Nature-derived lignan compound VB-1 exerts hair growth-promoting effects by augmenting Wnt/β-catenin signaling in human dermal papilla cells

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Background. Vitexin is a kind of lignan compound which has been shown to possess a variety of pharmacological effects, such as anti-inflammatory, anti-oxidative and anti-cancer activities. However the effect of vitexin on hair regeneration has not been elaborated. Methods. The proliferation of human dermal papilla cells (hDPCs) was examined by cell counting and continuous cell culture after vitexin compound 1 (VB-1) treated. The expression of lef1, wnt5a, bmp2, bmp4, alpl and vcan was examined by RT-PCR. The expression of dkk1, tgf-β1, active-β-Catenin, and AXIN2 was examined by RT-PCR or immunoblotting. Hair shaft growth was measured in the absence or presence of VB-1.

Results. We demonstrated that VB-1 significantly promotes the proliferation of hDPCs in a concentration-dependent manner within a certain concentration range. Among the hair growth-related genes investigated, dkk1 was clearly down-regulated by in hDPCs treated with VB-1. The increased active β-Catenin and decreased AXIN2 protein levels suggest that VB-1 facilitates Wnt/β-catenin signaling in hDPCs in vitro. The expression of DP signature genes was also upregulated after VB-1 treatment. We further study indicated that VB-1 promotes human hair follicle (HF) growth by HF organ culture assay. Discussion. VB-1 may exerts hair growth-promoting effects via augmenting Wnt/β-catenin signaling in hDPCs.
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Abstract

Background. Vitexin is a kind of lignan compound which has been shown to possess a variety
of pharmacological effects, such as anti-inflammatory, anti-oxidative and anti-cancer activities. However the effect of vitexin on hair regeneration has not been elaborated.

**Methods.** The proliferation of human dermal papilla cells (hDPCs) was examined by cell counting and continuous cell culture after vitexin compound 1 (VB-1) treated. The expression of *lef1, wnt5a, bmp2, bmp4, alpl* and *vcan* was examined by RT-PCR. The expression of *dkk1, tgf-β1*, active-β-Catenin, and AXIN2 was examined by RT-PCR or immunoblotting. Hair shaft growth was measured in the absence or presence of VB-1.

**Results.** We demonstrated that VB-1 significantly promotes the proliferation of hDPCs in a concentration-dependent manner within a certain concentration range. Among the hair growth-related genes investigated, *dkk1* was clearly down-regulated by in hDPCs treated with VB-1. The increased active β-Catenin and decreased AXIN2 protein levels suggest that VB-1 facilitates Wnt/β-catenin signaling in hDPCs *in vitro*. The expression of DP signature genes was also upregulated after VB-1 treatment. We further study indicated that VB-1 promotes human hair follicle (HF) growth by HF organ culture assay.

**Discussion.** VB-1 may exerts hair growth-promoting effects via augmenting Wnt/β-catenin signaling in hDPCs.

**Introduction**

The hair follicle (HF) is a complex mini-organ composed of epidermal and mesenchymal (dermal) components which undergoes cycles of degeneration (catagen), rest (telogen), and growth (anagen) throughout adult life [1]. This hair cycle is based on the capacity of hair follicle stem cells (HFSCs), which are slow-cycling, label-retaining cells located at a niche known as the bulge, to transiently exit the quiescent status to launch the growth phase [2, 3]. Activation and differentiation of HFSCs are mainly governed by a cluster of specialized mesenchymal cells residing in the base of hair follicles, known as the dermal papilla (DP) [4]. With anagen initiation, stem cells in the bulge are activated that fuel the growth of new hair follicle in response to DP
signals [5, 6].

During the postnatal hair cycle, dermal papilla cells (DPCs) act as a signaling center to control the proliferation, migration, and differentiation of the surrounding epithelial stem/progenitor cells to complete the process of hair regeneration. Moreover, DPCs possess hair follicle-inducing ability via interacting with neighboring epithelial stem cells [8-10]. Several signaling pathways, particularly Wnt/β-catenin signaling, have been shown to play a key role in the development of new hair follicles and initiation of hair growth [2, 11, 12,43-45]. Numerous Wnt ligands and inhibitors expressed in DPCs are crucial for regulating hair growth [13-15,46-48]. The proper crosstalk between the mesenchyme and epithelium facilitates the activation of HFSCs by overcoming the repressive signals that maintain HFSCs in a quiescent state [4, 16, 17]. Genetic deletion of β-catenin in the DP results in premature induction of catagen and prevents regeneration of HFss [7].

