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Ginsenoside Rb1 promotes the growth of mink hair follicle via PI3K/AKT/GSK-3β signaling pathway

Haihua Zhang[a,b], Yongmei Su[a], Jiantao Wang[c], Ying Gao[d], Fuhe Yang[b], Guangyu Li[b,*], Qiumei Shi[a,**]

[a] College of Animal Science and Technology, Hebei Normal University of Science and Technology, Qinhuangdao 066004, People's Republic of China
[b] State Key Laboratory of Special Economic Animal Molecular Biology, Institute of Special Animal and Plant Sciences, Chinese Academy of Agricultural Sciences, Changchun 130112, People's Republic of China
[c] Tangshan Municipal Station of Animal Husbandry, Tangshan 063000, People's Republic of China
[d] Tangshan Animal Health Supervision Institute, Tangshan 063004, People's Republic of China

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ABSTRACT

Aims: Hair follicles play a critical role in the process of hair growth. The dermal papilla cells (DPCs) are an important component in the hair follicle regeneration and growth. This study investigated the effects of ginsenoside Rb1 on the growth of cultured mink hair follicles and DPCs.

Main methods: The mink hair follicles were treated with ginsenoside Rb1 for 9 days and their lengths were measured every three days. Real-time PCR was used to determine the mRNA expression of vascularization endothelial growth factor A (VEGF-A), VEGF receptor 2 (VEGF-R2) and TGF-β1. In addition, the levels of proteins were detected by western blot. Cell proliferation was determined by immunofluorescence staining of proliferation marker Ki-67 and cell cycle analysis was performed on flow cytometry. Moreover, cell migration was evaluated by wound healing assay.

Key findings: Ginsenoside Rb1 promoted the growth of hair follicles, and proliferation and migration of DPCs. Ginsenoside Rb1 improved the expression levels of VEGFA and VEGF-R2, while attenuated the TGF-β1 expression both in hair follicles and DPCs. Furthermore, ginsenoside Rb1 facilitated the activation of PI3K/AKT/GSK-3β signaling pathway in hair follicles and DPCs.

Significance: The results reveals a crucial role of PI3K/AKT/GSK-3β signaling pathway in ginsenoside Rb1-induced growth of hair follicles and DPCs.

1. Introduction

Hair follicles have the ability of periodically regeneration and regulate the hair cyclic process, including anagen, catagen and telogen [1]. The dermal papilla cells (DPCs) are dermis mesenchymal cells located at the base of the hair follicles, and have the functions of controlling hair growth cycle and inducing hair follicle regeneration [2,3]. Therefore, DPCs are often used in in vitro cell model for studying hair follicle growth. Many studies have reported the effects on hair follicles and DPCs activity, such as cell proliferation and migration, which affect the hair follicle regeneration and growth. It is reported that regeneration of hair follicles in mice is activated by increasing the hair follicle stem cell proliferation through AKT and Wnt/β-catenin signaling in vivo [3]. Study also presented that the proliferation of human DPCs could be upregulated via activating ERK and AKT and stimulating phosphoinositide 3-kinase (PI3K)/AKT pathway [4]. Cell migration is also a critical property of DPCs for hair follicle growth [5], and it has been known that the cell migration could be improved by stimulating the activation of PI3K and AKT pathway [6,7].

Several associated growth factors and signaling proteins were also involved in the growth of hair follicle and DPCs. On the one hand, the expression levels of vascularization endothelial growth factor A (VEGFA) and VEGF receptor 2 (VEGF-R2) normally are increased when the hair follicle growth is promoted, because VEGF is needed to provide adequate nutrition for cell division of the hair follicles in anagen phase for hair growth and it is able to regulate the perifollicular

* Correspondence to: G. Li, State Key Laboratory of Special Economic Animal Molecular Biology, Institute of Special Animal and Plant Sciences, Chinese Academy of Agricultural Sciences, 4899 Juye Avenue, Changchun 130112, People's Republic of China.
** Correspondence to: Q. Shi, College of Animal Science and Technology, Hebei Normal University of Science and Technology, 360 West Section, Hebei Avenue, Qinhuangdao 066004, People's Republic of China.
E-mail addresses: li_guangyu123@sina.com (G. Li), 94195601@qq.com (Q. Shi).

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vascularization and vessel size, leading to changing the hair follicle size [8,9]. VEGF-R2 stimulates VEGF signaling to promote cell cycle progression in endothelial cells through several downstream signaling pathways [10]. In addition, cell migration also could be upregulated by VEGF binding to its receptors [11]. On the other hand, TGF-β1 is a secreted polypeptide with multifunctions, such as regulation of cell proliferation, cell differentiation and inhibition of the cell growth by inducing cell cycle arrest [12,13]. Both in vitro and in vivo studies have shown that TGF-β1 is a possible negative regulator in hair growth [13,14]. In contrary, glycogen synthase kinase-3β (GSK-3β) is a protein kinase for glycogen metabolism, and it can affect the cell proliferation, apoptosis and migration via GSK-3β related signaling-pathway, especially in carcinomas [15,16]. There are very close correlations between these associated growth factors and signaling proteins. It has been suggested that VEGFR2 can be autophosphorylated after binding VEGF-A, leading to the increase in VEGFR2 tyrosine kinase activity and the recruitment of downstream molecules, including PI3K/AKT [17]. In addition, GSK-3β integrates extracellular and intracellular signals and regulates the expression of various growth factors such as VEGF [18].

Ginseng Radix et Rhizoma (GRR), mainly found in East Asia and North America, is a well-known and traditionally used herbal drug. Ginsenosides (such as Rb1, Rb2, Rb3, Rd, Re, Rg1, Rg3, Rh2) are one kind of main active compounds in GRR and confirmed to have multiple pharmacological effects, such as anticarcinoma [19], immunoregulation [20], antioxidant [21], lowering serum lipid [22] and anti-aging [23]. Up to now, > 200 ginsenosides have been isolated and identified. Ginsenoside Rb1 is one of the representative components of ginsenosides in ginseng roots and berries from different species, such as *Panax ginseng* and *P. quinquefolius* [24,25]. Large numbers of studies have reported that ginsenoside Rb1 presents a variety of properties including anti-oxidation, anti-tumor, anti-apoptosis, anti-autophagy and other biological activities [26–28]. More importantly, it has been reported that ginsenoside Rb1 has the potency to promote hair growth through enhancing hair follicle cells proliferation by promoting AKT activation in human DPCs, and up-regulating p63 expression in follicular keratinocytes ([29,30].

