J Ginseng Res 44 (2020) 168–177

Contents lists available at ScienceDirect

Journal of Ginseng Research

journal homepage: http://www.ginsengres.org

Research Article

Effects of a gintonin-enriched fraction on hair growth: an *in vitro* and *in vivo* study

Na-Eun Lee [1,☆], Sang-Deuk Park [1,☆], Hongik Hwang [2], Sun-Hye Choi [1], Ra Mi Lee [1], Sung Min Nam [1], Jong Hee Choi [3], Hyewhon Rhim [2], Ik-Hyun Cho [3], Hyoung-Chun Kim [4], Sung-Hee Hwang [5,**], Seung-Yeol Nah [1,*]

[1] *Ginsentology Research Laboratory and Department of Physiology, College of Veterinary Medicine, Konkuk University, Seoul, Republic of Korea*
[2] *Center for Neuroscience, Korea Institute of Science and Technology, Seoul, Republic of Korea*
[3] *Department of Convergence Medical Science, Department of Science in Korean Medicine, and Brain Korea 21 Plus Program, Graduate School, Kyung Hee University, Seoul, Republic of Korea*
[4] *Neuropsychopharmacology and Toxicology program, College of Pharmacy, Kangwon National University, Chunchon, Republic of Korea*
[5] *Department of Pharmaceutical Engineering, College of Health Sciences, Sangji University, Wonju, Republic of Korea*

ARTICLE INFO

Article history:
Received 11 March 2019
Received in Revised form
18 May 2019
Accepted 31 May 2019
Available online 7 June 2019

Keywords:
Ginseng
Gintonin-enriched fraction
Hair growth
Human hair follicle dermal papilla cells
Mouse

ABSTRACT

Background: Ginseng has been widely used as a health-promoting tonic. Gintonin present in ginseng acts as a lysophosphatidic acid (LPA) receptor ligand that activates six LPA receptor subtypes. The LPA6 subtype plays a key role in normal hair growth, and mutations in the LPA6 receptor impair normal human hair growth. Currently, human hair loss and alopecia are concerning issues that affect peoples' social and day-to-day lives.
Objective: We investigated the *in vitro* and *in vivo* effects of a gintonin-enriched fraction (GEF) on mouse hair growth.
Methods: Human hair follicle dermal papilla cells (HFDPCs) and six-week-old male C57BL/6 mice were used. The mice were divided into the four groups: control, 1% minoxidil, 0.75% GEF, and 1.5% GEF. The dorsal hair was removed to synchronize the telogen phase. Each group was treated topically, once a day, for 15 days. We analyzed hair growth activity and histological changes.
Results: GEF induced transient $[Ca^{2+}]_i$, which stimulated HFDPC proliferation and caused 5-bromo-2′-deoxyuridine (BrdU) incorporation in a concentration-dependent manner. GEF-mediated HFDPC proliferation was blocked by the LPA receptor antagonist and Ca^{2+} chelator. HFDPC treatment with GEF stimulated vascular endothelial growth factor release. Topical application of GEF and minoxidil promoted hair growth in a dose-dependent manner. Histological analysis showed that GEF and minoxidil increased the number of hair follicles and hair weight.
Conclusion: Topical application of GEF promotes mouse hair growth through HFDPC proliferation. GEF could be one of the main components of ginseng that promote hair growth and could be used to treat human alopecia.

1. Introduction

Panax ginseng Meyer is a popular representative herbal medicine, generally used as a tonic, in far Eastern Asian countries [1]. In traditional medicine, ginseng is decocted with hot water. Recently, ginseng has been decocted with hot water to obtain more active ingredients, including main water-soluble components [2]. In addition, ginseng extract has also been used for diverse purposes in cosmetology to obtain health benefits [4], such as the prevention of hair loss or promotion of hair growth. Ginseng extract and ginsenosides, such as ginsenoside Rb1, have been shown to promote the growth of *in vitro* cultured human hair follicle cells [5,6].

* Corresponding author. Ginsentology Research Laboratory and Department of Physiology, College of Veterinary Medicine, Konkuk University, Seoul 05029, Republic of Korea
** Corresponding author. Department of Pharmaceutical Engineering, College of Health Sciences, Sangji University, Wonju, 26339, Republic of Korea
E-mail addresses: sunghhwang@sangji.ac.kr (S.-H. Hwang), synah@konkuk.ac.kr (S.-Y. Nah).
☆ These two authors contributed equally to this work.

https://doi.org/10.1016/j.jgr.2019.05.013
p1226-8453 e2093-4947/$ – see front matter

Subcutaneous injection, but not topical administration of ginsenoside, promotes hair follicle growth [7]. However, ginseng extract and individual components have not been fully explored for the molecular mechanism(s) responsible for the ginseng extract- or component-mediated beneficial effects on hair growth because ginseng extract consists of a variety of components such as ginseng saponins, acidic polysaccharides, and other components. Although ginsenoside is one of the primary components of ginseng, the mode of action underlying its hair growth–promoting effect is still unclear because it does not have a specific extracellular membrane or intracellular target protein [4].

Recently, a line of evidence showed that Korean Red Ginseng and Korean white ginseng contain a novel component called gintonin [8], which differs from the ginseng saponin; gintonin or gintonin-enriched fraction (GEF) consists of several cell growth–related factors such as lysophosphatidic acid (LPA), which is a specific lipid-derived growth factor, phosphatidic acid (PA), which is also a regulator of cell growth and proliferation as a mitogen, and linoleic acid, an essential fatty acid [9]. Gintonin acts as a ginseng-derived exogenous G protein–coupled LPA receptor ligand and activates LPA1–LPA6 receptor subtypes [10]. Interestingly, recent studies have shown that mutation in the LPA6 receptor causes abnormal hair growth in humans, hypotrichosis simplex, indicating that the LPA6 receptor might play an important role in normal hair growth in humans [11,12]. In addition, PA acts as a strong mitogenic activator through the mammalian target of rapamycin (mTOR) signaling pathways [13]. A recent study also showed that topical application of PA also promoted *in vivo* hair growth in mouse models [14]. Although GEF contains LPAs and PAs, which regulate cell proliferation, relatively little is known about whether GEF contributes also to the promotion of hair growth in mouse models.

Head hair and the scalp play various important roles in animals or humans, including physical protections from exterior shock, sensory activity, and thermoregulation [15]. During postnatal development, hair follicles follow a cyclic pattern involving three phases: a growth phase (anagen), a regression phase (catagen), and a resting phase (telogen), based on histological morphology [16]. As the amount of hair present in humans acts as one of the factors affecting personal activity and social interactions, people pay a lot of attention to their hair conditions, such as hair loss, thickness, and changes in color with aging [15]. Two factors mainly affect hair loss (also called alopecia) in humans; one is psychoemotional stress and the other is the imbalance in male hormone secretions. These factors disrupt the normal patterns of the hair cycle [17]. Until now, two medicines—finasteride and minoxidil—have been used for hair loss treatment. However, these medicines have limited therapeutic value as they show some side effects and unsatisfactory hair growth rates [18,19]. Recent studies have shown that herbal medicines could be an alternative or complementary way to prevent hair loss or to promote hair growth, with fewer side effects [20]. Ginseng has been known as a candidate for hair growth [21], although most studies were performed using a simple ginseng total extract or several limited ginsenosides, and ginseng still contains unknown components. Moreover, most of the studies using ginsenosides were also carried out at the level of organ cultures, such as vibrissal hair follicles rather than the whole animal, or with subcutaneous injection of ginseng extract rather than direct topical application [5,7,22]. As mention previously, although gintonin or GEF acts as an exogenous LPA receptor ligand and exhibits the role of lipid-derived growth factors, the effect of GEF on hair growth has not yet been examined.

In the present study, we investigated the effects of GEF on *in vitro* and *in vivo* mouse hair growth. In the *in vitro* study, GEF transiently induced $[Ca^{2+}]_i$ in human hair follicle dermal papilla cells (HFDPCs) and stimulated HFDPC proliferation; HFDPCs are key for hair growth. GEF-mediated HFDPC proliferation was blocked by an LPA receptor antagonist and intracellular Ca^{2+} chelator. GEF treatment on HFDPCs stimulated vascular endothelial growth factor (VEGF) release. We found that the direct *in vivo* topical application of GEF promoted hair growth in a concentration-dependent manner and increased the number of hairs. In histochemical studies, topical treatment with GEF increased the number of hair follicles and 5-bromo-2′-deoxyuridine (BrdU) incorporation, compared with the observations in the control. These results suggest that GEF-mediated proliferation of HFDPCs may be coupled to hair growth and that GEF could be a main active ingredient in ginseng with hair growth–promoting effects. We further discuss the molecular mechanism underlying GEF-mediated hair growth and the possible application of GEF for the prevention of hair loss or promotion of hair growth in humans.

