and the cells were again cultured with udenafil, IL-4, or IL12B in a serum-free medium for 3 days. Cell migration into the scratched area (wound closure) was visualized using a ZEISS Observer.D1 microscope. Multiple images were acquired per well, and the average cell number within the wound were monitored over 3 days.

Transwell migration assay

ASCs were seeded into 60 mm plates and treated with udenafil, IL-4, or IL12B for 3 days. ASCs starved for 1 day (1.5×10⁴ cells/well) were suspended in a serum-free medium and seeded on the upper side of transwell membrane insert (BD Falcon, CA, USA), which was pre-coated with matrigel (1/60 dilution, BD Matrigel matrix, CA, USA). The normal serum with FBS was added in lower plate as chemoattractant. The cultures were incubated for 1 day to allow transwell migration. The inserts were then removed, and their upper surface was cleaned using cotton swabs and washed with PBS to remove non-migrating cells. The inserts were stained with 0.1% formalin/10% crystal-violet solution (Sigma-Aldrich) for 20 min, and cell number was analyzed under a ZEISS Observer.D1 microscope. Multiple images (15-20) were acquired per insert, and the average cell counts were calculated.

Tube formation assay using matrigel

For this experiment, 12-well plates were coated with matrigel (1/2 dilution, BD Matrigel matrix) and dried for 2 h at 37°C. ASCs treated with endothelial cell basal medium-2 (EBM-2, LONZA, MD, USA) and udenafil were plated in matrigel-coated wells and incubated for 16 h at 37°C. The number of tubes was analyzed under a ZEISS Observer.D1 microscope.

RNA extraction, cDNA synthesis, QPCR and QPCR array

Total RNA was extracted from ASCs using Trizol reagent (Invitrogen, NY, USA) and was subjected to complementary DNA (cDNA) synthesis using oligodT and the HelixCRIPT™ Thermo Reverse Transcription System (NANOHELIX, WI, USA) according to the manufacturer's instructions. Bright-Green QPCR master mix-ROX (abm, NY, USA) was used for QPCR reactions. For the QPCR array, total RNA was extracted from ASCs or 1 µM udenafil-treated ASCs and subjected to cDNA synthesis, as described above. QPCR array for growth

factors was conducted using an RT² First Strand cDNA Synthesis Kit (QIAGEN, MD, USA).

Western blot

For western blotting of phospho-ERK, ASCs were treated with udenafil for 15 or 30 min. Next, the cells were treated with U0126 (10 μM, CALBIOCHEM, CA, USA) for 1 h and lysed with protein extraction solution (PRO-PREP™; iNtRON, Seoul, Korea) containing a phosphatase inhibitor (Na₃VO₄; Roche, CA, USA). Western blot analysis was performed as follows. Briefly, protein extracts were loaded on the acrylamide gel, blotted on the NC membrane, incubated with the following primary antibodies: mouse anti-phospho-p42/44 (1:1500; Cell Signaling Technology, MA, USA), and mouse anti-α-tubulin (1:2000; Santa Cruz Biotechnology, TX, USA) overnight at 4°C. Next, the membrane was incubated with HRP-tagged secondary antibodies (Jackson ImmunoResearch, PA, USA) for 1 h and blot images were obtained using ImageQuant LAS 4000 (GE Healthcare Life Science, PA, USA). For western blot of phospho-NFκB, siRNA for negative control or NFκB were treated for 2-3 days, then udenafil was treated for 15 or 30 min treated and were lysed with protein extraction solution. Western blot analysis was performed using the following primary

antibodies, rabbit anti-NFκB (1:1000; Santa Cruz Biotechnology) and blot images were obtained using ImageQuant LAS 4000.

Animal experiment

Mice were maintained and anesthetized according to a protocol approved by the US Pharmacopoeia and the Institutional Animal Care and Use Committee of Yonsei University (IACUC-A-201802-183-01). The dorsal area of 6.5-week-old C3H/HeN mice in the telogen stage of the hair cycle was shaved with an electric shaver. Naïve ASCs or 1 μM udenafil-treated ASCs (3×10⁴ cells/mice) were injected once into the dorsal skin of the shaved mice for 14 days. IL-4 or IL12B protein (1 μg/mL/day; PeproTech, NJ, USA) was applied on the dorsal skin of the shaved mice at 6-week-old every day for 16-17 days. The skin darkening (indicative of anagen cycle induction) was carefully monitored by photography. After that, the dorsal hair was shaved and weighted in order to estimate the growth rate (Buhl *et al.*, 1989)

Vibrissae follicle organ culture

For organ culture of vibrissae hair follicles, we cut the vibrissae hair follicles from 4 weeks c57b/6 mice, washed them