In this work, the effect of ginsenoside Rb1 on mink hair follicle growth and its potential mechanisms were studied.

2. Materials and methods

2.1. Compounds and antibodies

Ginsenoside Rb1 and goat serum were purchased from Solarbio (Beijing, China). PI3K inhibitor LY294002 was obtained from MCE (Monmouth Junction, NJ, USA). p-PI3K antibody and Ki-67 antibody were purchased from Abcam (Cambridge, UK). β-actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). PI3K antibody, p-GSK-3β antibody and GSK-3β antibody were obtained from Cell Signaling Technology (Danvers, MA, USA). p-AKT antibody and AKT antibody were purchased from Bioss (Beijing, China). Cyclin A antibody, VEGF-A antibody, VEGF-R2 antibody and TGF-β1 antibody were purchased from Wanleibio (Shenyang, China). Cyclin B1 antibody and Cyclin D1 antibody were purchased from Boster (Wuhan, China). Cy3-conjugated goat anti-rabbit IgG, goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP were obtained from Beyotime (Shanghai, China).

2.2. Culture of hair follicles and DPCs

Anagen hair follicles were acquired from 4-month-old, male neovison visons (American minks). Small pieces of dorsal skin (about 1 cm^2) without hair shafts and subcutaneous fat were harvested, rinsed in phosphate buffered saline (PBS) containing 100 U/ml penicillin and 0.1 mg/ml streptomycin, disinfected with iodine, discolored with 75% ethanol and then digested with Collagenase D (0.2 mg/ml; Sigma, St. Louis, MO, USA) at 4_C overnight. The complete hair follicles were selected and cultured in 35 mm culture dishes in a humid atmosphere at 37 °C with 5% CO_2 with Williams E medium (Gibco, Grand Island, NY, USA) supplemented with 10 μg/ml insulin, 10 ng/ml sodium selenite, 10 ng/ml hydrocortisone, 2 mM L-glutamine, 10 μg/ml transferring, 100 U/ml penicillin and 0.1 mg/ml streptomycin and all the chemicals were purchased from Sigma (St. Louis, MO, USA). Each group contained 15 hair follicles and they were cultured with or without 10 μg/ml of ginsenoside Rb1 in a humidified incubator at 37 °C and 5% CO_2 for 9 days. The culture medium was changed every two days and the measurement of lengths of the hair follicles were performed by blinding observer who did not know the experimental groups using a micrometer under an inverted microscope every three days.

DPCs were isolated from dorsal skin. Briefly, dorsal skin was cut into pieces, digested with Dispase II (0.5 mg/ml, Sigma) at 4 °C overnight, and then incubated at 37 °C for additional 30 min. Then the tissue pieces were minced and digested with Collagenase D (0.2 mg/ml) at 37 °C for 6 h to dissociate DPCs. Subsequently, the DPCs were rinsed with PBS, filtered through a 75-mm filter, and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Hyclone) in a humid atmosphere at 37 °C with 5% CO_2. Then the DPCs were seeded to 6 well-plates and cultured with 0 and 10 μg/ml ginsenoside Rb1 for 96 h. For studying the effects of ginsenoside Rb1 on PI3K/AKT/GSK-3β pathway, DPCs were also treated with the PI3K inhibitor 10 μM LY294002 or 10 μg/ml ginsenoside Rb1 + 10 μM LY294002, respectively. In the ginsenoside Rb1 and LY294002 combination group, the cells were pre-treated with 10 μM LY294002 for 1 h, then treated with 10 μg/ml ginsenoside Rb1 and cultured for another 96 h.

2.3. Real-time PCR (RT-PCR)

Total RNA was extracted from hair follicles or DPCs with TRIpure (BioTeke, Beijing, China) and the cDNA was generated from total RNA after reverse transcription. Quantitative detection was performed using 2 × Power Taq PCR MasterMix (BioTeke) in an Exicycler™ 96 fluorescence quantitative analyzer instrument (Bioneer, Daejeon, Korea). β-actin was chosen as an internal control. RT-PCR assays were carried out with the following primers synthesized by Sangon Biotech (Shanghai, China), and the sequences are as follow: VEGFA-forward, 5′-GGCAGA CTATTCAGCGGACTC-3′; VEGFA-reverse, 5′-CTCAAACCGTTGG.

CACGAT-3′; VEGF-R2-forward, 5′-TCTCCACCTTCAAAGTCTCA-3′; VEGF-R2-reverse, 5′-TCCCACTACCGAAAGCAAT-3′; TGF-β1-forward, 5′-GCA

ACAATTCCTGGCGTTACCT-3′; TGF-β1-reverse, 5′-GAAAGCCCTGT ATTCCG

TCTCC-3′; β-actin-forward, 5′-CTGTGCCCATCTACGAGGGCTAT-3′; β-actin-reverse, 5′-TTTGATGTCACGCA CGATTTCC-3′. The $2^{-\triangle\triangle}$CT method was used for analyzing the mRNA.

2.4. Western blot

Western blot was used to determine the protein expression in the hair follicles and DPCs. Briefly, total proteins of cells from each group were extracted by RIPA lysis buffer (Beyotime) and the protein concentrations were determined using BCA protein concentration assay kit (Beyotime). 40 μg protein per sample was separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The PVDF membranes for p-PI3K, p-AKT and p-GSK-3β detection were blocked and incubated with first and second antibodies in 1% BSA, and the other membranes were used 5% no-fat milk. The membranes were blocked at RT for 1 h and incubated with primary antibodies p-PI3K (1:1000, ab182651, Abcam), p-GSK-3β (1:1000, #9323, Cell Signaling Technology), p-AKT (1:500, bsm-33281M, Bioss), cyclin B1 (1:400, BA0766, Boster), cyclin D1 (1:400, BA0770, Boster), cyclin A (1:500, WL01841, Wanleibio), VEGF-A (1:500, WL00009b, Wanleibio), VEGF-

R2 (1:500, WL03212, Wanleibio), AKT (1:500, bs-0115R, Bioss), β-actin (1:500, sc-47778, Santa Cruz), TGF-β1 (1:500, WL01076a, Wanleibio), PI3K (1:1000, #4292, Cell Signaling Technology) or GSK-3β (1:1000, #12456, Cell Signaling Technology) respectively at 4 °C overnight. After washing with buffer, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000) at 37 °C for 45 min. Signal intensity was determined using a gel image processing system (Gel-Pro-Analyzer software).