2. Materials and methods

2.1. GEF preparation and drug treatment

GEF was prepared as previously described [9]. Briefly, 1 kg of 4-year-old ginseng (Korea Ginseng Corporation, Daejeon, Republic of Korea) was cut into small pieces (>3 mm) and refluxed with 70% fermented ethanol eight times, for 8 h each, at 80°C. The extracts (340 g) were concentrated, dissolved in distilled cold water at a ratio of 1:10, and stored at 4°C for 24–96 h. The supernatant and precipitate fractions, obtained by water fractionation after ethanol extraction of ginseng, were separated by centrifugation (3,000 rpm, 20 min), and the precipitate was lyophilized. GEF consists of carbohydrates, lipids, and ginseng proteins. The total content of carbohydrates, lipids, and proteins in GEF were approximately 30%, 20.2%, and 30.3%, respectively, and included other minor components [9]. The lipid composition of GEF, detected by LC-MS/MS, was as follows: fatty acids (7.53% linoleic acid, 2.82% palmitic acid, and 1.46% oleic acid), 0.60% lysophospholipids and phospholipids, and 1.75% phosphatidic acids. The total lipid content in GEF was approximately 14.2%. The qualitative assay showed that GEF contained diacylglycerols and triacylglycerols [9]. Other agents including minoxidil were purchased from Sigma-Aldrich (St. Louis, MI)

2.2. Animal experimental design

Male C57BL/6 mice (six weeks old, 18–20 g) were purchased from Orient Bio Inc., (Chuncheon, Korea) and maintained according to Institutional Animal Care and Use guidelines of Konkuk University (Permit Number: 16-206). All the experiments were conducted in a manner that minimized the number of animals used and their suffering. Animals were housed in plastic cages under controlled conditions: 50 ± 5% humidity, 12/12-h light–dark cycle, and free access to standard food and water. The mice were allowed 1 week to adapt to the conditions. Then, the dorsal skin of mice at 7 weeks of age, at which all hair follicles were in the telogen phase [23], was removed with a hair clipper and hair removal cream (Veet; Oxy Reckitt Benckiser, Chartes, France). The mice were randomly divided into four groups: group 1 (control vehicle group), group 2 (0.75% GEF group), group 3 (1.5% GEF), and group 4 (1% minoxidil). The concentrations of GEF used in present study were determined through the preliminary experiments. All treatments were topically applied once a day for 15 days. GEF and minoxidil were dissolved in 70% ethanol, 20% polyethylene glycol, and 10% H_2O. The back skin of mice was photographed at Day 1, 7, 9, 11, and 13. The hair regrowth efficacy scores used were as follows: 0 = no change, 1 = <30% of shaved area shows skin darkening, 2 = 30–70% of shaved area shows skin darkening, 3 = >70%

of shaved area shows skin darkening or <30% shows hair growth, 4 = >70% of shaved area shows skin darkening and 30% ~70% shows hair growth, 5 = >70% of shaved area shows skin darkening and >70% shows hair growth, and 6 = >90% of shaved area shows hair growth [23]. The dorsal skin of eight mice from each group was collected after 13 days of depilation, snap-frozen in liquid nitrogen, and stored at −80°C for further experiments. The final hair weight of each group was determined by clipping and weighing the hair on Day 13.

2.3. Culture of HFDPCs

HFDPCs were purchased from CEFO Co. Ltd (Seoul, South Korea) and cultured in HFDPC growth medium (cat no. CB-HDP-GM, CEFO Co. Ltd) supplemented with 2% cell culture supplements, 100 units/mL of penicillin, and 100 μg/mL of streptomycin, according to the manufacturer's instruction. Cultures were maintained at 37°C in humidified conditions under 5% CO_2. The cells at passages 3–7 were used for all experiments.

2.4. Measurement of intracellular Ca^{2+} levels

We examined intracellular Ca^{2+} levels in HFDPCs exposed to GEF. HFDPCs from each group were incubated for 40–60 min at room temperature with 5 μM Fura-2/AM (Molecular Probes, Eugene, OR, USA) and 0.001% pluronic F-127 (Molecular Probes) in a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered solution composed of the following: 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose, with the pH adjusted to 7.4 with NaOH. Cells were illuminated using a xenon arc lamp, and the excitation wavelengths (340 and 380 nm) were selected using a computer-controlled filter wheel (Sutter Instruments, Novato, CA, USA). The emitted fluorescence was reflected through a 515-nm long-pass filter to a frame transfer–cooled CCD camera (Olympus, Japan), and the ratios of the emitted fluorescence were calculated using a digital fluorescence analyzer and then converted to intracellular free Ca^{2+} concentrations $[Ca^{2+}]_i$. All imaging data were collected and analyzed using Universal Imaging software (Bedford Hills, New York, USA) [24].

2.5. 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5 carboxanilide and BrdU incorporation assays

Cell proliferation and viability were determined using 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5 carboxanilide (XTT) and BrdU assays, as previously described [10,25]. Briefly, the cells were seeded in 96-well plates, allowed to adhere overnight, and then treated with GEF or LPA in the absence or presence of inhibitors at the indicated concentrations. After 24 hours of incubation, cell proliferation was measured using an XTT assay or BrdU incorporation assay. After 2 hours of incubation with XTT, absorbance was measured at 450 nm using a microplate reader (SpectraMax® Plus 384 Microplate Reader, Molecular Devices, San Jose, CA, USA) [25]. For the BrdU incorporation assay, the BrdU labeling agent was added to the drug-treated cells and incubated for the last 16 h of the total incubation time. Then, BrdU incorporation was measured by enzyme-linked immunosorbent assay with a BrdU cell proliferation assay kit (Roche Applied Science, Mannheim, Germany), according to the manufacturer's instructions [10].

2.6. LPA receptor expression assay

Cells were incubated in a 150-mm culture dish, serum-starved for 6 h, and incubated with vehicle or GEF (30 μg/mL) for 24 h. The cells were lysed with modified Radioimmunoprecipitation assay buffer (RIPA buffer), and LPA receptor expression was detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting using rabbit anti-EDG2/LPA1 polyclonal antibody (Abcam, Cambridge, UK), rabbit anti-EDG7/LPA3 polyclonal antibody (Abcam), or rabbit anti-P2RY5/LPA6 polyclonal antibody (Origene Technologies, Inc., Rockville, MD, USA). The blotted membrane was stripped and reprobed with mouse anti-β actin monoclonal antibody–conjugated horseradish peroxidase (HRP) (Abcam). Image was visualized using Clarity Western ECL Substrate Bio-Rad (Hercules, CA, USA) using the luminescent image analyzer LAS-4000 (Fujifilm, Japan).

2.7. Histological analysis

Dorsal skin from each group was fixed in 4% formaldehyde solution and embedded in paraffin to obtain longitudinal sections. The longitudinal sections were stained with hematoxylin and eosin (H&E), and digital photomicrographs were taken from representative areas using a digital camera (Paxcam, Villa Park, IA, USA). The number of hair follicles in the anagen phase (A) and the telogen phase (T) was counted, and the A/T ratio was determined. For nuclear (replicating DNA) pulse labeling experiments, mice were administered with BrdU intraperitoneally (20 mg/kg/day) 120 minutes before skin excision. BrdU-labeled cells were detected in paraffin wax–embedded sections that had been treated sequentially with 2N HCl for 1 hour at 37°C, using a rat monoclonal antibody to BrdU (1:400; Biosource International Inc., Camarillo, CA, USA), biotinylated immunoglobulin G (1:400; Vector, Burlingame, CA, USA) and the streptavidin peroxidase complex (1:400; Vector). They were then visualized using a reaction with 3,3′-diaminobenzidine tetrachloride (Sigma-Aldrich) in 0.1 M Tris-HCl buffer (pH 7.2) and mounted on gelatin-coated slides. The sections were finally dehydrated and mounted in a toluene-based mounting medium (Richard-Allan Scientific, Thermo Scientific, MI, USA).

