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Novel effect of sildenafil on hair growth

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ABSTRACT

Background: Sildenafil, a phosphodiesterase 5 (PDE5) inhibitor, is known to increase the intracellular level of cyclic guanosine monophosphate (cGMP), which causes vasodilation. However, the effect of sildenafil on human hair follicles (hHFs) is unknown.
Objective: The purpose of this study was to determine the role of sildenafil in hair growth.
Methods: We investigated the expression of PDE5 in human dermal papilla cells (hDPCs) and hHFs. The effects of sildenafil on hDPC proliferation were evaluated using BrdU assays. The mRNA expression of growth factors and extracellular signal-regulated kinase (ERK) phosphorylation were investigated using real-time PCR and western blotting, respectively. Additionally, anagen induction and perifollicular vessel formation were evaluated using an *in vivo* mice model.
Results: We confirmed high expression of PDE5 in hDPCs and hHFs. Sildenafil enhances proliferation of hDPCs and up-regulates the mRNA expression of vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF), which are responsible for hair growth. Additionally, sildenafil up-regulates the levels of phosphorylated ERK and accelerates anagen induction by stimulating perifollicular vessel formation after topical application in mice.
Conclusion: Our study demonstrates for the first time, the significant therapeutic potential of sildenafil on hair growth and its potential use in treatment of alopecia.

1. Introduction

Hair follicle formation and hair growth are regulated by mesenchymal-epithelial interactions between dermal papilla (DP) and dermal sheath cells derived from the mesenchyme and the inner and outer root sheaths, matrix, and hair shaft cells derived from the epithelium [1]. The DP transports nutrients and growth factors to enhance new hair growth, is necessary for induction and maintenance of follicular development, and mediates the hair cycle [2].

Phosphodiesterases (PDEs) are enzymes that are responsible for breakdown of cyclic nucleotides including cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) [3]. PDE5 is highly specific for cGMP hydrolysis in various types of tissues or cells [4].

Sildenafil is a selective inhibitor of PDE5 and was originally developed as an anti-anginal drug due to its vasodilatory and anti-platelet coagulation properties [5]. It has been approved for the treatment of erectile dysfunction and works by increasing penile blood flow by relaxing smooth muscles in the corpus cavernosum [6,7]. Sildenafil has resulted in an increased interest in the therapeutic potential of PDE5 inhibitors for other cardiovascular indications and multiple clinical trials have demonstrated its benefit in treatment of pulmonary arterial hypertension [8,9]. Sildenafil works by dilating the blood vessels in the lungs, reducing pulmonary blood pressure and improving cardiac function. Additionally, sildenafil promotes angiogenesis and adipogenesis, which is accompanied by increased glucose uptake through the PKG pathway [10]. However, there is only one open-pilot comparison study on the effects of sildenafil on hair loss [11].

Stimulating blood flow in the human bald scalp promotes microcirculation in the surrounding HFs and can lead to promotion

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of hair growth and hypertrichosis [12–14]. Our previous study showed that cilostazol, a PDE3 inhibitor, promotes hair growth by stimulating hDPC proliferation, enhancing hair shaft elongation and acceleration of anagen induction in C57BL/6 mice [15].

On the basis of previous studies, it is hypothesized that sildenafil may promote hair growth because of its vasodilatory properties. However, there are no reported studies on the potential effects of sildenafil on hHFs. Therefore, in this study, we investigated the effect of sildenafil on hair growth *in vivo*, confirmed using the anagen induction assay in C57BL/6 mice as well as in cultured hDPCs.

2. Materials and methods

2.1. Ethics statement

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

2.2. Isolation and culture of hDPCs

Scalp tissue samples (1.5 × 1.0 cm) from the occipital region were taken from healthy male volunteers without current or prior scalp diseases. hDPCs were isolated from the bulbs of dissected HFs and cultured as described previously [16]. Briefly, candlelight-shaped DPs were dissociated and incubated in Dulbecco's modified Eagle medium (DMEM; Welgene, Daegu, Republic of Korea) supplemented with 10% fetal bovine serum (FBS, Welgene) and 1× antibiotic/antimycotic solution (Gibco BRL, Gaithersburg, MD, USA) containing penicillin and streptomycin at 37 °C in a 5% CO_2 atmosphere.