2.5. Cell cycle analysis

The DPCs were seeded and treated with DMSO, ginsenoside Rb1 and LY294002 as mentioned above. After 96 h incubation, the cells from each group were harvested and fixed with ice-cold 70% ethanol at 4 °C for 2 h. The fixed cells were washed with PBS and centrifuged at 310*g* for 5 min. 500 μl binding buffer was used to resuspend cells, then 25 μl propidium iodide (PI) and 10 μl RNase A was added to cells, followed by incubation at 37 °C for 30 min in darkness. The flow cytometry analysis of the samples was performed after incubation.

2.6. Wound healing assay

The DPCs from each test group were treated with serum-free medium containing 1 μg/ml mitomycin C (Sigma) for 1 h. A wound was gently made in the cell monolayer by scratching with a 200 μl sterile pipette tip and the cells were washed with serum-free medium once. The cells were cultured with serum-free medium for 48 h in a humidified incubator at 37 °C and 5% CO_2. The wounded area was photographed under microscope and the migration distances of the cells were calculated at 0 and 48 h.

2.7. Immunofluorescence

Cells were fixed with 4% paraformaldehyde for 15 min and washed with PBS to remove paraformaldehyde. 0.1% tritonX-100 was added to the cells at RT for 30 min to break the cell membrane. After washing with PBS, the cells were incubated with goat serum (Solarbio) at RT for 15 min, followed by incubation with Ki-67 antibody (1:200) at 4 °C overnight. The cells were incubated with 200 times dilution Cy3-conjugated goat anti-rabbit IgG at RT for 1 h. After removing the secondary antibody, the cells were added with DAPI (Sigma) to counterstain the cell nucleus. The cells were observed and photographed using the microscope (Olympus, Tokyo, Japan).

2.8. Statistical analysis

All values were expressed as mean ± standard deviation (SD), and analyzed with the GraphPad Prism software. The unpaired student's *t*-test (two-tailed) was used to analyze the data between two groups, while one-way ANOVA followed by Bonferroni post hoc test was performed when data among multiple groups. Statistical significance was considered as p value < 0.05.

3. Results

3.1. Effects of ginsenoside Rb1 on cell proliferation and migration of DPCs

We determined the effects of ginsenoside Rb1 on the proliferation of DPCs. As presented in Fig. 1A, cell viability was enhanced be treatment with various concentrations of Rb1. 10 μg/ml Rb1 showed the strongest promoting effect on proliferation. To evaluate the effect of ginsenoside Rb1 on the DPC migration, wound healing assay was performed. Based on the results in Fig. 3B, the wound distance was decreased with treatment of ginsenoside Rb1 after 48 h, and the cell migration rate was increased approximately 50% by Rb1 (10 μg/ml) comparing with the control cells (**$p < 0.01$). As illustrated in Fig. 3C, ginsenoside Rb1 with various doses increased proportion of the cells in the S phase comparing with control group (for 5 μg/ml, *$p < 0.01$; for 10 μg/ml, **$p < 0.01$). The cells in G2-M phase of the cell cycle were also significantly decreased (*$p < 0.05$ for all). As assessed by immunofluorescence staining of Ki-67 and shown in Fig. 3D, the expression level of Ki-67 in the DPCs was increased after ginsenoside Rb1 treatment, and the proportion of Ki-67 positive cells was significantly raised comparing with the control group (**$p < 0.01$).

The expressions of cyclin B1, cyclin D1 and cyclin A in the DPCs with or without ginsenoside Rb1 treatment were detected by western blot (Fig. 3E). We found that the expressions of cyclin B1, cyclin D1 and cyclin A were significantly increased in ginsenoside Rb1 group comparing with control group (***$p < 0.001$). According to the above experimental results, ginsenoside Rb1 could facilitate the proliferation and migration of DPCs.

3.2. Effects of ginsenoside Rb1 on the expressions of VEGF-A, VEGF-R2, and TGF-β1 in DPCs

The mRNA and protein levels of VEGF-A, VEGF-R2 and TGF-β1 with ginsenoside Rb1 treatment were detected using RT-PCR and western blot. As presented in Fig. 2A, compared with the control group, VEGF-A and VEGF-R2 mRNA expression levels were significantly increased (***$p < 0.001$) after ginsenoside Rb1 treatment, while TGF-β1 mRNA expression was markedly reduced (***$p < 0.001$). As shown in Fig. 2B, VEGF-A and VEGF-R2 protein levels were remarkably upregulated in ginsenoside Rb1 group compared with control group (**$p < 0.01$, ***$p < 0.001$), whereas TGF-β1 protein level was markedly reduced (***$p < 0.001$).

3.3. Effects of ginsenoside Rb1 on the activation of PI3K/AKT/GSK-3β signaling pathway in DPCs

The protein levels of related proteins in PI3K/AKT/GSK-3β signaling pathway were determined. After ginsenoside Rb1 treatment, the levels of p-PI3K, p-AKT and p-GSK-3β were increased significantly comparing with control group (**$p < 0.01$) (Fig. 3). The results indicated that ginsenoside Rb1 improved the activation of PI3K/AKT and inactivation of GSK-3β signaling pathway.