2.8. Statistical analysis

The data were expressed as mean ± standard deviation (SD). The statistical significance of the difference between mean values was examined using analysis of variance followed by unpaired Student *t* test. A $p < 0.05$ value was considered as a significant difference.

3. Results

3.1. GEF induces $[Ca^{2+}]_i$ transient in HFDPCs

In previous reports, we have shown that gintonin or GEF induces transient elevation of cytosolic Ca^{2+} levels for neurotransmitter release and cell proliferation [2,24]. Based on the previous studies, we first examined whether HFDPC treatment with GEF induces transient $[Ca^{2+}]_i$. As shown in Fig. 1A, GEF treatment elevated $[Ca^{2+}]_i$ transiently, and the effects of GEF were concentration dependent at the concentration range of 10–100 μg/mL and saturated above 100 μg/mL of GEF. However, the low concentration of GEF (0.1, 0.3, 1, and 3 μg/mL) had no effects on transient $[Ca^{2+}]_i$ (data not shown). We also examined whether ginsenosides can exhibit any transient effects on $[Ca^{2+}]_i$ in HFDPCs. For this, we used representative ginsenosides, such as ginsenoside Rb1 and Rg1, and found that they had no transient effects on $[Ca^{2+}]_i$ in HFDPCs (Fig. 1B), indicating that GEF, rather than ginsenosides, is a main active component of ginseng on the induction of transient $[Ca^{2+}]_i$ in HFDPCs. Then, we examined the involvement of the LPA receptor in GEF-mediated transient $[Ca^{2+}]_i$ in HFDPCs. Because there is no available and selective LPA6 receptor subtype antagonist, we used Ki16425, an LPA1/3 receptor antagonist. Interestingly, GEF-

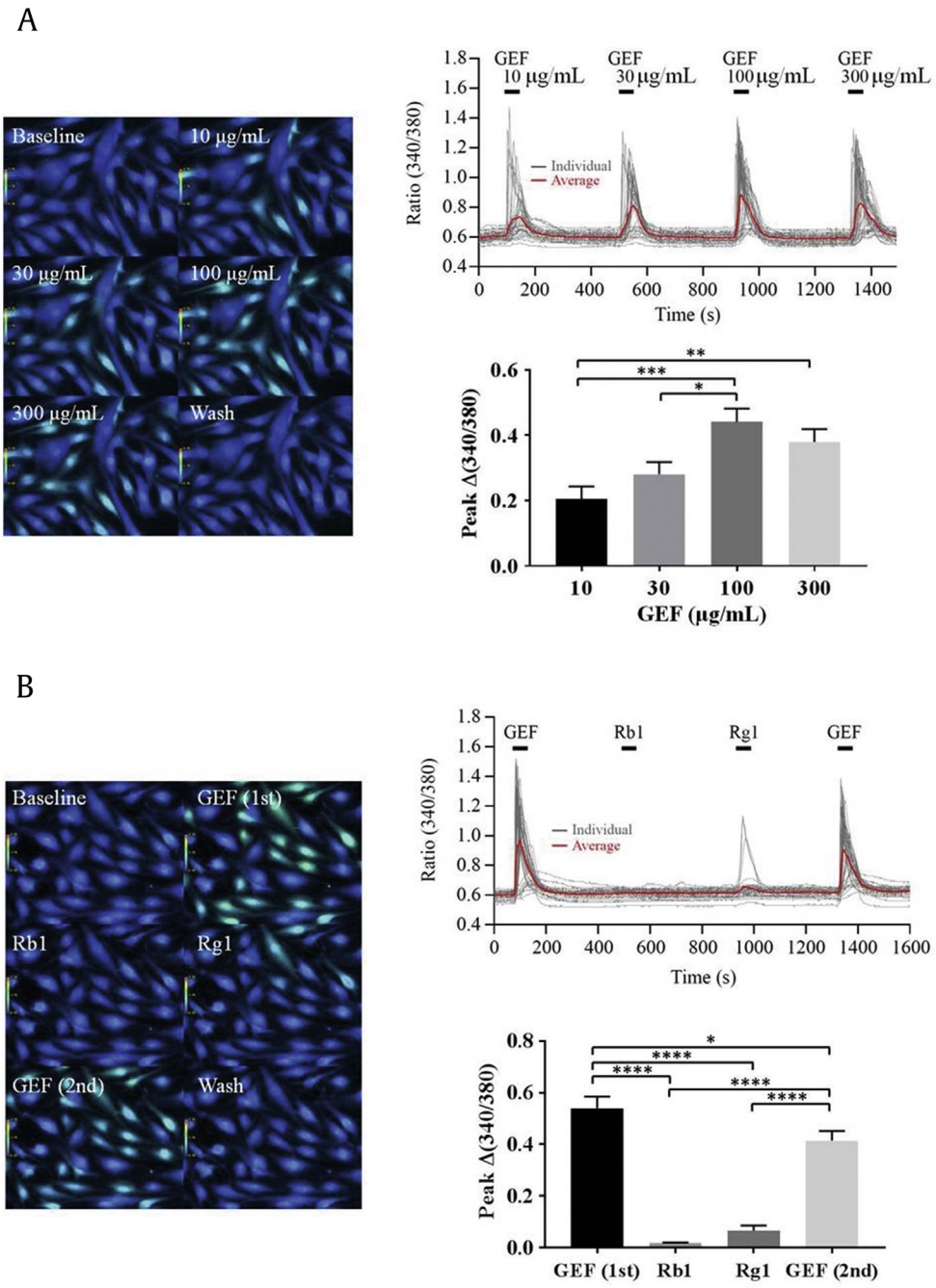

Fig. 1. Effects of the gintonin-enriched fraction (GEF) and ginsenosides on transient $[Ca^{2+}]_i$ in human hair follicle dermal papilla cells (HFDPCs). Analyses of the localization of calcium signals via confocal microscopy were performed with Fura-2/AM and a nuclear dye. We examined intracellular Ca^{2+} levels in HFDPCs exposed to GEF (10, 30, 100, or 300 μg/mL) (A) or ginsenosides such as ginsenoside Rb1 or Rg1 (50 μM each) (B). HFDPCs from each group were incubated for 40–60 min at room temperature with 5 μM Fura-2/AM (Molecular Probes, USA) and 0.001% pluronic F-127 (Molecular Probes) in a HEPES-buffered solution composed of the following: 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose, with the pH adjusted to 7.4 with NaOH. Cells were illuminated using a xenon arc lamp, and the excitation wavelengths (340 and 380 nm) were selected using a computer-controlled filter wheel (Sutter Instruments, USA). *Left panel (A and B)*: the representative pictures of HFDPCs on the $[Ca^{2+}]_i$ level in the absence or presence of various concentrations of GEF or ginsenosides (50 μM each). *Upper right panel (A and B)*: the representative peaks of $[Ca^{2+}]_i$ with different concentrations of GEF. *Lower right panel (A and B)*: summary histograms on the dose–responses of GEF. The data were obtained from 45–50 different cells in three independent experiments. Data represent the mean ± SD. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with 10 μg/mL GEF (A); $^*p < 0.05$, compared with the 2nd GEF application; $^{****}p < 0.001$, compared with the 1st or 2nd applications of GEF (B). SD, standard deviation.

mediated transient $[Ca^{2+}]_i$ in HFDPCs was blocked by Ki16425 and by the cell-permeable Ca^{2+} chelator, 1,2-Bis(2-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM), by demonstrating that GEF-mediated $[Ca^{2+}]_i$ transients were much lower in the second peak than in the first peak (Fig. 2). Thus, these results indicate that GEF-mediated transient $[Ca^{2+}]_i$ in HFDPC is achieved through LPA receptors.