2.3. Conventional RT-PCR and quantitative real-time PCR

Total RNA was isolated from hDPCs using RNAiso Plus reagent (Takara Bio, Shiga, Japan). Approximately 2 μg of total RNA was used for cDNA synthesis reaction using First Strand cDNA Synthesis Kit (Fermentas, Sankt Leon-Rot, Germany) according to the manufacturer's instructions. Subsequently, 50–100 ng of the cDNA was used in PCR amplification. For the expression analysis of PDE5A genes, forward, 5′-AGAGGCCACACCGAATCTTG-3′ and reverse 5′-AACAGGGAATAGCGGTCAGC-3′ primer were used. To normalize for differences in cDNA loading, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5′-ATTGTTGCCATCAATGACCC-3′ and reverse 5′- AGTAGAGGCAGGGATGATGT-3′ primer were used. The conditions for PCR were 3 min at 94 °C, 30 s at 94 °C, 30 s at 60 °C, 45 s at 72 °C, and 1 min at 72 °C for 35 cycles. For quantitative expression level of VEGF and PDGF-A, Real-time PCR was performed on a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using SYBR Premix Ex Taq (Takara Bio Inc.) according to the manufacturer's instructions, with the following primer pairs: 36B4 (forward, 5′-TGGGCTCCAAGCAGATGC-3′; reverse, 5′-GGCTTCGCTGGCTCCCAC-3′), VEGF (forward, 5′-ACTTCTGGGCTGTTCTCG-3′; reverse, 5′-TCCTCTTCCTTCTCTTCTTC-3′), PDGF-A (forward, 5′-GCCCATTCGGAGGAAGAG-3′; reverse, 5′-TTGGCCACCTTGACGCTGCG-3′). The PCR conditions were 50 °C for 2 min, 95 °C for 2 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. After being normalized versus 36B4, relative mRNA expression levels were determined.

2.4. Western blot analysis

hDPCs were lysed in radioimmunoprecipitation assay buffer (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Equal amounts of total proteins were separated by electrophoresis using 10% sodium dodecyl sulfate-polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane (Amersham, Buckinghamshire, UK) using semi-dry *Trans*-Blot® Turbo™ Transfer System (Bio-Rad, CA, USA). The blotted membranes were incubated with the respective primary antibodies at 4 °C. The following antibodies were used: anti-phospho extracellular signal-regulated kinase (ERK, #9101; Cell signaling Technology, Danvers, MA, USA) anti-total ERK (#9102; Cell signaling Technology) and anti-α tubulin (#SC-8035; Santa Cruz Biotechnology, CA, USA) antibodies. Membranes were probed with anti-rabbit, anti-mouse and anti-goat-IgG-horseradish peroxidase conjugates (GeneTex Inc., Irvine, CA, USA) for 1 h at 25 °C. Antibody-antigen complexes were detected using an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Little Chalfont, UK).

2.5. Immunofluorescence staining

Immunofluorescence staining of hDPC, hHF and mouse skin were previously described [15]. Cultured hDPCs were incubated with anti-PDE5A (#NBP1-86139; Novus Biologicals, CA, USA) and proliferating cell nuclear antigen (PCNA, #M0879; DAKO, Carpinteria, CA, USA) antibodies at 4 °C overnight and then with Alexa Fluor 594-labeled goat anti-rabbit IgG (Invitrogen, Kyoto, Japan). A 4′,6-diamidino-2-phenylindole (DAPI) mounting media kit was used to counterstain nuclei. Immunofluorescence staining for PDE5A and MECA32 (#553849; BD Pharmingen, San Diego, CA, USA) was performed on 5 -μm frozen sections of hHFs and 8-μm frozen sections of mouse skin. The following antibodies were used: anti-PDE5A (Novus Biologicals), anti-PCNA (Novus Biologicals) and anti-MECA32 (DAKO, Carpinteria, CA, USA) antibodies. Immunofluorescence images were acquired using a digital fluorescence microscope (Nikon, Tokyo, Japan). The number of MECA32 positive vessels per square millimeter and average vessel size were measured using NIS-Elements BR software.

2.6. Proliferation assays

Cell proliferation was measured by the bromodeoxyuridine (BrdU) assays. hDPCs (5×10^3 cells/well) were seeded into 96-well plates, serum-starved for 24 h, and then treated with sildenafil for 2 days. For the BrdU assay, Cell Proliferation ELISA (BrdU chemiluminescent) (Roche, Mannheim, Germany) was used according to the manufacturer's instructions. After the addition of BrdU labeling solution, the cells were incubated for 4 h at 37 °C in 5% CO_2. After the final washing step, 100 μl/well substrate solution was added to the cells, followed by analysis using a luminometer (Victor 3; PerkinElmer, Waltham, MA, USA).