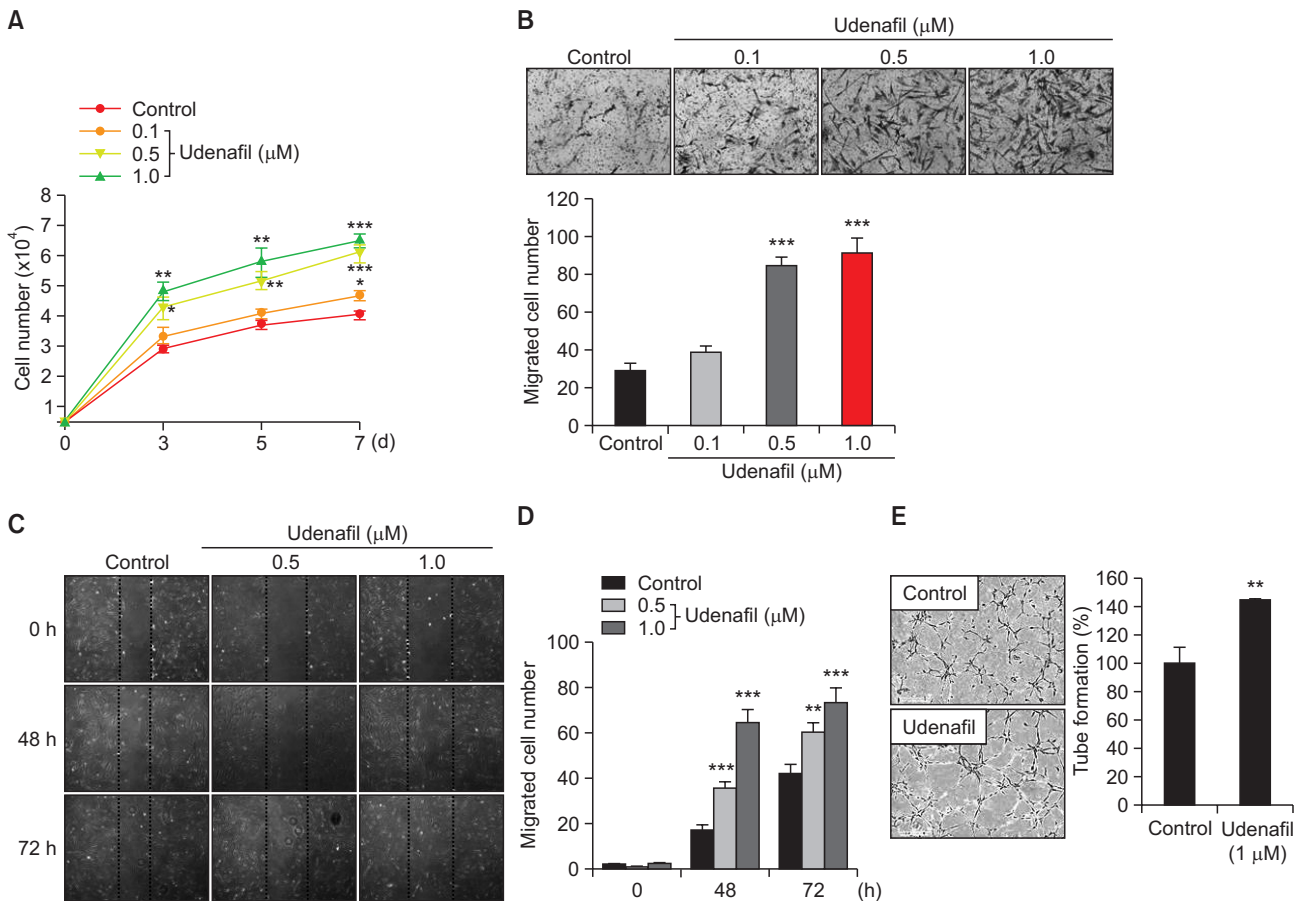


Fig. 1. Udenafil promotes ASC proliferation and migration. (A) Cell growth was measured after udenafil treatment in ASCs for 7 days. (B-D) Cell migration was measured after udenafil treatment using transwell migration assay (B) and scratch wound-healing assay (C, D). (E) Tube formation assay was carried out to show the enhanced tube formation by udenafil-treated ASCs. **p*<0.05; ***p*<0.01; ****p*<0.001. Three independent experiments were conducted per all data point. All error bars indicate SEM.

with PBS and cultured them in a defined medium (williams E medium supplemented with 2 mM L-glutamine, 10 μ g/mL insulin, 10 ng/mL hydrocortisone, 100 U/mL penicillin and 100 μ g/mL streptomycin, without a serum) including IL-4 or IL12B (5 and 20 ng/mL) for 3 days at 37°C in a humidified 5% CO₂ incubator. Images were obtained using Nikon SMZ800N microscope (Nikon, Tokyo, Japan) at 0 and 3 days, and analyzed using Adobe Photpshop CS6 extended program (Adobe, CA, USA).

HE and immunofluorescence staining

HE staining, paraffin sections were de-waxed using xylene for 30 min, hydrated in 100%, 90%, 80% and 70% ethyl alcohol (EtOH), dipped into Mayer's hematoxylin (Sigma-Aldrich) for 8 min, and then rinsed in water for 10 min. The slides were

again dipped into eosin Y (Sigma-Aldrich) for 80 s, dehydrated with 70%, 80%, 90% and 100% EtOH, washed with fresh xylene for 30 min and dried and mounted with a mounting medium. Immunofluorescence staining was performed using standard protocols: Briefly, paraffin sections were de-waxed using xylene for 30 min. and hydrated in 100%, 90%, 80%, and 70% EtOH, and antigen retrieval was performed by boiling the sections in an antigen retrieval buffer using a microwave (Dako, CA, USA) for 2 min 20 sec. The sections were then treated with rabbit Ki67 antibody (1:300) (Abcam, Cambridge, UK) overnight at 4°C and then incubated with secondary antibodies, Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen), for 1 h at room temperature with 4,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich). Immunofluorescence staining was performed and images obtained using a ZEISS LSM700 confocal micro-