3.2. Effect of GEF on LPA1, LPA3, or LPA6 expression in HFDPCs

To examine the effects of GEF on LPA receptor expression level in HFDPCs, LPA receptor (LPA1, LPA3, and LPA6) expression was first determined by immunoblotting assay. As shown in Figure S1, expressions of LPA receptor subtypes LPA1, LPA3, and LPA6 were conformed in HFDPCs. Then, we treated HFDPCs

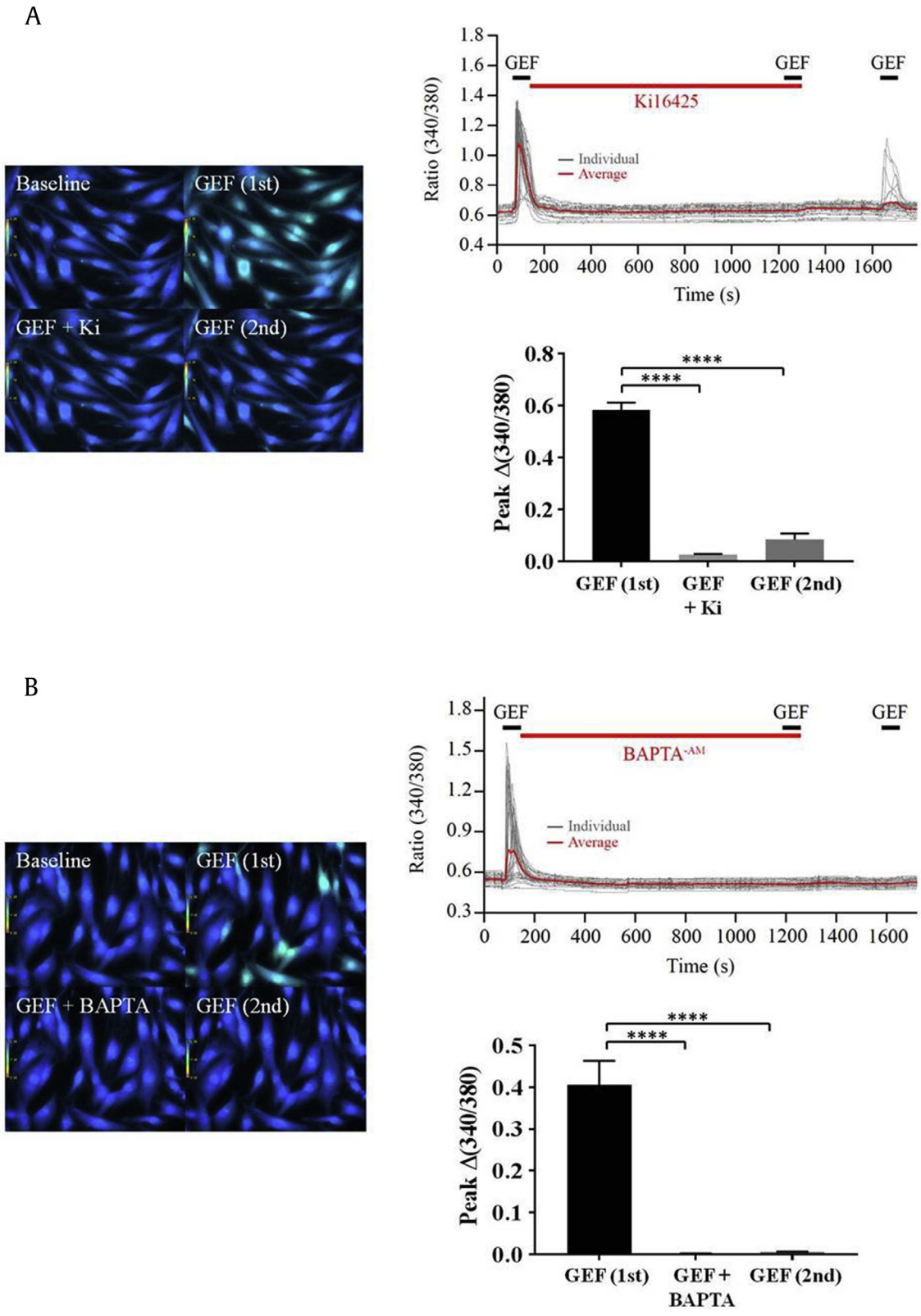

Fig. 2. Effects of the LPA1/3 receptor antagonist Ki16425 and the calcium chelator BAPTA-AM on GEF (100 μg/mL)-mediated transient $[Ca^{2+}]_i$ in HFDPCs. Analyses of the localization of calcium signals were performed as described in Fig. 1. *Left panel (A and B)*: the representative pictures of HFDPCs on the $[Ca^{2+}]_i$ level in the absence or presence of Ki16425 (10 μM) or BAPTA-AM (10 μM). *Upper right panel*: the representative peaks of transient $[Ca^{2+}]_i$ in the absence or presence of Ki16425. *Lower right panel (A and B)*: summary histograms on the effects of Ki16425 or BAPTA-AM on GEF (100 μg/mL)-mediated transient $[Ca^{2+}]_i$. The data were obtained from 40–50 different cells in three independent experiments. Data represent the mean ± SD. ****$p < 0.001$, compared with the presence of Ki16425 (Ki); ****$p < 0.001$, compared with the presence of BAPTA-AM. SD, standard deviation; HFDPCs, human hair follicle dermal papilla cells; GEF, gintonin-enriched fraction.

with GEF and found that GEF treatment to HFDPCs did not induce significant changes in LPA receptor subtype expression levels corrected over β-actin expression compared with control cells.

3.3. Effects of GEF on cell viability and proliferation of HFDPCs

Because the transient increase of cytosolic Ca^{2+} levels is closely related to cell proliferation [3,24], we examined the effects of GEF-mediated cell proliferation on HFDPC growth. For this, we performed the XTT assay and BrdU incorporation assays, which assessed cell viability and proliferation, as a function of cell number based on metabolic activity, and DNA synthesis, respectively. As shown in Fig. 3A, treatment of HFDPCs with GEF increased cell proliferation in a concentration-dependent manner in the XTT-based assay. In the BrDU incorporation study, HFDPC treatment with GEF also increased BrdU incorporation into a newly synthesized DNA in a concentration-dependent manner (Fig. 3B), indicating GEF-stimulated cell proliferation. Interestingly, GEF-mediated cell proliferation was partially attenuated by the LPA1/3 receptor antagonist, Ki16425, whereas LPA-mediated cell proliferation was completely attenuated by the LPA1/3 receptor antagonist, raising a possibility that other components in GEF, besides LPA, might be involved in the increased cell