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

We performed an anagen induction assay as previously described [17]. The back skin of 8-week-old C57BL/6 female mice in the telogen phase was shaved with a clipper. Vehicle (70% polyethylene glycol + 30% ethanol), sildenafil (0.1 and 1%), and minoxidil (2%) were then topically applied to the shaved skin every weekday for 3 weeks. At day 21, skin samples were obtained from the treated site for histological analysis by hematoxylin and eosin (H&E) staining. Skin thickness and anagen induction score were assessed using a slightly modified version of previously described

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methods [18–20]. Anagen induction scores were calculated using assigned score values (telogen = 1, anagen I-VI = 2–7) [18–20]. The resulting values were added and divided by the number of HFs (n = 70 HFs/group). In the histological analysis by H&E staining, skin thickness was measured as the distance from the epidermis to the subcutaneous fat using the Image J software (National Institutes of Health, Bethesda, MD, USA).

2.8. Statistical analysis

The statistical analyses were done by using the IBM SPSS Statistics 21.0 software package (IBM Co., Armonk, NY, USA). Statistical significance was determined by Student's t-test. The results are presented as means ± standard deviation (SD). *P*-values were two-tailed, and statistical significance was considered at $P < 0.05$.

3. Results

3.1. PDE5A expression in hDPCs and hHFs

The expression level of mRNA and protein in hDPC were measured using RT-PCR and western blotting, respectively. PDE5A was expressed in cultured hDPCs, which were isolated from four individuals (Fig. 1A), and the protein was expressed in cultured hDPCs (Fig. 1B). PDE5A is known to be localized in the cytoplasm. Therefore, we confirmed the expression and localization of PDE5A in hDPC using immuno-fluorescence staining and confirmed that it was predominantly expressed in the cytoplasm (Fig. 1C). We also confirmed the expression of PDE5A in hHF. PDE5A was highly expressed in the DP, the matrix keratinocyte and the lower part of the dermal sheaths of the HFs (Fig. 1D).