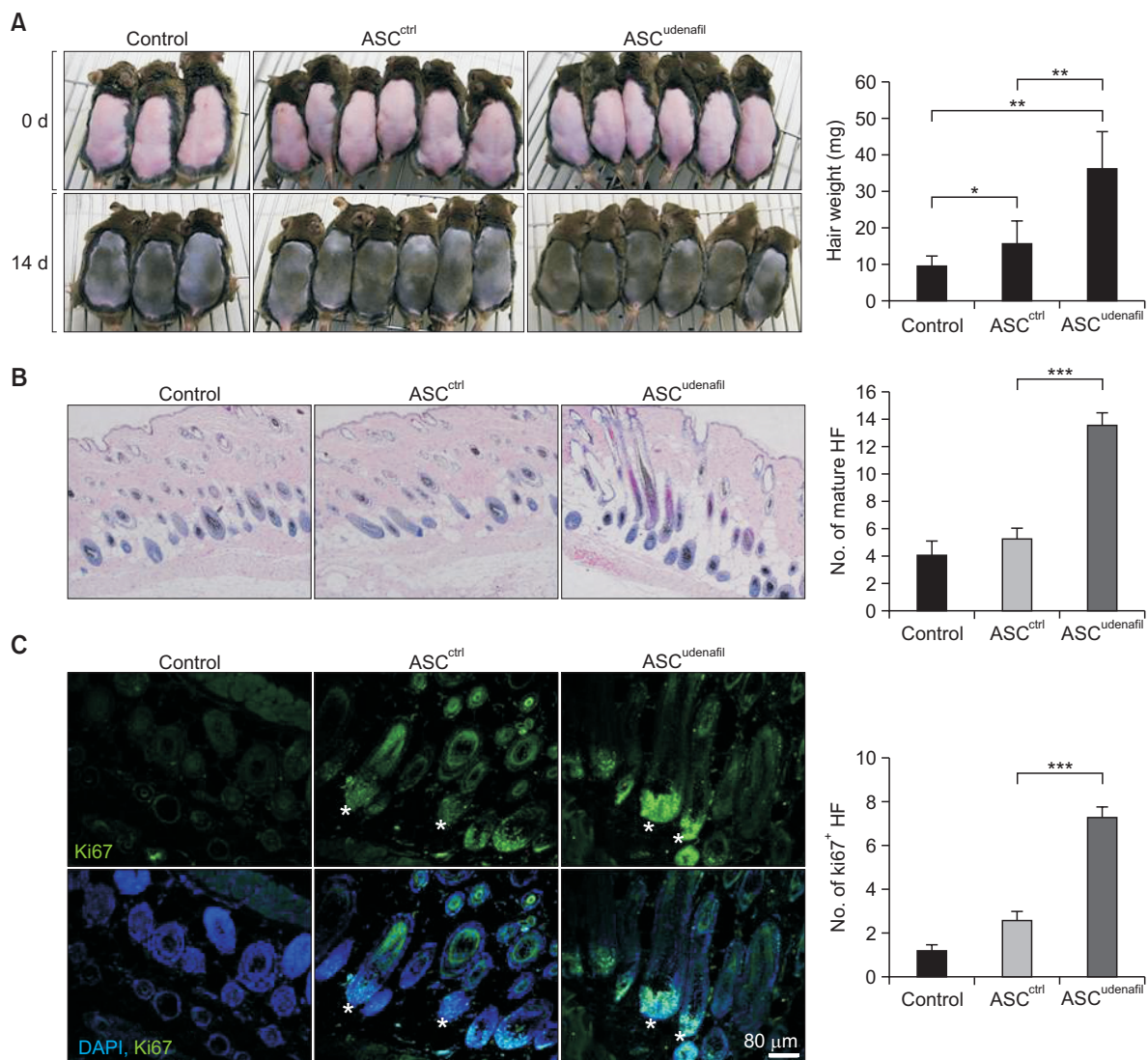


Fig. 2. Udenafil-pretreated ASCs promote hair growth *in vivo*. Udenafil-treated ASCs or untreated ASCs were injected into the dorsal skin of shaved mice. Photographs were taken and the hair weight measured (A) 14 days later. (B) A skin section was analyzed by HE staining, and the number of mature hair follicle was measured. (C) A hair follicle with Ki67⁺ cells in cortex region is shown by immunostaining. Asterisks indicate hair follicles with Ki67⁺ cortex cells. * p <0.05; ** p <0.01; *** p <0.001. N=6 or 7 mice per group. All error bars indicate standard error of the mean (SEM).

scope (Ca Zeiss, Oberkochen, Germany).

Statistical analysis

All experiments were performed more than three times using independent cultures. Data were presented as mean ± standard error of the mean. The means were compared by Student's *t*-test. For all statistical tests, a 0.05 level of confidence was accepted as being statistically significant.

RESULTS

Udenafil-pretreated ASCs induce cell proliferation and migration

Although ASCs secrete various growth factors that promotes hair growth (Won *et al.*, 2010; Festa *et al.*, 2011; Jeong *et al.*, 2013b; Kim *et al.*, 2014a, 2015; Jin and Sung, 2016), it is still unmet that promotion of hair growth using precondition-

ing of ASCs with trichogen. First, we investigated the effect of udenafil on growth and migration of human ASCs. We counted of the live cell number for 7 days after udenafil treatment and found that udenafil increases ASC growth in a time- and dose-dependent manner (Fig. 1A). To investigate the effect of udenafil on ASC migration, we conducted transwell migration assay using insert and scratch wound-healing assay. The results showed that udenafil increases ASC migration into both assays in a dose-dependent manner (Fig. 1B-1D). Initially, it was believed that application of preconditioned udenafil in ASCs stimulates vasodilatation and increases blood flow by inhibiting the cGMP degradation (Gopal *et al.*, 2001). Therefore, we examined whether udenafil affects blood vessel formation in ASCs. The result showed that udenafil increases the number of nascent tubes after 16 h (Fig. 1E), indicating an increase in the ASC tube-forming activity. Collectively, these results suggested that udenafil may promote hair growth by enhancing ASC proliferation and migration. Considering the previous re-