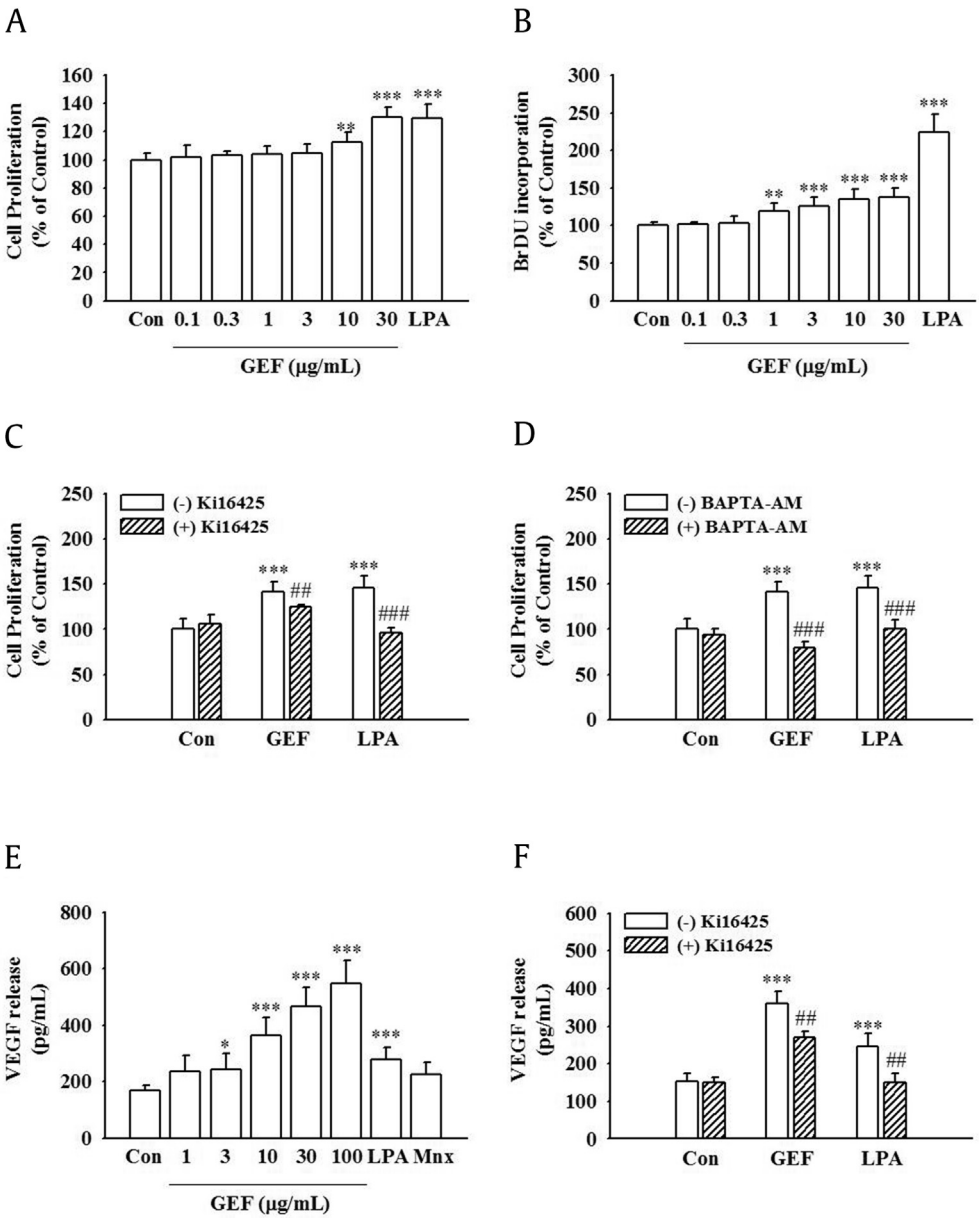

Fig. 3. Effects of gintonin-enriched fraction (GEF) on cell proliferation and VEGF release in HFDPCs. (A, B) HFDPCs were incubated with the control vehicle (control) or various concentrations of GEF (0.1, 0.3, 1, 3, 10, and 30 μg/mL) and 10 μM lysophosphatidic acid (LPA), the positive control. After 24 h of incubation, cell proliferation was determined by an XTT assay (A) or BrdU incorporation assay (B). GEF (30 μg/mL)-induced cell proliferation was also assessed 24 h later in the absence or presence of the LPA1/3 receptor antagonist Ki16425 (final concentration 10 μM) (C), or the intracellular calcium chelator BAPTA-AM (final concentration 10 μM) (D). (E) HFDPCs were incubated with the control vehicle or various concentrations of GEF (1, 3, 10, 30, and 100 μg/mL), 10 μM LPA, and 50 μM minoxidil (M), a positive control, on VEGF release. The degree of VEGF concentration was determined 24 h later. Ki16425 (final concentration 10 μM) attenuates GEF (30 μg/mL)-mediated VEGF release (F). Data represent the mean ± SD of four to six independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with (−)Ki16425 or (−)BAPTA. VEGF, vascular endothelial growth factor; SD, standard deviation; HFDPCs, human hair follicle dermal papilla cells; XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5 carboxanilide.

proliferation (Fig. 3C). In addition, cotreatment of 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM), which is a cell membrane–permeable Ca^{2+} chelator, with GEF-attenuated GEF-mediated cell proliferation (Fig. 3D). These results indicate that GEF-mediated transient $[Ca^{2+}]_i$ is coupled to cell proliferation by LPA receptors.

3.4. GEF enhances VEGF release from HFDPCs

In previous reports, we showed that GEF increased VEGF release in nonneuronal and neuronal cells [25,26], and previous reports also showed that VEGF is one of the growth factors involved in the regulation of hair morphogenesis and hair growth [24]. In the present study, we examined whether GEF also stimulated VEGF release from HFDPCs. As shown in Fig. 6, HFDPC treatment with GEF HFDPCs increased VEGF release, in a concentration-dependent manner, but GEF-mediated VEGF release was attenuated by BAPTA and Ki16425, indicating that GEF-mediated VEGF release is associated with LPA receptor–transient $[Ca^{2+}]_i$.

3.5. Effects of GEF on mouse hair growth and hair weight

To evaluate the hair growth–promoting activities of GEF, we topically treated telogenic dorsal skins of C57BL/6 mice with the control vehicle, different doses of GEF, or 1% minoxidil once a day for 15 days. C57BL/6 mouse dorsal hair is known to have a 7-week, time-synchronized hair growth cycle [23]. We considered using skin pigmentation as evidence for hair growth, which is homogenously bright pink, indicating that the cycle is in the telogen phase and turns into gray/black in the anagen phase (Fig. 4). As shown in Fig. 4, GEF and minoxidil treatment seemed to promote hair regeneration in mice. Thus, GEF or minoxidil treatment started to reveal gray skin at Day 7, but the control vehicle group still maintained pink color on the dorsal region after depilation; short hair shafts were seen on Day 9 after depilation, and their dorsal skins were completely covered by visible hair shafts at Day 13 to 15. The control vehicle group had large areas of skin that had no pigmentation until Day 15. The hair scoring and total hair weight of the GEF and minoxidil groups were also significantly different from those of the control group (Fig. 4B). These results indicate that GEF has hair growth–promoting effects in the mouse model.