3.4. Ginsenoside Rb1 promotes proliferation and migration of DPCs via PI3K/AKT/GSK-3β signaling pathway

To investigate whether ginsenoside Rb1 affects DPC proliferation and migration through the PI3K/AKT/GSK-3β signaling pathway, the DPCs were co-treated with Rb1 and PI3K inhibitor LY2940002, or pretreated with Rb1 before LY2940002. As shown in Fig. 4A, B and C, compared with the control group, VEGFA, VEGF-R2 and p-AKT protein levels were significantly increased (***$p < 0.001$) in ginsenoside Rb1-treated cells, which were significantly reduced (*$p < 0.05$) after co-treatment with LY2940002 (^$p < 0.01$, ^^$p < 0.001$).

As presented in Fig. 4D, compared with control group, the expression of Ki-67 in ginsenoside Rb1 group was increased (***$p < 0.001$), which was suppressed in ginsenoside Rb1 + LY2940002 group (^$p < 0.05$). Flow cytometry analysis was used to detect the cell cycle progression and the results were showed in Fig. 4E. Ginsenoside Rb1-induced decrease in G0-G1-arrested cells and increase in S phase were significantly inhibited after co-treatment with LY2940002. As shown in Fig. 4F, cell migration ability was significantly higher in ginsenoside Rb1 treatment cells (**$p < 0.01$), which was reduced in Rb1 + LY2940002 treatment cells (^$p < 0.01$). The incubation with LY2940002 after the pretreatment with Rb1 also restrained Rb1-induced cell cycle change and increased migration ability (Supplementary Fig. 1A & B). The above results implied that ginsenoside Rb1 promoted the proliferation and migration of DPCs through regulating the PI3K/AKT/GSK-3β signaling pathway.

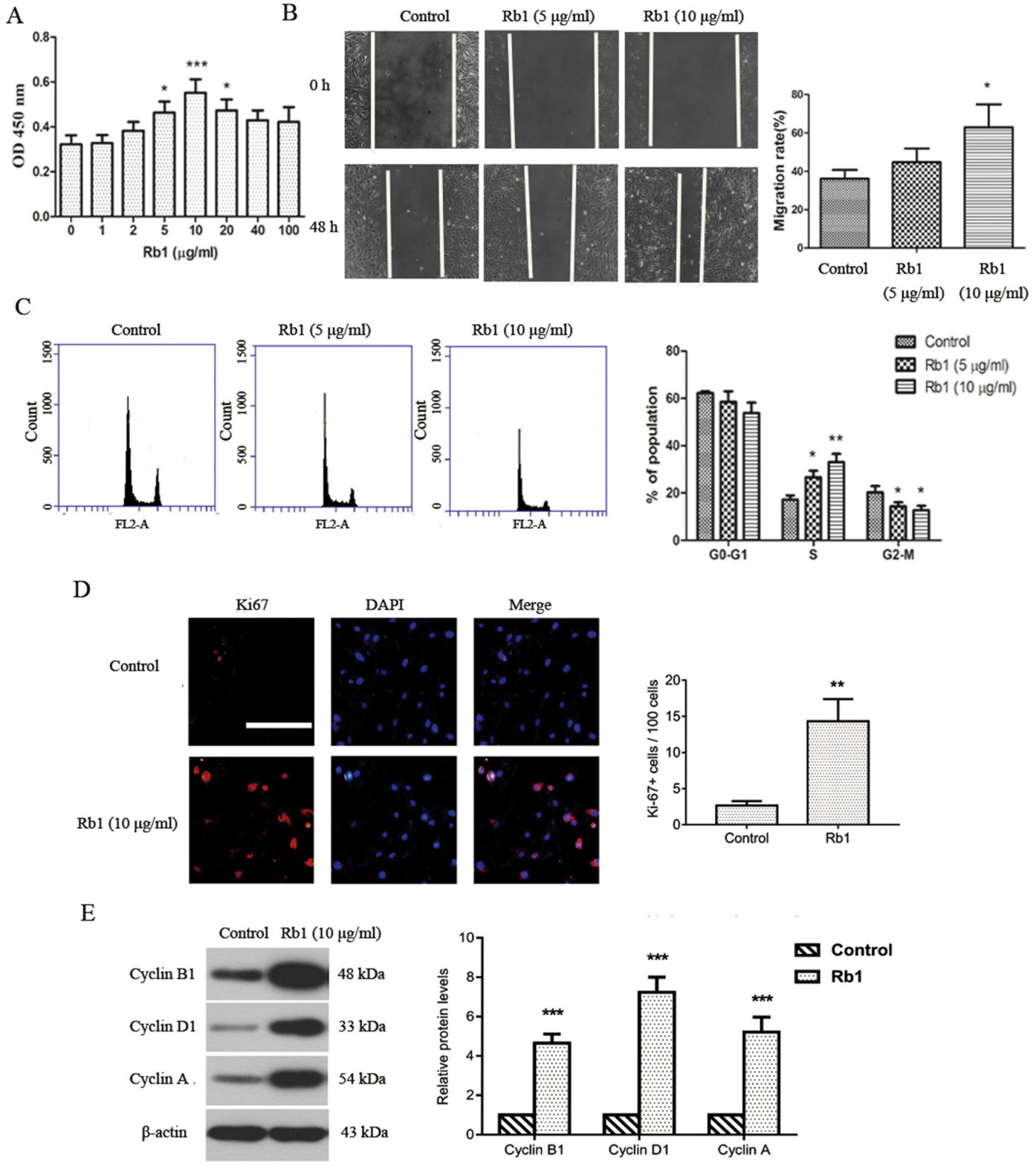

Fig. 1. Ginsenoside Rb1 promoted cell proliferation and migration of DPCs. (A) The proliferation of DPCs was detected by CCK8 assay. (B) The migration rate of DPCs was detected by wound healing assay. (C) The cell cycle of Rb1-treated DPCs was determined by flow cytometry. (D) Representative immunofluorescence staining results for Ki-67 of DPCs incubated with Rb1. (E) Western blot was employed to measure the levels of cyclins in DPCs. Data were expressed as mean ± SD (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 versus control group.