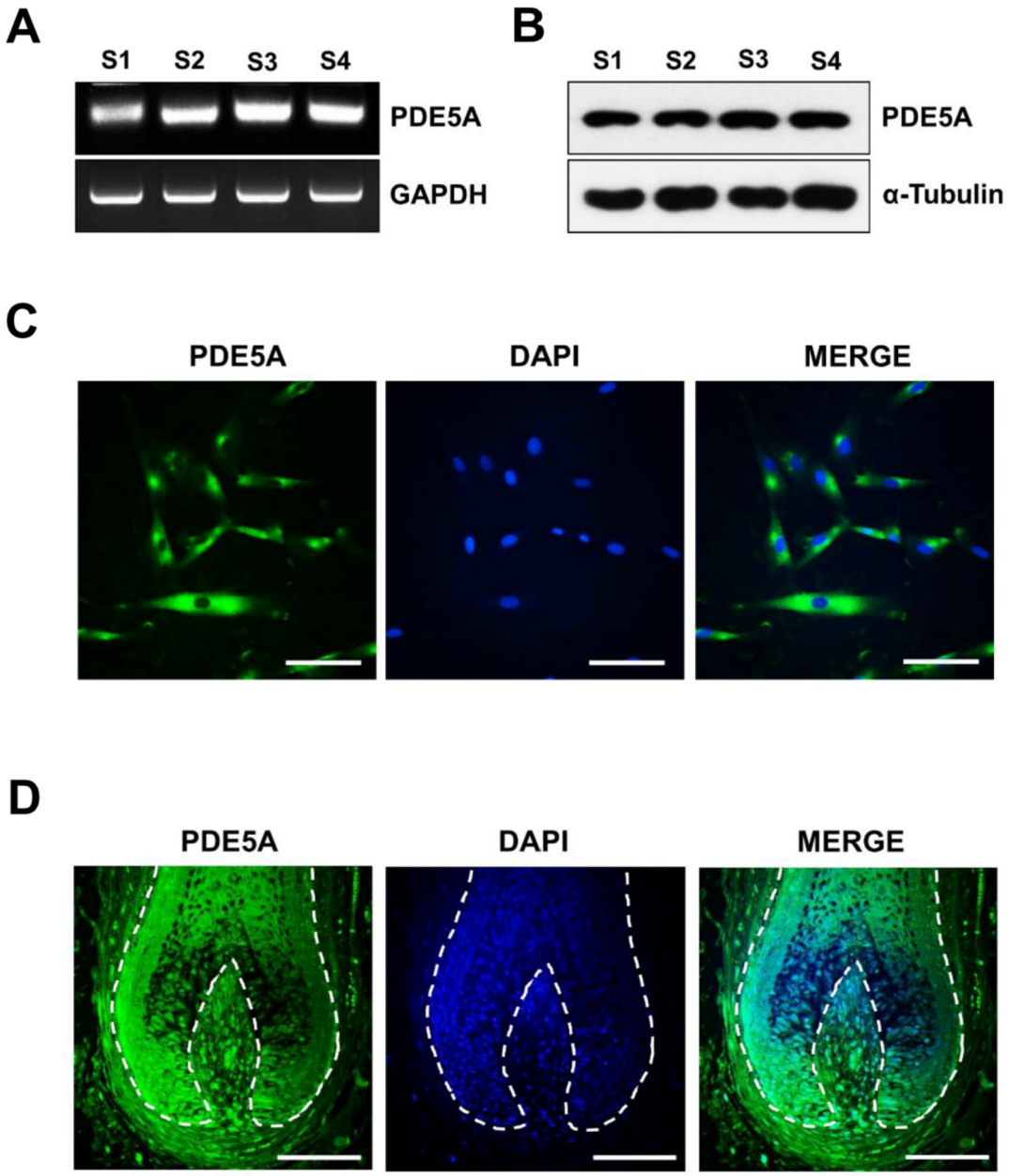

Fig. 1. Expression of PDE5A in hDPCs and hHFs. Expression levels of PDE5A mRNA (A) and protein (B) in hDPCs were determined using conventional RT-PCR and western blotting, respectively (S, subject; n = 4). GAPDH and α-Tubulin were determined as a loading controls. Immunofluorescent staining of PDE5A was performed in (C) cultured hDPCs and (D) hHFs. Nuclei were counterstained with DAPI. Scale bar = 100 μm.

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3.2. Sildenafil enhances the proliferation of hDPCs

To investigate the effect of sildenafil on hair growth, we treated hDPCs for 48 h with various concentrations of sildenafil (0.1–10 μM) and evaluated cell proliferation using the BrdU assay. As shown in Fig. 2A, cell proliferation was higher in hDPCs treated with sildenafil than in vehicle-treated control cells. We also analyzed the expression of PCNA, a marker of cellular proliferation using immunofluorescence staining after treating hDPCs with sildenafil for 48 h. Expression of PCNA in the nucleus was increased for the sildenafil-treated cells compared with control cells (Fig. 2B).

3.3. Sildenafil enhances expression of growth factors and activates ERK phosphorylation

hDPCs secrete diverse growth factors, which stimulate the growth of HFs. We evaluated the mRNA expression of VEGF, a key growth factor, in hair growth. The mRNA expression of VEGF exhibited a dose-dependent increase after treatment with sildenafil. In addition, the mRNA expression of PDGF, another potent inducer of hair growth, also significantly increased in hDPCs after treatment with sildenafil (Fig. 2C). It has been reported that the ERK pathway is regulated by sildenafil in endothelial cells. Therefore, we examined whether sildenafil regulates ERK activation in hDPCs. Sildenafil up-regulated the phosphorylation of ERK in a time-dependent manner. This response was suppressed by the specific ERK inhibitor U0126 (Fig. 2D).

3.4. Sildenafil promotes hair growth in C57BL/6 mice

To investigate whether treatment with sildenafil can promote spontaneous anagen entry *in vivo*, we topically applied 0.1% and 1% sildenafil onto the shaved back of telogen phase C57BL/6 mice. Treatment with 2% minoxidil was the positive control. Typically, the skin color of the C57BL/6 mice is pink during the telogen phase and darkens due to anagen initiation [21]. We observed a color change from pink to light gray on day 7 for the sildenafil-treated mice, indicating a transition from the telogen phase to the anagen phase. The control group showed no significant color changes. At day 21, the areas of black skin and hair coat were significantly larger for the sildenafil-treated group, whereas the hair coat was poorly regenerated for the control group (Fig. 3A). Histological studies showed that the number and size of hair follicles were significantly increased for the sildenafil-treated group than for the control group (Fig. 3B). The thickness of the interfollicular whole skin and the HF size significantly increased after treatment with sildenafil (Fig. 3C). The anagen HFs were scored according to the anagen phase of each HF and were determined to be significantly higher for the sildenafil-treated group than for the control group (Fig. 3D).