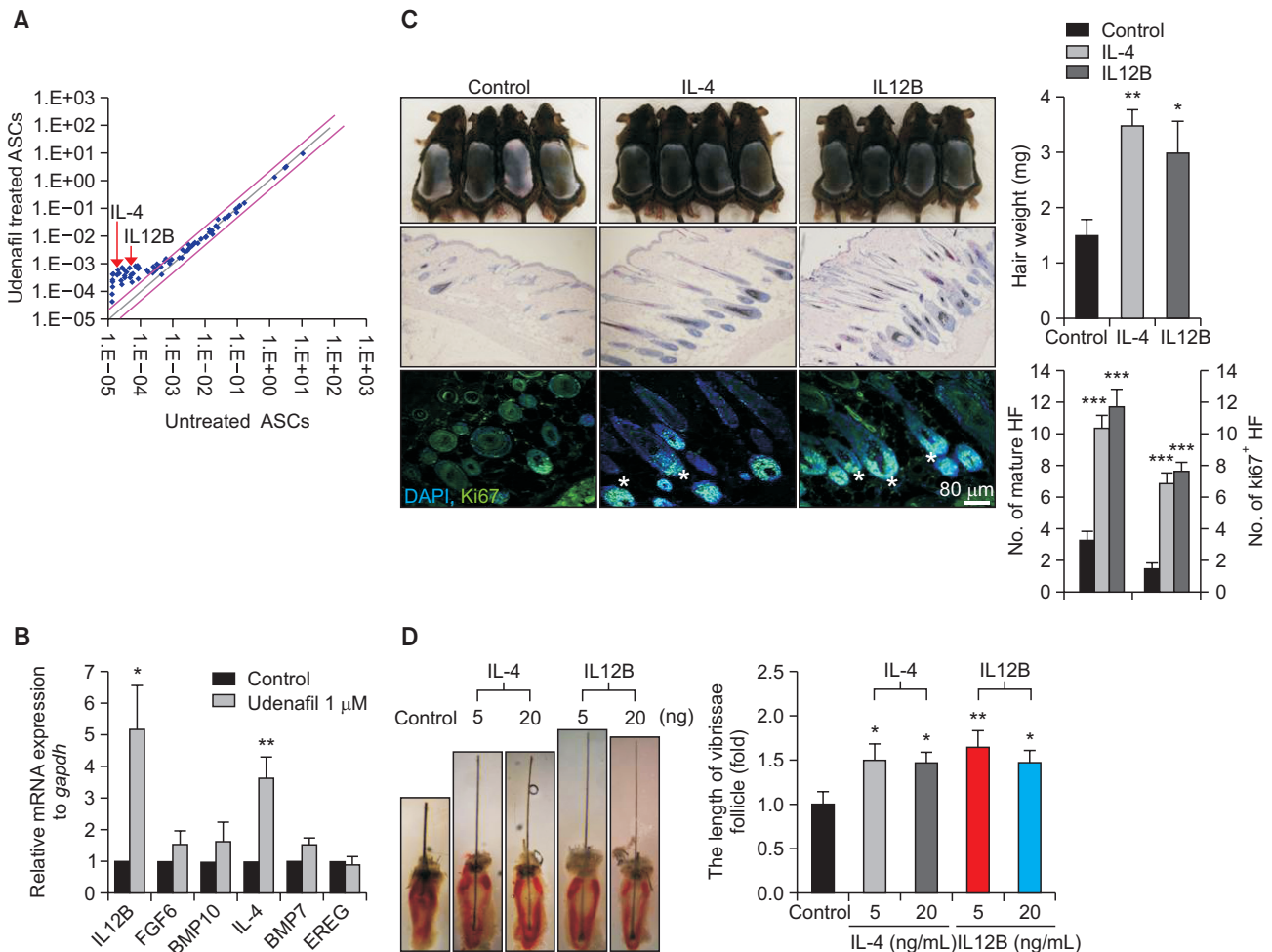


Fig. 3. Udenafil upregulates the expression of hair growth-promoting factors IL-4 and IL12B in ASCs. (A) Upregulation of many growth factors in udenafil-treated ASCs compared to untreated ASCs was analyzed by QPCR array for growth factors. Pink straight lines indicate ± 2 fold change, and arrows indicate the two genes, IL-4 and IL12B, analyzed in (B). (B) Confirmation of six upregulated genes, IL12B, FGF6, BMP10, IL-4, BMP7 and EREG, between untreated and udenafil-treated ASCs using QPCR. (C) Human IL-4 and IL12B proteins each facilitate hair growth and increase the number of mature proliferating hair follicle after application on the dorsal skin of shaved mice. N=7-8 mice per group. (D) IL-4 and IL12B proteins each facilitate mouse vibrissal hair follicle growth. N=10 samples per treatment group. **p*<0.05; ***p*<0.01; ****p*<0.001. All error bars indicate SEM.