3.5. Effects of ginsenoside Rb1 on the growth of mink hair follicles

To study the effects of ginsenoside Rb1 on hair follicle growth, hair follicles were treated with or without 10 μg/ml of ginsenoside Rb1 for 9 days. Hair follicle growth was measured every 3 days and expression levels of related cell factors were determined. From the results of Fig. 5A, after ginsenoside Rb1 treatment, the growth lengths of hair follicles were significantly increased, especially on the day 3 and 5,

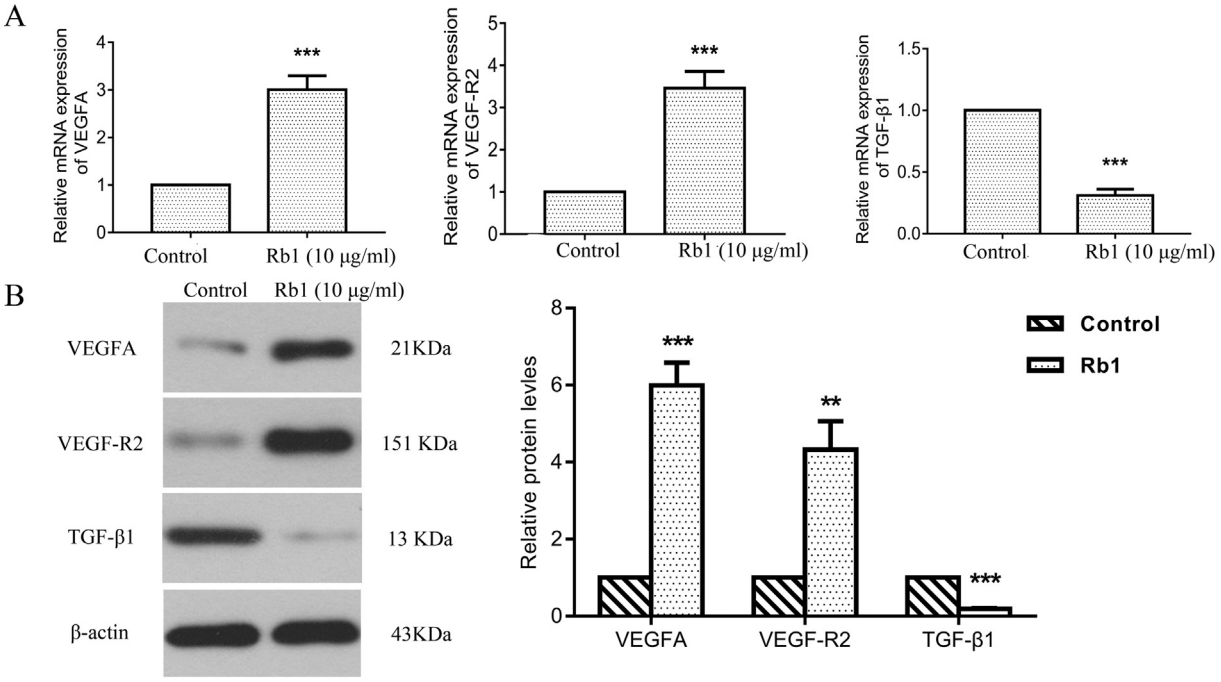

Fig. 2. Ginsenoside Rb1 treatment induced the up-regulation of VEGFA and VEGF-R2 and the down-regulation of TGF-β1. (A) Real-time PCR was used to detect the mRNA levels of VEGFA, VEGF-R2 and TGF-β1 in DPCs treated with Rb1. (B) Representative western blot and analysis for VEGFA, VEGF-R2 and TGF-β1. Data were expressed as mean ± SD (n = 3). **p < 0.01, ***p < 0.001 versus control group.

compared with the control group (***p < 0.001). In Fig. 5B, compared with the control group, the expression levels of VEGFA and VEGF-R2 mRNA in ginsenoside Rb1 group were remarkably elevated (**p < 0.01), while TGF-β1 mRNA expression was markedly reduced (**p < 0.01). Consistent with their transcriptional level results, the protein expression levels of VEGFA and VEGF-R2 in ginsenoside Rb1 group was significantly increased (***p < 0.001), and the expression of TGF-β1 protein was markedly decreased (***p < 0.001) in western blot (Fig. 5C).

Western blot was also used to analyzed the effect of ginsenoside Rb1 on the activation of PI3K/AKT/GSK-3β signaling pathway in hair follicles. Based on the results from Fig. 6, the levels of p-PI3K, p-AKT and p-GSK-3β all were remarkably increased after ginsenoside Rb1 treatment in hair follicles comparing with the control group (*p < 0.05, **p < 0.01). According to the above experimental results, ginsenoside Rb1 promoted the growth of hair follicles.

4. Discussion

The effects of ginsenoside Rb1 on hair follicle growth and its possible mechanism have been studied in this work. According to the results, ginsenoside Rb1 promoted the growth of hair follicles. In addition, the expression levels of VEGFA and VEGF-R2 were markedly up-regulated both in hair follicles and DPCs, while the TGF-β1 expression was decreased. Ginsenoside Rb1 also stimulated the expressions of cyclin B1, cyclin D1 and cyclin A, increased the proportion of cells in S phase and improved the cell migration. Furthermore, the phosphorylation of PI3K, AKT, and GSK-3β in ginsenoside Rb1-treated hair follicles and DPCs were increased. The above results suggested that ginsenoside Rb1 promoted hair follicle growth and improved DPC proliferation and migration via PI3K/AKT/GSK-3β signaling pathway.