3.5. Sildenafil promotes perifollicular vascularization

To investigate whether sildenafil promotes angiogenesis, we performed immunofluorescence staining of the blood vessel marker, MECA32. To ensure proper selection of the control group, we used mice that had naturally entered the anagen phase as controls and excluded mice in the telogen phase on day 21. As shown in Fig. 4A, large and elongated perifollicular vessels surrounding the HFs and bulb were observed after sildenafil treatment. An approximately 2-fold increase in the perifollicular vessel size was observed for the sildenafil-treated group (average size of 973 μm^2 and 900 μm^2 for 0.1% and 1% sildenafil, respectively) as

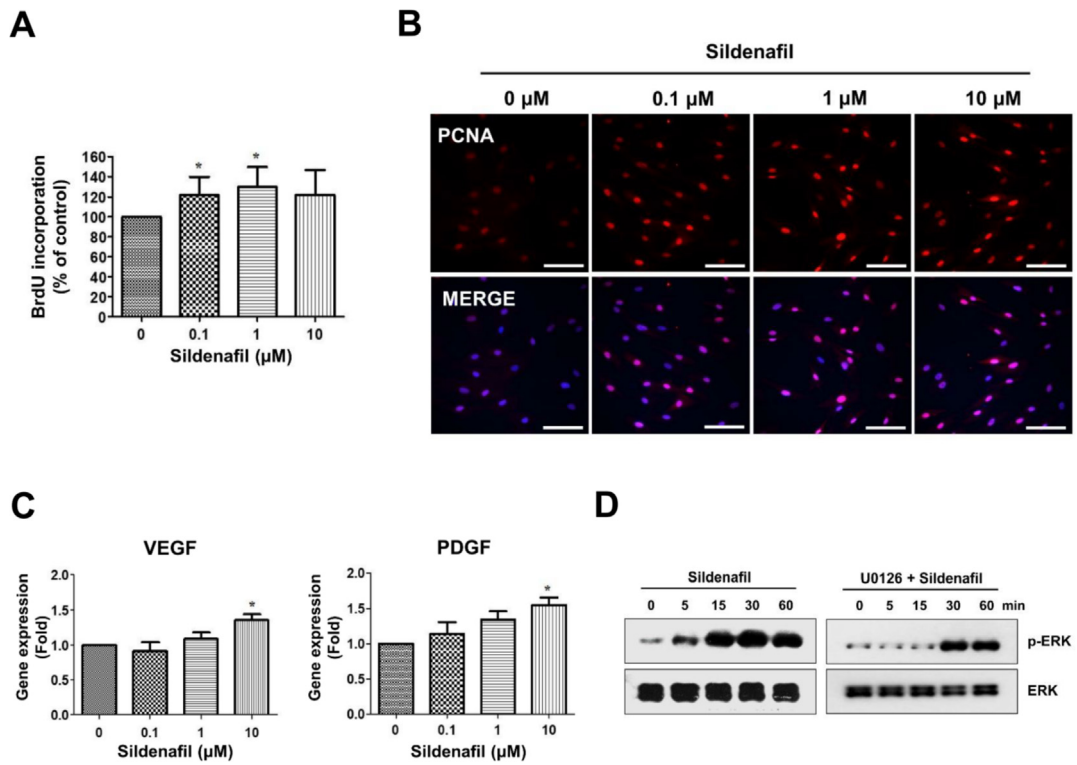

Fig. 2. Effect of sildenafil on cultured hDPCs. (A) The proliferation of hDPCs was measured using the BrdU incorporation assay after treatment of the cells with 0–10 μM sildenafil for 48 h. The results are shown as mean ± standard error values (n = 5). * indicates $P < 0.05$ when compared to the control group. (B) Immunofluorescent staining of PCNA was performed in hDPCs. Nuclei were counterstained with DAPI. (C) hDPCs treated with 0–10 μM sildenafil for 6 h were analyzed for gene expression of VEGF and PDGF-A using real-time PCR. The results are shown as mean ± standard error values (n ≤ 6). (D) hDPCs were pre-incubated with U0126 (10 μM) for 1 h prior to sildenafil (10 μM) treatment. Changes in phosphorylation of ERK and total ERK were analyzed using western blotting.