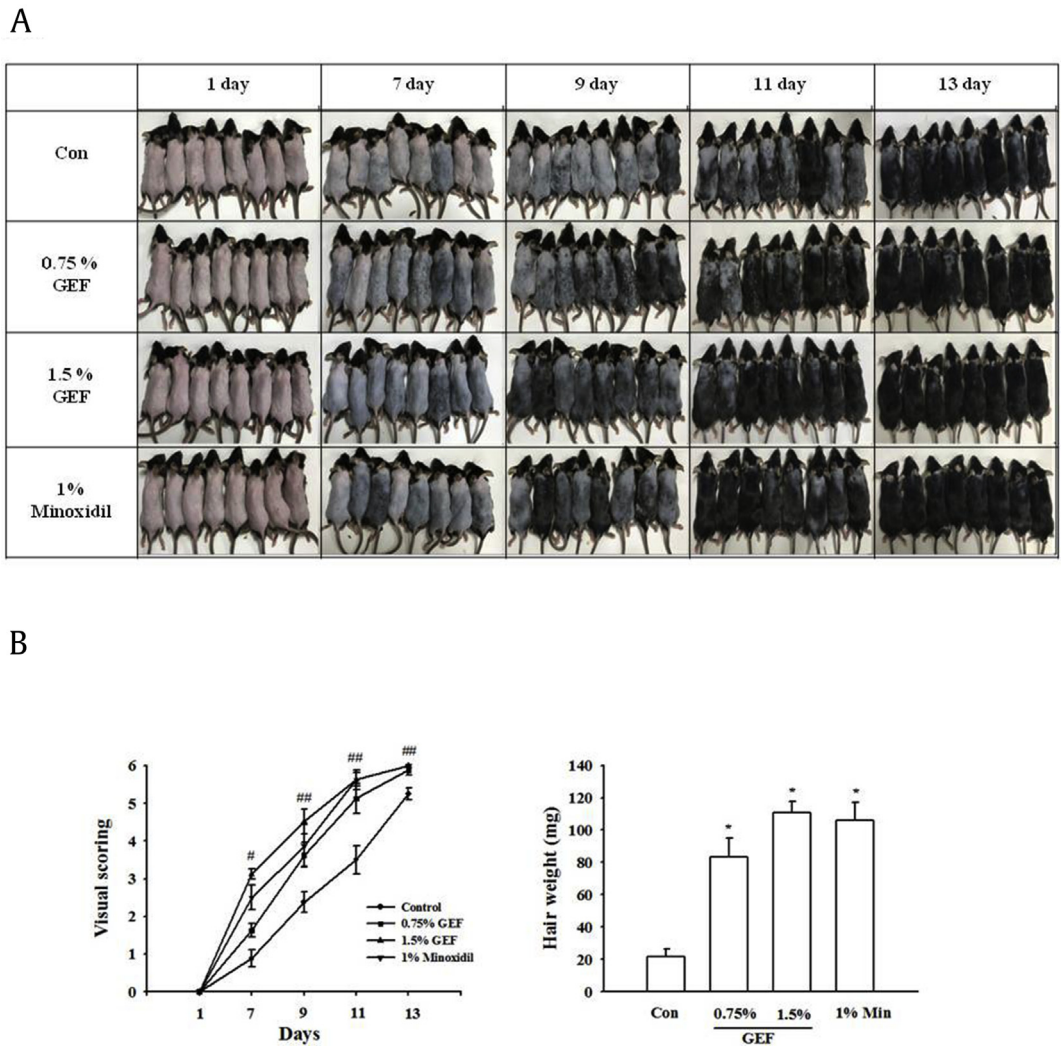

Fig. 4. Effect of gintonin-enriched fraction (GEF) on hair growth in C57BL/6 mice. (A) After synchronizing the telogen phase in mice, the dorsal skin of C57BL/6 mice was treated with the vehicle control, 0.75 or 1.5% GEF, or minoxidil (positive control) once a day for 13 days. GEF and minoxidil treatment facilitated hair growth unlike the control vehicle from Day 7. These pictures are representative samples. The volume used for topical treatment was 200 μL. (B) *Left panel*: visual scoring of the hair growth–promoting effect of GEF by using the scoring guideline shown in the study by Park et al. [23]. Data represent the mean ± SD. $^{\#}p < 0.01$, $^{\#\#}p < 0.001$ compared with the control vehicle. *Right panel*: hair growth in each group was determined by measuring total hair weight at Day 13. $^{*}p < 0.001$ compared with the control vehicle (n = 8). SD, standard deviation.

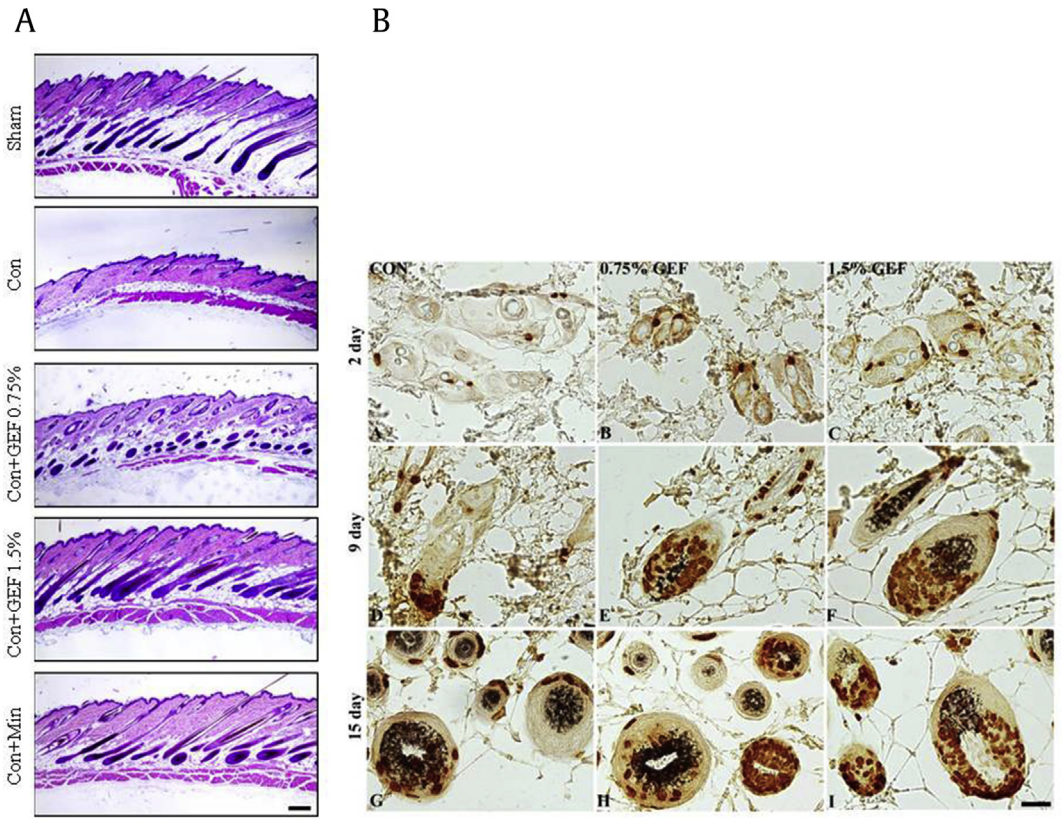

Fig. 5. The representative H&E staining and BrdU incorporation of skin sections from C57BL/6 mice in each group. (A) The part dyed in strong purple color is the hair follicle. GEF and minoxidil treatment showed stronger H&E staining in hair follicles than the control vehicle. The scale bar is 100 μm. (B) The representative BrdU-labeled hair follicle cells of skin sections from C57BL/6 mice at Day 2, 9, and 15 in each group. The part dyed in strong brown color is BrdU-incorporated mitotic cells in hair follicles. GEF treatment on Day 9 showed stronger BrdU staining in hair follicle cells than that after control vehicle treatment. The scale bar is 25 μm. H&E, hematoxylin and eosin; GEF, gintonin-enriched fraction.

3.6. Effects of GEF on the movement from the telogen to anagen phase

Then, we examined the effects of GEF and minoxidil on hair follicle formation, which shows whether GEF or minoxidil induces movement from the telogen to the anagen phase. As shown in Fig. 5A, topical application of GEF or minoxidil significantly increased the number of hair follicles compared with that in the control vehicle group, thereby promoting the hair growth cycle into the anagen phase from the telogen phase. Histological studies also showed that GEF markedly increased the depth and size of the hair follicles compared with those observed with the control treatment (Fig. 5A). Then, we examined the degree of time-dependent BrdU incorporation among treatment groups. As shown in Fig. 5B, we observed that there were a low number of BrdU-labeled mitotic cells in hair follicles at Day 2 in the treatment groups. On Day 9, we observed that the topical GEF treatment group had increased BrdU-labeled hair follicle cells compared with those of the control vehicle group. However, on Day 15, the degree of BrdU-labeled cells was not different between the treatment groups, suggesting the notion that GEF induces the early onset of anagen, from telogen, and stimulates hair growth.

4. Discussion

Many people, even young men and women, suffer from hair loss and hair color changes. Although recent studies have shown that there are many reasons for human hair loss, one of the most likely causes is stresses derived from a competitive society [27,28]. Current treatment for hair loss or alopecia includes direct implantation of hair follicles or therapeutic intervention with drugs such as finasteride or minoxidil [29]. Although these therapeutic drugs show clinical efficacy, they cause potential adverse effects; the long-term use of finasteride can cause erectile dysfunction and depression. Minoxidil has been used for a long time, but its molecular mechanism of action is still unknown, and it induces skin-related side effects. Thus, the use of current therapeutic agents is limited, and it is necessary to develop new therapies to prevent hair loss or to enhance hair growth to mitigate hair loss. A line of evidence showed that ginseng extract could be a candidate to substitute chemical compounds to promote hair growth, but its active component and the underlying mechanisms are still unknown [21]. In the present study, we investigated the effects of GEF on *in vitro* and *in vivo* hair growth using HFDPCs and experimental animals, respectively.

We first examined the *in vitro* effects of GEF on HFDPCs, which are known as the main mesenchymal components for hair growth, are located at the deepest end of the hair follicle, and are thought to play an essential role in the induction of new hair follicles and maintenance of human hair growth [30,31]. We found that GEF, but not ginsenoside, treatment induced transient $[Ca^{2+}]_i$ and stimulated cell proliferation in HFDPCs. GEF-mediated HFDPC proliferation was attenuated by BAPTA, indicating that GEF-mediated, but not ginsenoside-mediated, transient $[Ca^{2+}]_i$ is linked to the stimulation of *in vitro* proliferation of HFDPCs. GEF-mediated HFDPC proliferation was only slightly, but significantly, blocked by the LPA1/3 receptor antagonist Ki16425, indicating that HFDPCs express LPA1/3 receptor and LPA6 receptor and that GEF stimulates