Hair follicles play an important role in hair growth and the DPCs are the critical components of the hair follicles for controlling the hair growth cycle and inducing hair follicle regeneration [2,3]. Many efforts have worked on improving hair follicle growth and DPCs activity. VEGF and VEGF-R2 are reported as the positive regulator for promoting the

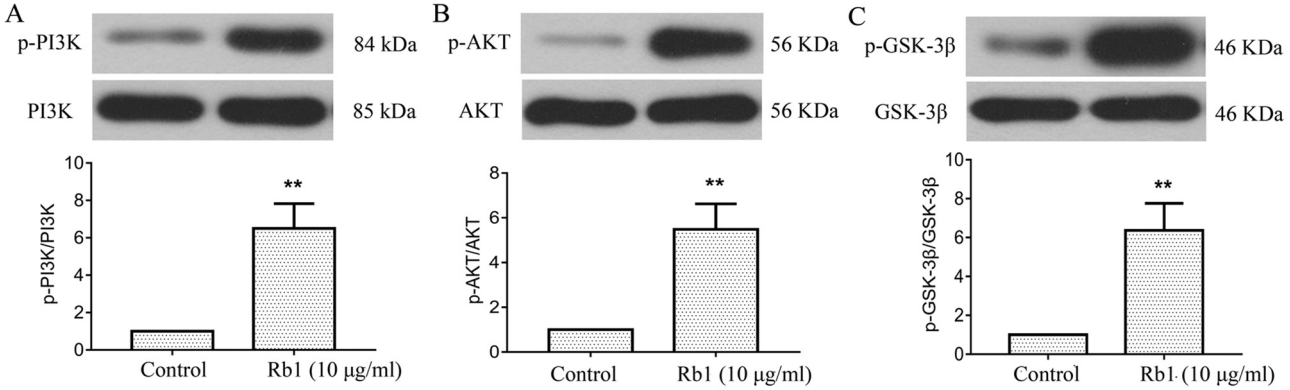

Fig. 3. Ginsenoside Rb1 treatment activated the PI3K/AKT/GSK-3β signaling pathway in DPCs. The phosphorylation levels of (A) PI3K, (B) AKT and (C) GSK-3β in DPCs were measured by western blot. Data were expressed as mean ± SD (n = 3). **p < 0.01versus control group.

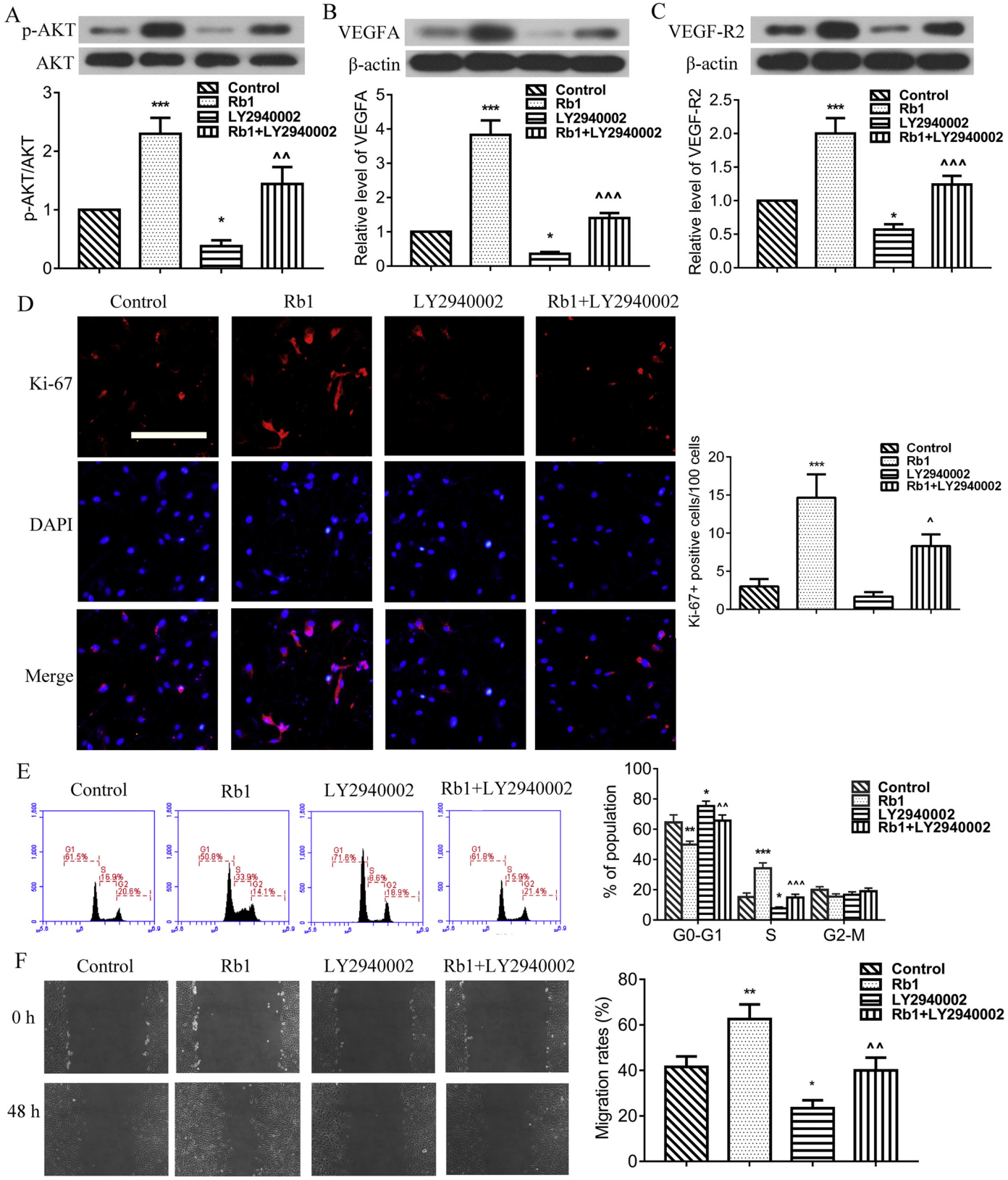

Fig. 4. The activation of PI3K/AKT/GSK-3β signaling pathway was essential for Ginsenoside Rb1-mediated proliferation and migration of DPCs. (A) LY2940002 deactivated the AKT pathway in DPCs. The expression levels of (B) VEGFA and (C) VEGF-R2 in Rb1-treated DPCs were decreased in the present of LY2940002. (D) The Ki-67 positive DPCs were detected by immunofluorescence staining. (E) The cell cycle of DPCs in different group was measured by flow cytometry. (F) The LY2940002 repressed Rb1-induced increased migration rate of DPCs. Data were expressed as mean ± SD (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 versus control group; ^p < 0.05, ^^p < 0.01, ^^^p < 0.001 versus Rb1 group.