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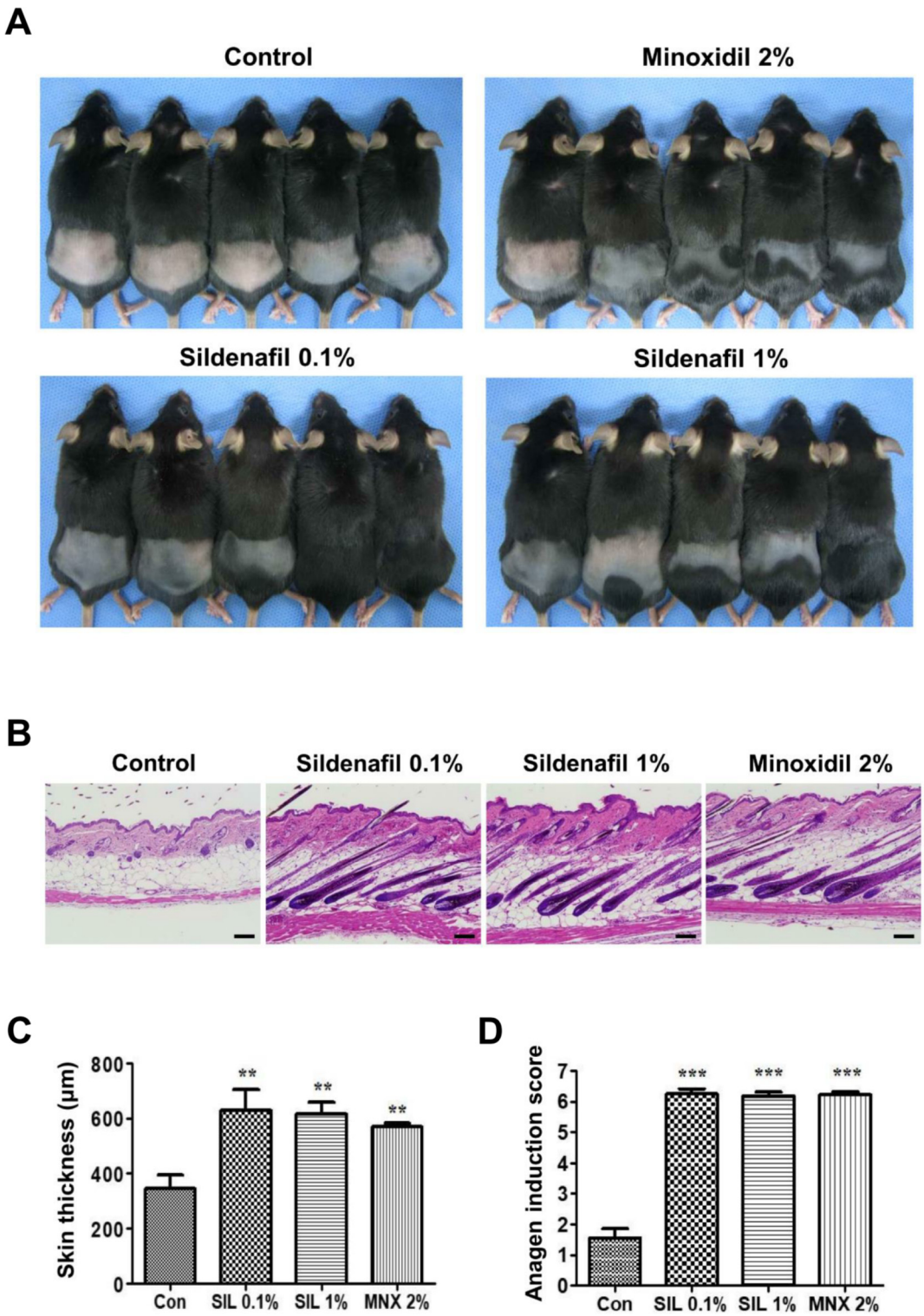