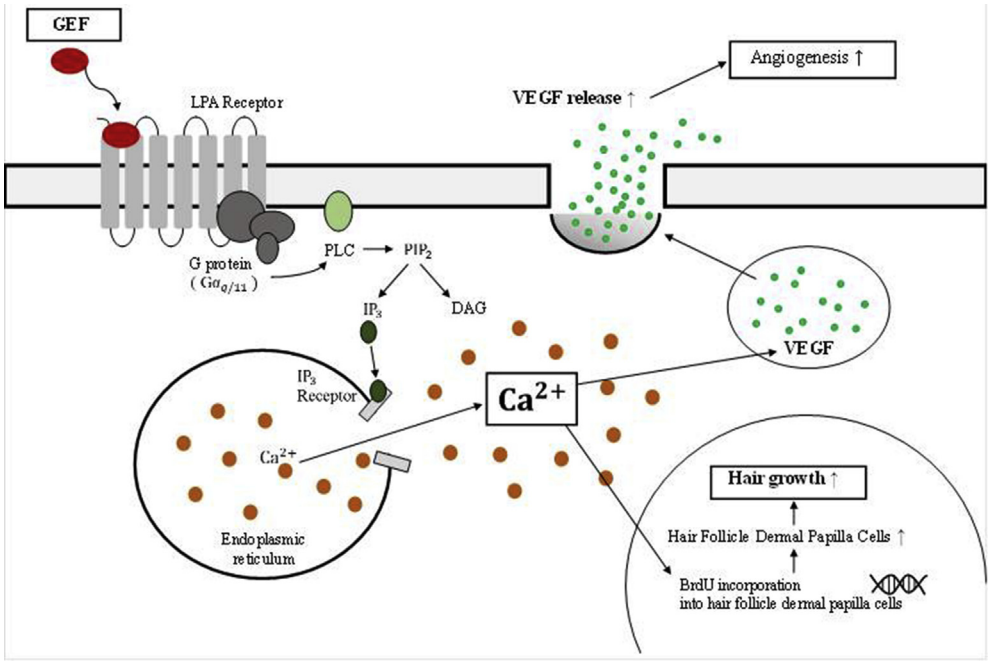

Fig. 6. A schematic illustration of GEF action on the promotion of hair growth. GEF-mediated activation of the LPA receptors such as LPA1, LPA3, and LPA6 is coupled to the induction of transient $[Ca^{2+}]_i$ in HFDPCs and induces cell proliferation and VEGF release. These dual actions of GEF might contribute to the promotion of mouse hair growth. GEF, gintonin-enriched fraction; HFDPCs, human hair follicle dermal papilla cells; LPA, lysophosphatidic acid; VEGF, vascular endothelial growth factor; BrdU, 5-bromo-2′-deoxyuridine; PLC, phospholipase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; IP_3, Inositol-1,4,5-trisphosphate.

the *in vitro* proliferation of HFDPCs partially through LPA1/3 receptors without effects on LPA1/3 receptor expression levels (Figs. 1–3 and Fig. S1).

VEGF plays an important role in angiogenesis and new blood vessel formation [32]. Recent studies showed that the hair follicle cycle is also closely associated with hair growth vascular networks [33–37]. For example, in the anagen phase, VEGF mRNA is expressed strongly in an HFDPC, whereas VEGF expression is decreased in the catagen and telogen phases [35]. Thus, VEGF plays a role as a growth factor for HFDPCs [36]. Interestingly, although mechanisms involved in minoxidil-mediated promotion of hair growth are relatively unexplored, one molecular mechanism is to enhance the expression of VEGF in normal human hair follicles, which may contribute to initiate the anagen phase from the telogen phase [37]. In the present study, we also found that GEF increased VEGF release from HFDPCs (Fig. 3E). Thus, the effect of GEF on VEGF release from HFDPCs may contribute to twofold benefits via the LPA receptor signaling pathway (Fig. 3F): One is to stimulate HFDPC proliferation, and the other is to enhance VEGF release for the conversion of HFDPCs into the anagen phase.

We further investigated whether GEF-mediated stimulation of HFDPC proliferation is closely coupled to *in vivo* hair growth. As shown in Fig. 4A, *in vivo* topical application of GEF promoted hair growth in the synchronized telogen phase mice compared with that in the control. The stimulating effect of GEF on hair growth was similar to that of minoxidil, which was used as a positive control for hair growth. We found that the visual scores for hair growth were significantly higher than those of the control vehicle group and that the actual hair weight measured after 13 days was much higher than that of control vehicle group (Fig. 4B). In addition, histological studies showed that topical application increased the number of hair follicles and BrdU incorporation (Fig. 5). These results suggest that direct topical application of GEF on mouse skin could promote hair growth.

In previous reports, we have shown that gintonin and GEF exhibit many beneficial effects in animal models of neurodegenerative diseases, such as Alzheimer disease and Parkinson disease [38–41]. Oral administration of gintonin or GEF mitigated Alzheimer disease neuropathies by inhibiting the accumulation of amyloid plaques in mice via the activation of the nonamyloidogenic pathway and improved cognitive functions in human dementia [39,40]. One of the molecular mechanisms of anti-Alzheimer disease by GEF is to stimulate the proliferation of *in vitro* hippocampal neural progenitor cells and *in vivo* hippocampal neurogenesis in the hippocampus [22]. Interestingly, oral administration of GEF also attenuated 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced symptoms of Parkinson disease and its molecular mechanisms included GEF-mediated antioxidant and antiinflammatory activities in addition to activation of LPA receptors [40,41]. In central and peripheral nervous systems, GEF stimulated VEGF release from cortical astrocytes and protected astrocytes from hypoxic damage via the VEGF receptor. GEF also stimulated peripheral proliferation and migration of human umbilical vein endothelial cells (HUVECs) and stimulated the release of VEGF for angiogenesis [25,26]. The underlying ginseng-induced angiogenic effects were achieved through gintonin-mediated proliferation, migration, and VEGF release in HUVECs. In the present study, in addition to beneficial effects on brain diseases, we further extended the biological roles of GEF to HFDPC proliferation and hair growth, which is clinically applicable to treat human alopecia (Fig. 4). Taken together, GEF-mediated stimulation of HFDPCs to induce transient $[Ca^{2+}]_i$, cell proliferation, and VEGF release could be some of underlying molecular mechanisms of GEF-mediated promotion of mouse hair growth (Fig. 6).

In summary, we have shown that GEF stimulates *in vitro* transient $[Ca^{2+}]_i$, proliferation of HFDPC, VEGF release, and *in vivo* hair growth in synchronized telogen phase mice. These results indicate that GEF might be an active component involved in hair growth in

Panax ginseng. Finally, GEF can be applied to humans for the prevention of hair loss or the promotion of hair growth.

Conflicts of interest

All authors have no conflict of interest to declare

Acknowledgments

This work was supported by the Konkuk Univeristy in 2019.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2019.05.013.