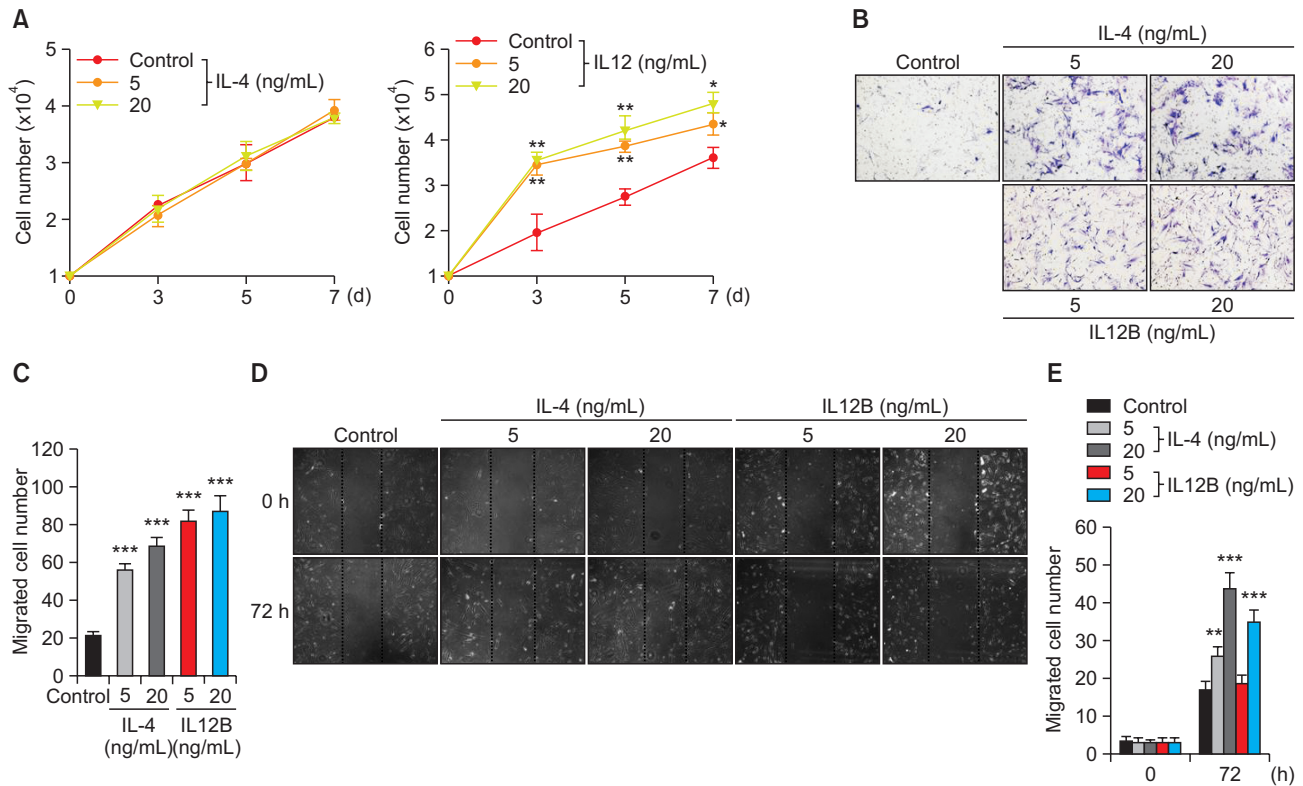


Fig. 4. IL12B induce ASC proliferation and migration, but IL-4 induces migration (A) Cell growth was measured after treatment by IL-4 or IL12B in ASCs for 7 days. Three independent experiments were conducted. (B-E) IL-4 or IL12B enhances ASC migration, as evidenced by both (B, C) transwell migration and (D, E) scratch wound-healing assays. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Three independent experiments were conducted per data point. All error bars indicate SEM.

ports that sildenafil enhances melanoma growth by promoting MAPK signaling (Dhayade *et al.*, 2016), we examined the effect of sildenafil which is one of the other PDE₅ inhibitors on ASCs growth and migration. The various concentration of sildenafil couldn't effect on ASCs growth and migration (Supplementary Fig. 1), suggesting that sildenafil might not be stimulator of hair growth due to no effect on ASCs growth and migration.

Udenafil-pretreated ASCs promote hair growth *in vivo*

When we investigated the hair growth effect of udenafil-pretreated ASCs *in vivo*, we found that subcutaneous injection of untreated ASCs (ASC^{Ctrl}) slightly increased the rate of hair growth in mice, while udenafil-pretreated ASCs (ASC^{udenafil}) induce marked the rate of hair growth (Fig. 2A). To examine the effect of udenafil-pretreated ASCs on hair follicles, we performed hematoxylin and eosin (HE) staining and immunofluorescence staining for Ki67, a proliferating cortex cell marker. The skin section of ASC^{udenafil}-treated mice showed a higher number of mature hair follicles compared to vehicle- or ASC^{Ctrl}-treated mice (Fig. 2B). In addition, most hair follicles of ASC^{udenafil}-treated mice showed cortex region with Ki67⁺ cells compared to vehicle- or ASC^{Ctrl}-treated mice (Fig. 2C). These results indicated that most hair follicle cycling of ASC^{udenafil}-treated mice is in anagen phase compared to vehicle- or ASC^{Ctrl}-treated mice. All results suggested that udenafil-preconditioned ASCs can promote telogen-to-anagen induction, thereby promoting hair growth.

IL-4 and IL12B induce hair growth

It has been reported previously that growth factors secreted by ASCs, such as VEGF, b-FGF, PDGF-A, PDGF-D chemokine (C-X-C motif) ligand 1 (CXCL1), PD-ECGF, and PDGF-C, regulate hair follicular stem cell activity and induce the anagen phase of the hair cycle *in vivo* (Won *et al.*, 2010; Festa *et al.*, 2011; Kim *et al.*, 2015; Choi *et al.*, 2018). In addition, preconditioning of PDE₅ inhibitors with ASCs improves the efficacy of ASCs in myocardial infarction, releasing VEGF, b-FGF, and IFG (Hoke *et al.*, 2012). We hypothesized that udenafil may indirectly promote hair growth by enhancing growth factor release from ASCs. Therefore, we compared the expression pattern of cell growth-related genes between untreated and udenafil-treated ASCs by qPCR array (Fig. 3A). After QPCR array analysis, we found that the expression of many growth factor genes were upregulated in udenafil treated ASCs compared to control (Supplementary Fig. 2). Among top-notch genes, we selected 6 genes and confirmed their expression by QPCR. We finely selected interleukin 4 (IL-4) and subunit beta of interleukin (IL12B) as potential target genes of udenafil because their consistent upregulation was confirmed by QPCR (Fig. 3B). To investigate whether IL-4 and IL12B can induce the telogen-to-anagen phase of the hair cycle faster than the control *in vivo*, we applied human IL-4 or IL12B protein on the back skin of shaved mice. We observed that the application of both IL-4 and IL12B proteins significantly induced the telogen-to-anagen phase of the hair cycle and increased the number of mature hair follicles and Ki67⁺ hair follicles (Fig.