Fig. 3. Effect of sildenafil on anagen induction in 8-week-old female C57BL/6 mice. (A) The back skin of mice (n = 5 in each group) was shaved and treated with topical application of vehicle, 0.1% sildenafil, 1% sildenafil, or 2% minoxidil for 3 weeks. (B) Histological analysis of paraffin-embedded skin samples by H&E staining. Scale bar = 100 μm. (C) Skin thickness was measured as the distance from the epidermis to subcutaneous fat. (D) Anagen induction scores (telogen = 1, anagen I-VI = 2–7, n = 70 HFs/group). The results are shown as mean ± standard error values. *** indicates $P < 0.001$, ** indicates $P < 0.01$, and * indicates $P < 0.05$ when compared to the control group.

compared with the control group (average size of 511 μm^2) (Fig. 4B). Additionally, the number of MECA32-positive vessels was significantly increased more than a 2-fold in the sildenafil-treated HFs compared with the control group (Fig. 4C).

4. Discussion

The hair follicles undergo a cycle of anagen, catagen, and telogen and re-arrangement of the skin vasculature during hair cycling

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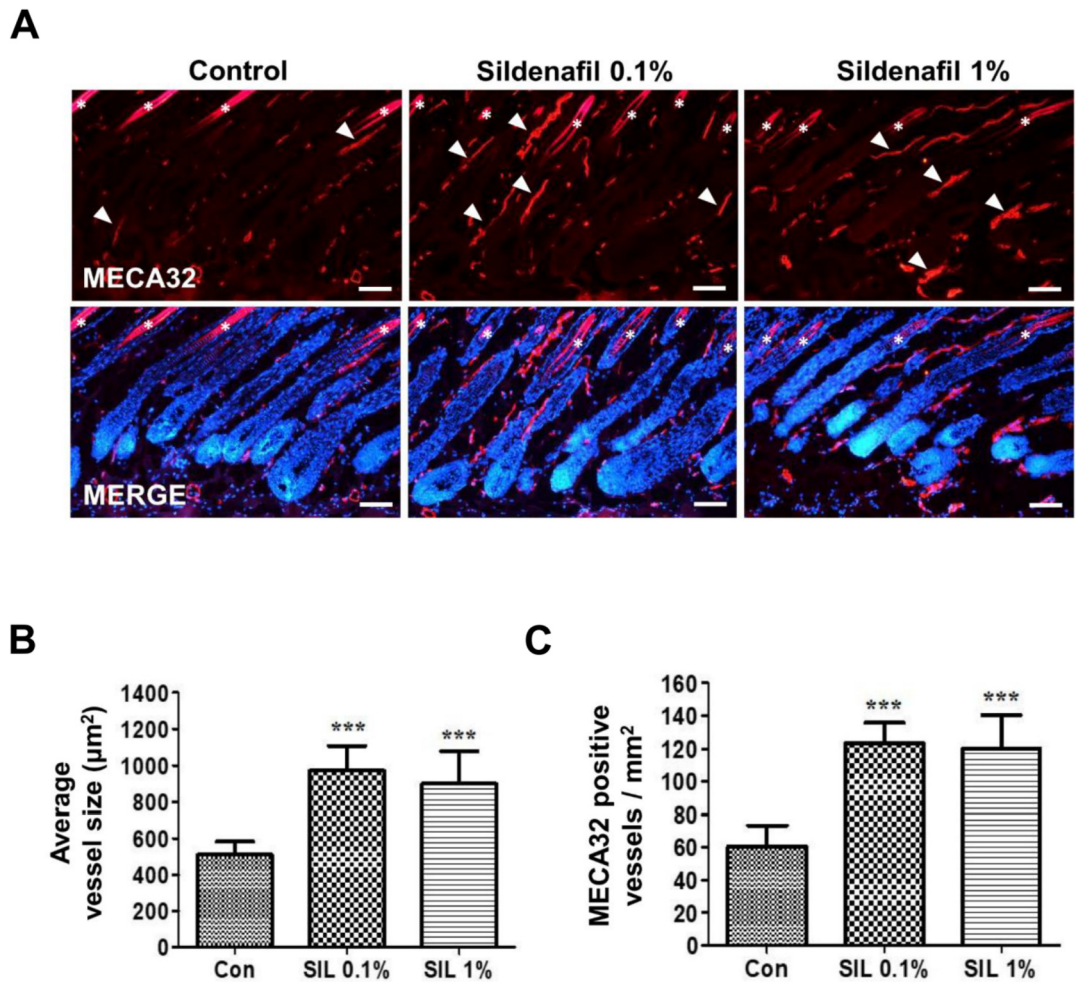