References

[1] Nah SY. Ginseng ginsenoside pharmacology in the nervous system: involvement in the regulation of ion channels and receptors. Front Physiol 2014;5:98.
[2] Choi SH, Jung SW, Lee BH, Kim HJ, Hwang SH, Kim HK, Nah SY. Ginseng pharmacology: a new paradigm based on gintonin-lysophosphatidic acid receptor interactions. Front Pharmacol 2015;6:245.
[3] Munaron L, Antoniotti S, Lovisolo D. Intracellular calcium signals and control of cell proliferation: how many mechanisms? J Cell Mol Med 2004;8:161–8.
[4] Frederico EHFF, Sá-Caputo DC, Dionello CF, Cardoso ALB, Morel DS, Maiworm A, Guimarães CAS, Santos R, Guedes-de-Aguiar EO, Gonçalves CR, et al. Is There Scientific Evidence that Panax Promotes Hair Growth. Trichol Cosmetol 2016;1:1–7.
[5] Matsuda H, Yamazaki M, Asanuma Y, Kubo M. Promotion of hair growth by ginseng radix on cultured mouse vibrissal hair follicles. Phytother Res 2003;17:797–800.
[6] Kim SN, Kim S, Hong YD, Park H, Shin SH, Kim AR, Park BC, Shin SS, Park JS, Park M, et al. The ginsenosides of Panax ginseng promote hair growth via similar mechanism of minoxidil. J Dermatol Sci 2015;77:132–4.
[7] Park GH, Park KY, Cho HI, Lee SM, Han JS, Won CH, Chang SE, Lee MW, Choi JH, Moon KC, et al. Red ginseng extract promotes the hair growth in cultured human hair follicles. J Med Food 2015;18:354–62.
[8] Pyo MK, Choi SH, Shin TJ, Hwang SH, Lee BH, Kang J, Kim HJ, Lee SH, Nah SY. A simple method for the preparation of crude gintonin from ginseng root, stem, and leaf. J Ginseng Res 2011;35:209–18.
[9] Cho HJ, Choi SH, Kim HJ, Lee BH, Rhim H, Kim HC, Nah SY. Bioactive lipids in gintonin-enriched fraction from ginseng. J Ginseng Res 2019;43:209–17.
[10] Hwang SH, Shin TJ, Choi SH, Cho HJ, Lee BH, Pyo MK, Lee JH, Kang J, Kim HJ, Park CW, et al. Gintonin, newly identified compounds from ginseng, is novel lysophosphatidic acids-protein complexes and activates G protein-coupled lysophosphatidic acid receptors with high affinity. Mol Cells 2012;33:151–62.
[11] Pasternack SM, von Kügelgen I, Al Aboud K, Lee YA, Rüschendorf F, Voss K, Hillmer AM, Molderings GJ, Franz T, Ramirez A, et al. G protein-coupled receptor P2Y5 and its ligand LPA are involved in maintenance of human hair growth. Nat Genet 2008;40:329–34.
[12] Shimomura Y, Wajid M, Ishii Y, Shapiro L, Petukhova L, Gordon D, Christiano AM. Disruption of P2RY5, an orphan G protein-coupled receptor, underlies autosomal recessive woolly hair. Nat Genet 2008;40:335–9.
[13] Fang Y, Vilella-Bach M, Bachmann R, Flanigan A, Chen J. Phosphatidic acid-mediated mitogenic activation of mTOR signaling. Science 2001;294:1942–5.
[14] Takahashi T, Kamimura A, Hamazono-Matsuoka T, Honda S. Phosphatidic acid has a potential to promote hair growth in vitro and in vivo, and activates mitogen-activated protein kinase/extracellular signal-regulated kinase kinase in hair epithelial cells. J Invest Dermatol 2003;121:448–56.
[15] Hadshiew IM, Foitzik K, Arck PC, Paus R. Burden of hair loss: stress and the underestimated psychosocial impact of telogen effluvium and androgenetic alopecia. J Invest Dermatol 2004;123:455–7.
[16] Alonso L, Fuchs E. The hair cycle. J Cell Sci 2006;119:391–3.
[17] Cash TF, Price VH, Savin RC. Psychological effects of androgenetic alopecia on women: comparisons with balding men and with female control subjects. J Am Acad Dermatol 1993;29:568–75.
[18] Messenger AG, Rundegren J. Minoxidil: mechanisms of action on hair growth. Br J Dermatol 2004;150:186–94.
[19] Hirshburg JM, Kelsey PA, Therrien CA, Gavino AC, Reichenberg JS. Adverse effects and safety of 5-alpha reductase inhibitors (finasteride, dutasteride): a systematic review. J Clin Aesthet Dermatol 2016;9:56–62.
[20] Patel S, Sharma V, Chauhan NS, Thakur M, Dixit VK. Hair growth: focus on herbal therapeutic agent. Curr Drug Discov Technol 2015;12:21–42.
[21] Sabouri-Rad S, Sabouri-Rad S, Sahebkar A, Tayarani-Najaran Z. Ginseng in dermatology: a review. Curr Pharm Des 2017;23:1649–66.
[22] Shin DH, Cha YJ, Yang KE, Jang IS, Son CG, Kim BH, Kim JM. Ginsenoside Rg3 up-regulates the expression of vascular endothelial growth factor in human dermal papilla cells and mouse hair follicles. Phytother Res 2014;28:1088–95.
[23] Park PJ, Moon BS, Lee SH, Kim SN, Kim AR, Kim HJ, Park WS, Choi KY, Cho EG, Lee TR. Hair growth-promoting effect of Aconiti Ciliare Tuber extract mediated by the activation of Wnt/β-catenin signaling. Life Sci 2012;91:935–43.
[24] Kim HJ, Kim DJ, Shin EJ, Lee BH, Choi SH, Hwang SH, Rhim H, Cho IH, Kim HC, Nah SY. Effects of gintonin-enriched fraction on hippocampal cell proliferation in wild-type mice and an APPswe/PSEN-1 double Tg mouse model of Alzheimer's disease. Neurochem Int 2016;101:56–65.
[25] Hwang SH, Lee BH, Choi SH, Kim HJ, Won KJ, Lee HM, Rhim H, Kim HC, Nah SY. Effects of gintonin on the proliferation, migration, and tube formation of human umbilical-vein endothelial cells: involvement of lysophosphatidic-acid receptors and vascular-endothelial-growth-factor signaling. J Ginseng Res 2016;40:325–33.
[26] Choi SH, Kim HJ, Cho HJ, Park SD, Lee NE, Hwang SH, Rhim H, Kim HC, Cho IH, Nah SY. Gintonin-mediated release of astrocytic vascular endothelial growth factor protects cortical astrocytes from hypoxia-induced cell damages 1. J Ginseng Res 2019;43:305–11.
[27] Herman A, Herman AP. Mechanism of action of herbs and their active constituents used in hair loss treatment. Fitoterapia 2016;114:18–25.
[28] Peters EM, Arck PC, Paus R. Hair growth inhibition by psychoemotional stress: a mouse model for neural mechanisms in hair growth control. Exp Dermatol 2006;15:1–13.
[29] Lee SW, Juhasz M, Mobasher P, Ekelem C, Mesinkovska NA. A systematic review of topical finasteride in the treatment of androgenetic alopecia in men and women. J Drugs Dermatol 2018;17:457–63.
[30] Oliver RF. The induction of hair follicle formation in the adult hooded rat by vibrissae dermal papillae. J Embryol Exp Morphol 1970;23:219–36.
[31] Jahoda CAB, Reynolds AJ. Induction of follicle formation and hair growth by vibrissa dermal papillae implanted into rat ear wounds: vibrissae-type fibers are specified. Development 1992;115:1103–9.
[32] Dvorak HF, Brown LF, Dermar M, Dvorak AM. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. Am J Pathol 1995;146:1029–39.
[33] Ellis RA, Moretti G. Vascular patterns associated with catagen hair follicles in the human scalp. Ann N Y Acad Sci 1959;83:448–57.
[34] Stenn KS, Fernandez LA, Tirrell SJ. The angiogenetic properties of the rat vibrissa hair follicle associate with the bulb. J Invest Dermatol 1988;90:409–11.
[35] Yano K, Brown LF, Detmar M. Control of hair growth and follicle size by VEGF-mediated angiogenesis. J Clin Invest 2001;107:409–17.
[36] Lachgar S, Charveron M, Ceruti I. Distribution of VEGF mRNA indifferent hair follicle stages by fluorescence in situ hybridization combined with confocal laser microscopy. In: Van Neste DJJ, Randall VA, Baden H, Ogawa H, Oliver R, editors. Hair research for the next millennium. Amsterdam: Elsevier; 1996. p. 407–12.
[37] Lachgar S, Moukadiri H, Jonca F, Charveron M, Bouhaddioui N, Gall Y, Bonafe JL, Plouët J. Vascular endothelial growth factor is an autocrine growth factor for hair dermal papilla cells. J Invest Dermatol 1996;106:17–23.
[38] Hwang SH, Shin EJ, Shin TJ, Lee BH, Choi SH, Kang J, Kim HJ, Kwon SH, Jang CG, Lee JH, et al. Gintonin, a ginseng-derived lysophosphatidic acid receptor ligand, attenuates Alzheimer's disease-related neuropathies: involvement of non-amyloidogenic processing. J Alzheimer's Dis 2012;31:207–23.
[39] Moon J, Choi SH, Shim JY, Park HJ, Oh MJ, Kim M, Nah SY. Gintonin administration is safe and potentially beneficial in cognitively impaired elderly. Alzheimer Dis Assoc Disord 2018;32:85–7.
[40] Choi JH, Jang M, Oh S, Nah SY, Cho IH. Multi-target protective effects of gintonin in 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine-Mediated model of Parkinson's disease via lysophosphatidic acid receptors. Front Pharmacol 2018;9:515.
[41] Jo MG, Ikram M, Jo MH, Yoo L, Chung KC, Nah SY, Hwang H, Rhim H, Kim MO. Gintonin mitigates MPTP-induced loss of nigrostriatal dopaminergic neurons and accumulation of α-synuclein via the Nrf2/HO-1 pathway. Mol Neurobiol 2019;56:39–55.