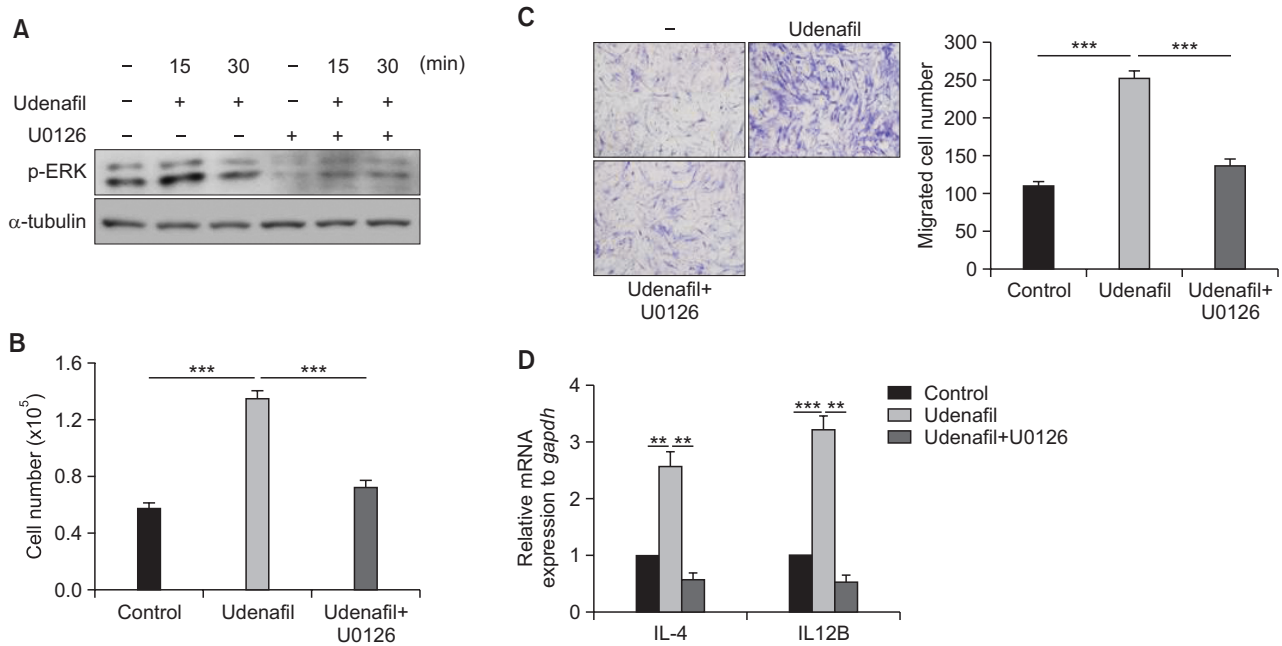


Fig. 5. Udenafil upregulates IL-4 and IL12B expression through the ERK pathway in ASCs. (A) The MEK inhibitor U0126 reversed udenafil-induced ERK phosphorylation. (B, C) U0126 suppressed udenafil-increased growth and migration of ASCs. (D) U0126 also suppressed udenafil-induced upregulation of IL-4 and IL12B expression in ASCs. Three independent experiments were carried out per every experiment. ***p*<0.01; ****p*<0.001. All error bars indicate SEM.

3C), suggesting that udenafil may induce anagen by triggering IL-4 and IL12B release from ASCs. Moreover, treatment with IL-4 and IL12B also increased the length of isolated mouse vibrissal hair follicles in organ culture (Fig. 3D). These results strongly suggested that udenafil promotes hair growth through the release of cytokines such as IL-4 and IL12B from ASCs.

IL12B induce ASC proliferation and migration, but IL-4 induces migration

To examine whether IL-4 and IL12B affect ASC proliferation, we followed the live cell number for 7 days after IL-4 or IL12B treatment. We found that IL12B, not IL-4, increased ASC proliferation in a time- and dose-dependent manner (Fig. 4A). Furthermore, to explore whether IL-4 and IL12B affect ASC migration, we conducted transwell migration and scratch wound-healing assays. Both assays showed that IL-4 and IL12B increased ASC migration dose-dependently (Fig. 4B-4E). Collectively, these results suggested that IL-4 and IL12B may promote hair growth by enhancing ASC migration and partial proliferation. To clarify how increased IL-4 and IL12B in udenafil-treated ASCs is functioned in hair-compositing cells such as dermal papilla cells (DP cells), we examined the effect of IL-4 and IL12B on DP cells. Indeed, the treatment of each IL-4 or IL12B protein increased growth of DP cells (Supplementary Fig. 3). This result suggested that released IL-4 or IL12B from ASCs increased growth of DP cells thereby stimulating hair growth.

Udenafil regulates IL4 and IL12B expression in ASCs via the ERK pathway

Previous studies have reported that the MAPK pathway regulates growth factor expression in ASCs, including VEGF-A and FGF2 (Kim *et al.*, 2014a, 2014b). Therefore, in this study,

we examined whether udenafil regulates IL-4 or IL12B expression via the MAPK pathway. We found that udenafil upregulated ERK phosphorylation time-dependently, a response suppressed by U0126, a specific MEK inhibitor (Fig. 5A). U0126 suppressed udenafil-increased proliferation and migration of ASCs (Fig. 5B, 5C) and reversed udenafil-induced IL-4 and IL12B upregulation (Fig. 5D). These results suggested that udenafil upregulates IL-4 and IL12B expression in ASCs via the MAPK pathway.