Fig. 4. Effect of sildenafil on perifollicular vessel formation. (A) Immunofluorescent staining of MECA32. Nuclei were counterstained with DAPI. White arrowheads mark the vessels surrounding HFs. The white asterisk indicates non-specific staining of hair shaft. Scale bars = 100 μm. Quantification of average vessel size (B) and MECA32 positive vessel numbers (C) in vehicle- or sildenafil-treated mice (n = 6 random fields, n = 3 mice/group). Data are presented as mean ± standard error values. *** indicates $P < 0.001$, ** indicates $P < 0.01$ when compared to the control group.

[22,23]. As blood vessels are necessary to supply oxygen and nutrients to cells in the skin and hair follicles, hair growth is dependent on the induction of angiogenesis to meet the increased nutritional needs of HFs during the anagen phase of rapid cell division. Certain vasodilators such as Minoxidil, increase the cutaneous blood flow to the scalp and stimulate hair growth [24]. Additionally, previous studies have shown that the well-known vasodilator, cilostazol, promotes hair growth through *in vitro*, *ex vivo* and *in vivo* experiments [15]. Therefore, we hypothesized that sildenafil would promote hair growth.

As there has been limited research on the effects of sildenafil on HFs, we measured the expression of PDE5A, which is known to be a target of sildenafil and found that PDE5A mRNA and protein were highly expressed in hDPCs and hHFs. Additionally, we confirmed that sildenafil significantly increased the proliferation of hDPCs.

We also found that sildenafil increases the mRNA expression of VEGF and PDGF in hDPCs, which are important growth factors for hair growth. Previous studies have reported that VEGF and VEGF receptor 2 were expressed in human anagen HFs, particularly in VEGF induced proliferation of hDPCs [25].

In addition, PDGF is also well known as a hair growth-promoting factor and is expressed in hDPCs and follicular keratinocytes [29]. PDGF isoforms have been reported to induce and maintain the anagen phase of the murine HF and promote hair regeneration [30]. In addition, the ability of two PDGF isoforms (PDGF-AA and PDGF-BB) to regulate hair dermal stem cell and maintain hair-inducing activity has been reported [31]. We assume that increased mRNA expression of VEGF and PDGF due to sildenafil stimulates secretion of VEGF and PDGF, and could promote proliferation of hDPCs.

ERK is an important member of the mitogen-activated protein kinase (MAPK) family, and is involved in the regulation of cell growth. ERK is activated by sildenafil in the endothelial cells [26]. We investigated whether sildenafil activates ERK signaling in hDPCs and demonstrated that sildenafil increases ERK phosphorylation in hDPCs and that U0126 blocks sildenafil-induced ERK activation. Therefore, it suggests that sildenafil affects signal transduction pathways that regulate cell proliferation in hDPCs.

We observed that topical application of sildenafil to the dorsal skin of C57BL/6 mice significantly promotes anagen induction because of stimulation of hair growth cycle. Several studies have shown that sildenafil has a role in angiogenesis [27–29]. Therefore, we hypothesized that sildenafil might affect perifollicular angiogenesis around HFs. We performed immuno-fluorescence staining and observed an increase in the number of large and elongated vessels surrounding the HFs, suggesting that sildenafil promotes HF vessel formation.

Growth factors, such as VEGF, PDGF and IGF-1, which are produced by vascular endothelial and non-endothelial cells are likely

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to play an important role in the paracrine mechanisms that regulate angiogenesis under physiological and pathological conditions [30,31]. In addition, the dermis around the anagen HFs is more vascularized than the dermis around the telogen follicles, and proliferating endothelial cells have been noted inside the follicular DP only during anagen [32]. Taken together, these results demonstrate that sildenafil increased perifollicular vessel formation near HFs, which allowed the effect of various growth factors secreted by dermal cells.

In summary, our results demonstrate that sildenafil significantly stimulates hair growth both *in vitro* and *in vivo*. In addition, it enhances proliferation of hDPCs and accelerates the anagen hair cycle by promoting perifollicular vessel formation. We propose that sildenafil plays an important role as an inducer of HF growth and may function as an additional therapeutic agent for alopecia.

Conflict of interests

The author(s) declare(s) that there is no conflict of interests regarding the publication of this article.

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