Several hair disorders are characterized by the inability to re-enter the regeneration phase (anagen) of the hair cycle. Particularly, in the case of androgenetic alopecia, ectopic activation of androgen receptor signaling responding to dihydrotestosterone in the HF, mainly in the DP, alters the expression of hair growth-related paracrine factors (such as DKK1, Wnts, and TGF-βs). Dysregulation of these paracrine factors impairs the proliferation and differentiation of hair follicle stem/matrix cells, causing shortening of the anagen phase and resulting in progressive HF miniaturization, a major characteristic of androgenetic alopecia [15, 18-21]. Therefore, DP is thought to be the primary therapy target for androgenetic alopecia. Current pharmacological treatment for androgenetic alopecia is mainly concentrated on the prevention of further hair loss [22]. However, the development of pharmacologic agents to activate the proliferation of HFSCs and reboot the hair cycle has been unsatisfactory.

Lignan is a group of complex polyphenolic antioxidants widely present in plant vitex negundo [23, 24]. Vitexin is a kind of lignan compound found in vitex negundo seeds, widely used herb medicine in China [25, 26]. Vitexin has been shown to possess a variety of pharmacological
effects, such as anti-inflammatory and anti-oxidative and anti-cancer activities [27]. Clinical studies indicated that lignans have a potential role in cancer prevention [28, 29]. Some clinical trials have confirmed that lignans can also inhibits the development of certain cancers. For example, some studies have shown that the low risk of ovarian cancer and prostate cancer correlated with high lignans intake diet. This may be the reason of mediterranean diet (high lignans content in olive) associated with a lower incidence of cancer. The anti-cancer properties of lignans have been studied in cancer cells culture in vitro. In these studies, purified vitexin compound-1(VB-1) prevented the proliferation of cancer cells in the G2/M phase of the cell cycle and induced apoptosis within a certain concentration range effectively [25,26]. Although VB-1 has been shown to exerts anticancer activities, but other pharmacological effects still unclear. It is worth further developing of pharmacological values urgently.

Here, we evaluated the effect of VB-1 on hair growth. We demonstrated that VB-1 facilitated the proliferation of hDPCs in a concentration-dependent manner within a certain concentration range. Among the hair growth-related genes investigated, dkk1 was significantly decreased in VB-1-treated hDPCs. By immunoblot analysis, we showed that active β-Catenin was increased and AXIN2 was decreased, suggesting that VB-1 promotes Wnt/β-catenin signaling in hDPCs. Furthermore, VB-1 enhances the expression of DP signature genes in hDPCs. Moreover we found that VB-1 promoted human HF growth in an organ culture assay. Taken together, these findings indicate that VB-1 promotes hair growth and may be a new therapy for hair loss treatment.

Materials and methods

Reagents

Vitexin compound-1(VB-1), was kindly provided by Prof. Yingjun Zhou and prepared as previously described [25]. VB-1 powder was produced in Prof. Jinsong Ding’s lab (Department of Medicinal Chemistry, Central South University, China) and used in this study.
Isolation and culture of human hair follicles

Punch scalp biopsy (5 mm) specimens were obtained from male non-balding occipital scalps of patients undergoing hair transplantation surgery for androgenetic alopecia. All human subjects involved procedures were approved by the institutional review board of Xiangya hospital (IRB NO. 201611609) in accordance the helsinki guidelines. Briefly, hair follicles were isolated with scissors and forceps under a binocular light microscope and cultured in 24-well dishes for 14 days in William’s E medium (Gibco, Grand Island, NY, USA) supplemented with 10 mg/mL insulin, 2 mM L-glutamine, 10 ng/mL hydrocortisone, and 100 U/mL streptomycin at 37°C in a 5% (v/v) CO₂ atmosphere[40]. Hair follicles were incubated in William’s E medium with VB1, they were photographed by immersing in PBS at 37°C, using a stereoscope every 48 h. In all experiments, VB-1 culture medium was refreshed every other day. A total of 150 anagen hair follicles were isolated from 3 different volunteers and cultured with each concentration of VB-1, the experiments were repeated for 5 times with 6 repetitions for each concentration group.