Udenafil regulates IL4, not IL12B, expression in ASCs via the NF-κB pathway

Previous studies have reported that nuclear factor kappa B (NFκB) inhibition suppresses both IL-4 and IFN-dependent increase in polymeric immunoglobulin receptor (pIgR) expression in human intestinal epithelial cells (HT29), suggesting immunity regulation (Ackermann and Denning, 2004). Therefore, in this study, we examined whether udenafil regulates the IL-4 or IL12B expression via the NFκB pathway. We found that udenafil upregulated NFκB phosphorylation at 15 min post-treatment, and this response was suppressed by NFκB knockdown, which was confirmed by messenger RNA (mRNA) and protein down-regulation (Fig. 6A, 6B). Furthermore, NFκB knockdown recovered udenafil-increased proliferation and migration in ASCs (Fig. 6C, 6D). Finally, NFκB knockdown also reversed udenafil-induced upregulation of IL-4 but not IL12B (Fig. 6E). These results suggested that udenafil upregulates IL-4, not IL12B, expression in ASCs via the NFκB pathway.

DISCUSSION

Udenafil, an inhibitor of the cGMP-degrading phosphodies-

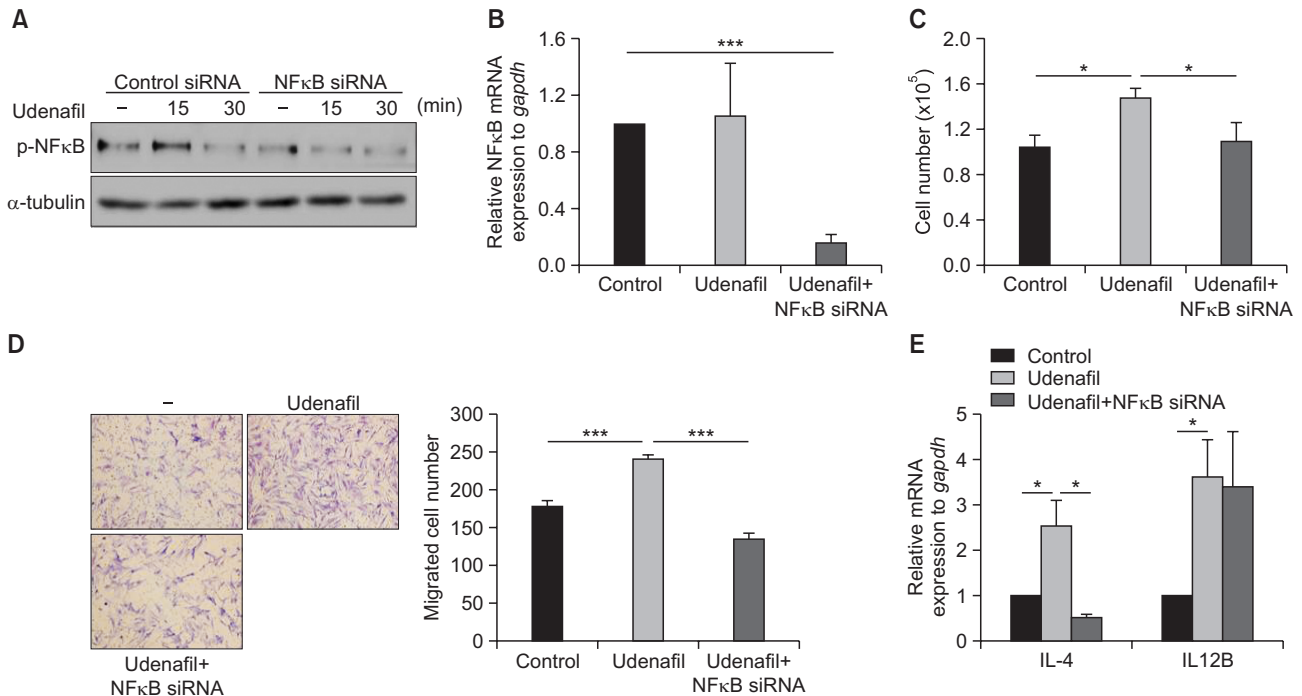


Fig. 6. Udenafil upregulates IL-4, not IL12B, expression in ASCs via the NFκB pathway. (A) NFκB knockdown reversed udenafil-induced NFκB phosphorylation. (B) NFκB down-regulation was confirmed by QPCR. (C, D) NFκB knockdown suppressed udenafil-increased growth and migration of ASCs. (E) NFκB knockdown also suppressed udenafil-induced upregulation of IL-4, not IL12B, expression in ASCs. Three independent experiments were carried out per every experiment. * $p < 0.05$; *** $p < 0.001$. All error bars indicate SEM.

terase 5, is used to treat erectile dysfunction. However, it was not understood the link between udenafil and hair promoting effects through ASC stimulation. Therefore, in this study, we investigated whether udenafil stimulates ASCs and enhances hair growth through growth factor release. First, we demonstrated that subcutaneous injection of udenafil-preconditioned ASCs accelerates telogen-to-anagen transition *in vivo*, increasing the number of proliferating hair follicles as well as hair weight. Second, udenafil increases ASC proliferation, migration, tube formation and secretion of cytokines such as IL-4 and IL12B via ERK or NFκB. Third, application of these cytokines enhances anagen induction in mice and also increases the growth of isolated mouse vibrissal hair follicles in organ culture.