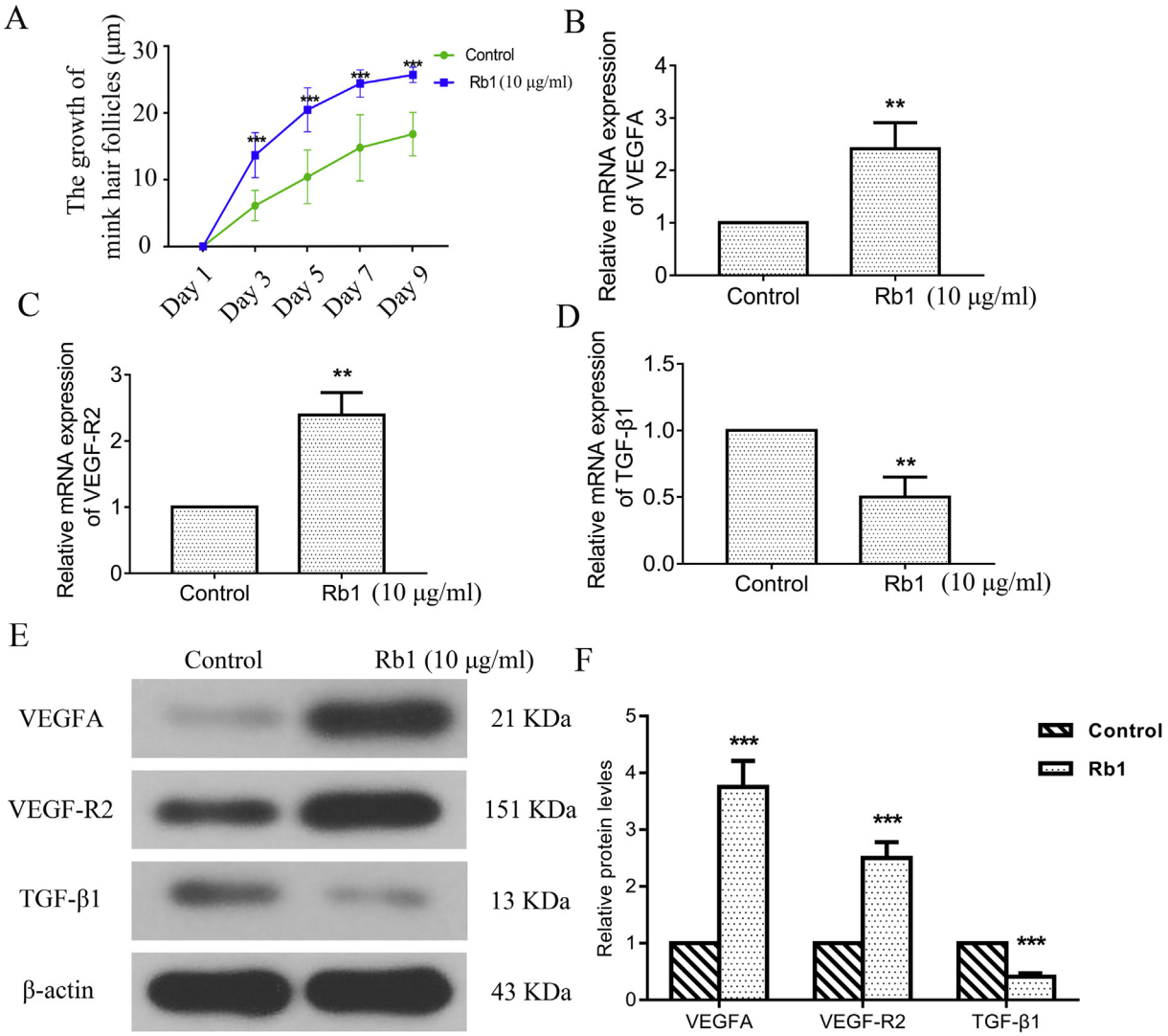

Fig. 5. Ginsenoside Rb1 treatment facilitated growth of the mink hair follicles. (A) The growth of mink hair follicles was enhanced in the present of Rb1. (B) The mRNA levels of VEGFA, VEGF-R2 and TGF-β1 was measured by RT-PCR. (C) The protein levels of VEGFA, VEGF-R2 and TGF-β1 was determined by western blot. Data were expressed as mean ± SD (n = 8). **p < 0.01, ***p < 0.001 versus control group.

hair growth by supporting nutrition for cell division of the hair follicles in anagen phase [8]. Li Wei et al. also found that VEGF binding with VEGF-R2 also could promote cell proliferation by VEGF-R2/ERK signaling pathway in human DPCs [31]. Another growth factor TGF-β1 is capable of down-regulating the cell growth and it has been proved that TGF-β1 inhibits hair follicles cycling and induces differentiation of rat DPCs into fibroblasts [13,32,33]. Large numbers of natural compounds or herbal extracts promote hair follicles growth and DPCs proliferation