Isolation and culture of hDPCs

The hDPCs were isolated and cultured as previously described [41]. Briefly, isolated hDPCs were cultured in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% (v/v) fetal bovine serum (Gibco), 100 U/mL penicillin, and 100 mg/mL streptomycin. The medium was changed every 2 days. Once cell outgrowth was sub-confluent, hDPCs were harvested with 0.25% (w/v) trypsin-EDTA (Invitrogen, Carlsbad, CA, USA) and passage cultured with a split ratio of 1:3. hDPCs at passages 3–5 were used in this study.

MTS assay:

MTS working solution was added as 20μl/well to the culture wells, after cell seeding for 4 hour, then shaken and mixed. The 96-well plates were incubated in a 37°C, 5% CO₂ incubator for 1 hour. Value of OD₄₉₀nm was recorded at the subsequent 12 h, 24 h, 48 h, and 72 h time points. the growth curve was plotted by OD₄₉₀nm value.
**Total RNA isolation, cDNA synthesis, and real-time PCR**

RNA was extracted from cells using TRIzol reagent (Invitrogen) and cDNA was prepared using the RevertAid First Strand cDNA Synthesis Kit (Gibco/Thermo Scientific, Waltham, MA, USA). Sequences of qPCR primers were from PrimerBank and queried in NCBI blast to check their specificity. *gapdh* was used as an internal reference. For RT-PCR 1 μl each of the forward primer (10 ng/μl) and Reverse Primer (10 ng/μl) per well, iTaq Universal Green Supermix (2×) 10 μl, Nuclease-Free H₂O 6 μl, and cDNA 2 μl were mixed to a final volume of 20 μl. The PCR procedure software recorded the average fluorescence value of each cycle of the reaction. The relative expression levels of different genes in the cells were obtained by comparing the Ct values. The experiment was repeated three times with three replicates were seted per reaction. PCR primer sequences are given in Supplemental Table 1.

**Western blot assays**

The collected cells were lysed in RIPA buffer (Thermo Scientific) with protease inhibitors (Thermo Scientific) after rinsing twice with PBS (precooled at 4°C). Proteins were quantified in the bicinchoninic acid assay (Pierce BCA Protein Assay, Rockford, IL, USA), and then separated by SDS–polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene fluoride membranes. After blocking in 5% nonfat milk 1 h at 25°C, the membranes were probed with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Dallas, TX, USA). Primary antibodies used were rabbit anti-active β-Catenin protein (1:1000, Cell Signaling Technology, Danvers, MA, USA), rabbit anti-AXIN2 (1:1000, Cell Signaling Technology) and mouse anti-Tubulin (1:1000, Cell Signaling Technology). Immunoreactive bands were visualized with horseradish peroxidase substrate (Luminata, Millipore, Billerica, MA, USA) using the ChemiDocTM XRS+ system (Bio-Rad, Hercules, CA, USA).

**Statistical analysis**
Statistical analyses were performed using GraphPad Prism 7.0 software (GraphPad, Inc., La Jolla, CA, USA) and using Student’s t-test as indicated in the individual figure legends. P values < 0.05 were considered significant. Error bars represent the standard error of the mean as noted in the individual figure legends.

Results

**VB-1 promotes the proliferation of human dermal papilla cells (hDPCs)**

To assess the effects of VB-1 on cultured hDPCs, we first examined the proliferation of cells treated with different doses of VB-1 (0, 0.01, 0.1, 1, and 10 µM). During the 8 days of culture, the number of expanded hDPCs was greater in the 0.1 µM VB-1 group than in the control group (0 µM), and there was no significant difference observed at 0.01 and 1 µM. However, a high concentration of VB-1 (10 µM) dramatically suppressed the proliferation of hDPCs, and may have increased the apoptosis of cells (Figure 1A and B). And these results were further confirmed by MTS assay in hDPCs treated with different doses of VB-1 (Figure 1D).

Interestingly, we also found that 0.1 µM VB-1 contributed to colony growth (Figure 1A), indicating an increase in the hair-inducing capacity of hDPCs [30]. Similarly, the number of culture days per passage for hDPCs were lower in medium supplemented with 0.1 µM VB-1 compared to in other groups (Figure 1C). Collectively, these results suggest that VB-1 facilitates the proliferation of hDPCs and improve hair-inducing abilities of these cells.