IL-4 is a cytokine that induces activated B-cell and T-cell proliferation and naïve helper T-cell differentiation, indicating it is a key regulator in humoral and adaptive immunity (Bao and Reinhardt, 2015). Although the role of IL-4 in hair growth or alopecia areata (AA) has not been reported yet, there is evidence of the relationship between AA and cytokines (Hoffmann, 1999; Gregoriou *et al.*, 2010). AA is a highly prevalent, chronic, and relapsing autoimmune disease that targets hair follicles. IL18 is a significant pro-inflammatory cytokine that is present in high levels in AA patients (Celik and Ates, 2018). Recent data have suggested the possible role of IL17 in AA pathogenesis (Ramot *et al.*, 2018). In addition, IL1 is a potent inducer of hair loss and a significant inhibitor of human hair growth *in vitro* (Hoffmann and Happel, 1995). Also, Groves *et al.* (1994) have shown that transgenic mice over-expressing IL1a in the epidermis have patchy hair loss resembling AA. IL-4-mediated apoptosis may participate in catagen formation

regulation in hair follicles, acting selectively on cultured keratinocyte and being independent of bcl-2 and bax expression (Mandt *et al.*, 2002). There is no evidence of the relationship of another cytokine, IL12B, which acts on T- and natural killer (NK) cells, with hair growth. Studies have only reported that cytokines such as IL12B, IL10, IL36RN, IL6, IL2, IL23, IL2RA, and IL4R are related to autoimmune diseases (Redler *et al.*, 2012).

Although ASC transplantation promotes hair growth *in vivo* experimentally and ASC-CM induces the proliferation of hair-compositing cells *in vitro*, ASCs and ASC-CM have shown limited effectiveness on hair growth. In addition, our many animal experiments revealed that only ASC or ASC-CM injection is not enough to promote hair growth significantly. ASC preconditioning is one strategy that can be used to enhance ASC efficacy. Our group has already shown preconditioning methods of enhancing hair growth using ASC stimulators, such as vitamin C, PDGF, hypoxia, UVB, and minoxidil (Kim *et al.*, 2011; Jeong *et al.*, 2013b; Kim *et al.*, 2014a, 2015). Especially, preconditioned ASCs with minoxidil, which is widely used for AA treatment, induce hair follicle cycling and hair growth-releasing growth factors, including PDGF-C (Choi *et al.*, 2018). In addition to hair loss treatment, minoxidil is also used as a vasodilator. Studies have reported that minoxidil induces the tube-forming activity of ASCs and expression of angiogenic-related genes (Choi *et al.*, 2018). Udenafil is also used as a vasodilator and also increases the tube-forming activity, proliferation and migration of ASCs (Fig. 2A) suggesting that udenafil may be used as a stimulator of ASCs to enhance hair growth. Although the role of other PDE₅ inhibitors, such as avanafil, sildenafil, tadalafil, and vardenafil, in hair growth has

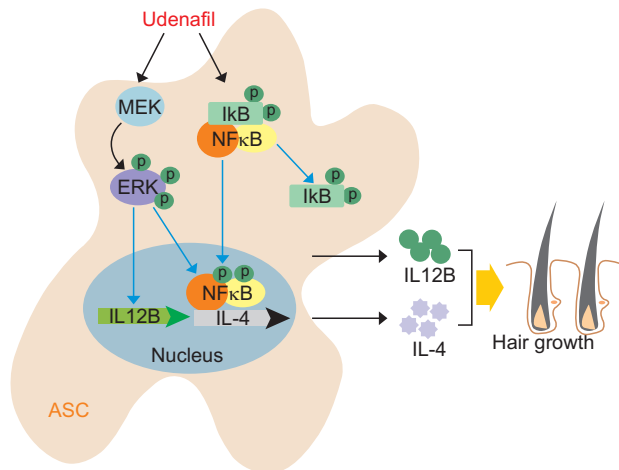


Fig. 7. Summary of hair promoting effect by udenafil. Udenafil upregulates the expression of IL-4 and IL12B via ERK or NFκB pathways, and releases two cytokines from ASC to promote hair growth.

not been investigated, it would be meaningful to investigate this function of theirs. Indeed, the use of a PDF₅ inhibitor such as sildenafil in preconditioned ASCs improved myocardial infarction by releasing growth factors (VEGF, b-FGF, and IGF) (Hoke *et al.*, 2012). However, as we examined the effect of sildenafil on ASCs growth and migration, various concentration of sildenafil couldn't effect on ASCs growth and migration (Supplementary Fig. 1). Although preconditioned ASCs with sildenafil can release some growth factors, it didn't stimulate ASCs. This means that sildenafil might not be stimulator of hair growth using ASCs.

We also investigated the increased cellular phenotype and upregulation of IL4 and IL12B by udenafil via the MEK-ERK and NFκB pathways. However, udenafil acts by inhibiting the degradation of cGMP to guanosine monophosphate (GMP), which relaxes the smooth muscle and allows increased blood flow for penile tumescence (Gopal *et al.*, 2001). Smooth muscle relaxation is, in part, mediated via protein kinase G (PKG) activation, subsequent potassium channel opening and reduction in intracellular calcium levels. Although we did not investigate the relationship between the cGMP-PKG pathway and hair growth in this study, it will be done in another project.

In summary, subcutaneous injection of udenafil-preconditioned ASCs accelerates telogen-to-anagen transition in mice, and direct udenafil treatment increases tube formation, proliferation, migration, and cytokine secretion by ASCs. The most strongly upregulated cytokines, IL-4 and IL12B, individually enhance anagen induction in mice and also increase the growth of isolated mouse vibrissal hair follicles in organ culture. In addition, upregulation of IL-4 by udenafil are mediated via both ERK and NFκB pathways, and upregulation of IL12B is mediated by ERK pathway. Therefore, udenafil can be used as a novel ASC-preconditioning agent for hair regeneration (Fig. 7).

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare and

have not received any payment for the preparation of this manuscript.

ACKNOWLEDGMENTS

This study was supported by a grant from the National Research Foundation (NRF-2016R1D1A1B03932050). NC was also supported by the National Research Foundation (NRF-2017R1A6A3A11035599) funded by the Korean government.

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