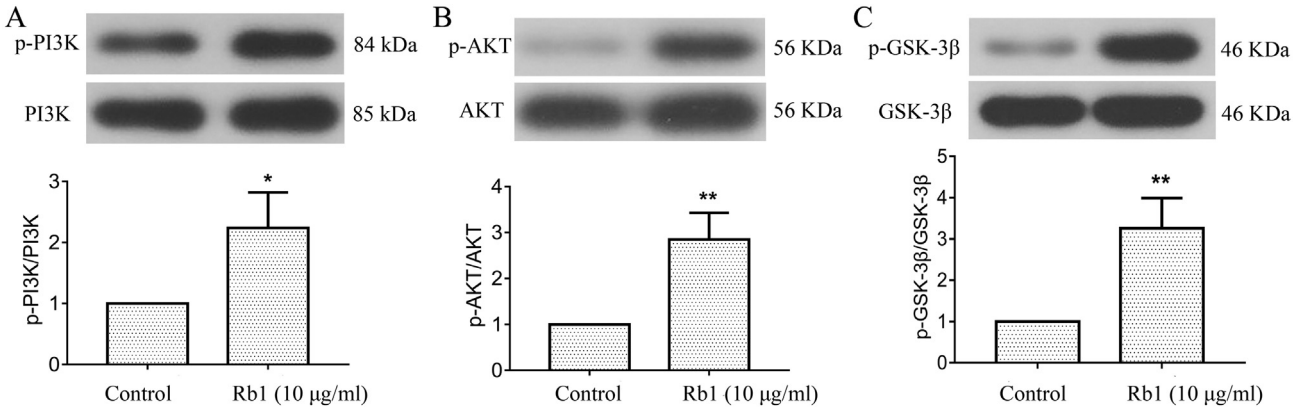

Fig. 6. Ginsenoside Rb1 treatment induced the activation of PI3K/AKT/GSK-3β axis in mink hair follicles. Western blot was used to detect the phosphorylation levels of (A) PI3K, (B) AKT and (C) GSK-3β. Data were expressed as mean ± SD (n = 3). *p < 0.05, **p < 0.01 versus control group.

and migration through AKT activation, which is the key factor for cell biological functions in multiple cell types [3,6,34]. The expression and activation of ERK, PI3K and GSK-3β, β-catenin related to AKT signaling pathway all become the important factors for hair growth. Hosein Rastegar et al. reported that hair follicle DPCs proliferation can be improved by ERK and AKT activation [35]. In addition, M. H. Kwack et al. found that PI3K upregulates L-Ascorbic acid 2-phosphate-inducible insulin-like growth factor-1 (IGF-1) to promote elongation of hair shafts of DPCs [36]. Moreover, GSK-3β acts as a downstream effector of the PI3K/AKT pathway and negatively regulates the β-catenin, a key factor for morphogenetic formation of the hair follicles and differentiation of stem cells, and therefore phosphorylation of GSK-3β is one of the critical reaction steps for hair follicle growth and DPCs activation [37,38]. These findings indicated that hair follicle growth and DPCs activation are mainly mediated by proteins of related with AKT signaling pathways, which is consistent with our results.

Ginsenoside Rb1, as a member of ginsenosides, presents various biological activities, such as inhibiting oxidative stress, preventing of apoptosis, and improving of endothelial dysfunction ([27,28,39]. It has been reported that ginsenosides Rb1 and Rd positively mediate p63 expression to promote cell proliferation of human hair follicles [29]. In addition, ginsenoside Rb1 is also considered having the ability to enhance keratinocyte migration for skin wound repair via the sphingosine-1-phosphate/ERK/NF-*k*B/MMP-2/-9 pathway [40]. The effects of ginsenoside Rb1 on expression and function of VEGF, VEGF-R2 and TGF-β1, as well as the signaling protein PI3K, AKT and GSK-3β have also been reported. Ginsenoside Rb1 could stimulate VEGFA and VEGF-R2 production to promote angiogenesis and reduce TGF-β1 to attenuate its fibrogenic activity skin wound healing [41]. Additionally, studies reported that ginsenoside Rb1 reduces myocardial ischemia and reperfusion injury and homocysteine-induced endothelial dysfunction by activating the PI3K/AKT pathway [39,42]. Furthermore, ginsenoside Rb1 also prevents the oxidative stress by activating the PI3K/Akt/Nrf2 pathway both in aged rats' skeletal muscle cells and human dopaminergic cells [43,44]. The results of the research of Kong Hongliang and his colleagues suggested that ginsenoside Rb1 significantly inhibits hypoxia-induced mitochondria permeability transition pore opening via promoting GSK-3β phosphorylation [27]. Therefore, according to the research reports and our experimental results, the possible mechanism of ginsenoside Rb1 promoting hair follicle growth, DPC proliferation and migration is by promoting the expressions of VEGFA and VEGF-R2, and directly or indirectly activating the PI3K/AKT/GSK-3β pathway. Briefly, ginsenoside Rb1 stimulated VEGF-R2-mediated VEGFA to promote cell proliferation and mitogen, and activated the PI3K/AKT pathway to improve cell survival [45]. Ginsenoside Rb1 also could increase the phosphorylation of PI3K and AKT, then GSK-3β phosphorylation is enhanced by p-AKT activation. The phosphorylated GSK-3β loses the ability to activate β-catenin phosphorylation, leading to accumulate β-catenin in nucleus to upregulate β-catenin-dependent transcription through Wnt/β-catenin pathway. Eventually, ginsenoside Rb1 promotes the DPC proliferation and migration and positively affects hair follicle growth [46,47].

In order to support our results and to prove our hypothesis, the effect of ginsenoside Rb1 on β-catenin need determined. Additionally, many studies have also reported the signaling protein ERK is involved in hair follicle growth by adjusting cell proliferation and hair shaft elongation [48,49]. Therefore, for the further studies, research on the effect of ginsenoside Rb1 on ERK activation and its related MAPK signaling pathway should be considered. Moreover, the other members of ginsenosides isolated from *Panax ginseng* including Rd, Re, Rg1 and Rg3 who share the similar structures with Rb1 are also able to improve the hair growth through elevating the VEGF expression [50,51]. Thus, the compounds with the structures like Rb1, Rd and Rg3 (protopanaxadiol) and Re and Rg1 (protopanaxatriol) possibly become the higher priority to be the candidates for promoting hair follicle growth. The further studies, for instance, study on which site of the target protein binds with compounds, or find better ways for the selection, identification and synthesis of compounds for hair follicle growth may be the next research directions.

5. Conclusion

In conclusion, this study suggests that ginsenoside Rb1 promotes growth of hair follicles and DPCs by enhancing VEGF and VEGF-R2 expressions and regulating the PI3K/AKT/GSK-3β signaling pathway.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.05.033.

Ethical approval

All animal experiments were accordance with international ethical guidelines and the National Institutes of Health guide for the care and use of Laboratory animals. All animal experiments were approved by Animal Ethics Committee of Chinese Academy Agricultural Sciences.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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Author contributions

HZ: conceived and designed the experiments, prepared the manuscript, performed experiments. YS, JW, YG: performed experiments and interpreted data. FY: interpreted data. GL and QS: conceived, designed the experiments, and revised the manuscript. All authors read and approved the manuscript.

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