**VB-1 improves hair-inducing properties of hDPCs**

Previous studies have suggested the indispensable roles of DPCs in hair follicle reconstruction assays *in vitro*. However, DPCs lose their hair-inducing properties quickly during culture [31-34], greatly limiting their applications for hair reconstitution. To determine whether VB-1 affects the hair-inducing ability of hDPCs, we treated cultured hDPCs with different concentrations of VB-1 (0, 0.01, 0.1, 1, and 10 µM). Our results showed that the Wnt signaling-associated signature
genes of DP, *lef1*, and *wnt5a*, *alpl* and *vcan* were clearly upregulated in hDPCs treated with 0.1 µM VB-1 compared to in other groups (*Figure 2A, B, E and F*). VB-1 also increased the expression of *bmp2* and *bmp4* (*Figure 2C and D*), two additional markers [10]. These findings indicate that VB-1 can promote the hair-inducing ability of hDPCs.

**VB-1 activates Wnt/β-catenin signaling in hDPCs**

To define the roles of VB-1 in hair growth, we investigated its effects on the expression of hair growth-related genes in hDPCs. We found that compared to control group, *dkk1* was significantly down-regulated by 0.1 µM VB-1 treatment and upregulated in the presence of 10 µM VB-1 (*Figure 3A*). However there was no statistically significant difference in the expression of *tgf-β1*, another hair growth-related gene (*Figure 3B*). By immunoblot analysis, we showed that the active β-Catenin protein level was increased in hDPCs treated with 0.1 µM VB-1, while it was decreased in hDPCs exposed to a high concentration of VB-1 (*Figure 3C*). Moreover, the mRNA level of *axin2*, a negative regulator of Wnt/β-catenin signaling [35], was reduced in hDPCs treated with 0.1 µM VB-1; high concentrations of VB-1 (1 and 10 µM) increased the expression of *axin2* (*Figure 3E*). Suppression of AXIN2 by VB-1 in a dose-dependent manner was confirmed by immunoblotting analysis (*Figure 3F and G*). These results suggest that VB-1 induces Wnt/β-catenin signaling activation in hDPCs in a concentration-dependent within a certain concentration range manner.

**VB-1 promotes hair shaft elongation in cultured human hair follicles**

Because DP is essential for the regulation of hair growth, we further explored the possible effects of VB-1 on hair shaft elongation. Human scalp hair follicles were isolated and cultured in the absence or presence of VB-1. We found that 0.1 µM VB-1 significantly facilitated the elongation of hair shafts in cultured human hair follicles (*Figure 4A and B*).
The number of individuals currently suffering from hair thinning or balding, such as androgenetic alopecia, is increasing. Although numerous products claim to be useful for treating hair loss, they have sexual-related side-effects and unpredictable efficacy [22, 36, 37]. Therefore, it is extremely important to develop new therapies for treating hair loss. In this study, we showed that VB-1 promotes the proliferation of hDPCs and partially restores hair-inducing properties. In HF organ culture, we demonstrated that VB-1 facilitates hair shaft elongation in cultured human scalp hair follicles, which may have resulted from the activation of Wnt/β-catenin signaling in hDPCs.

It has been shown that lignans exert anti-cancer activities by arresting cancer cells in the G2/M phase of the cell cycle and subsequently inducing apoptosis [25, 26]. Interestingly, our data showed that VB-1 can promote the proliferation of hDPCs at a low dose; however, high dose of VB-1 inhibits the growth of these cells, which may be resulted from the arrestment of cell cycle induced by high concentration of VB-1 as previous described in the cancer cells [25, 26], and the underlying molecular cues may need future study.

Adult human hair follicle reconstruction has become an attractive strategy for regenerative medicine, in which the roles of DP in epithelial-mesenchymal interactions that induce hair follicle neogenesis are indispensable [4, 9]. However, DPCs lose their hair-inducing properties quickly during culture in vitro, limiting their applications for hair follicle reconstitution [10]. In the present study, we demonstrated that VB-1 increases the expression of human DP signature genes, such as lef1, wnt5a, and bmp2. Further studies are required to focus on whether VB-1 is suitable for long-term culture of hDPCs while maintaining their hair-inducing abilities.

Wnt/β-catenin signaling has been shown to be essential for hair morphogenesis and cycling [38, 39], and its activation in the dermal papilla contributes to the proliferation and differentiation of hair follicle stem cells, thus initiating the anagen phase of the hair cycle [4, 7, 42]. Our data showed that VB-1 significantly upregulated Wnt/β-catenin signaling in hDPCs in a
certain dose-dependent manner range. These observations suggest that VB-1 promotes hair
growth by modulating Wnt/β-catenin signaling in hDPCs. Responding to active androgen
receptor signaling, hDPCs produce a variety of paracrine factors such as dkk1 and tgf β-1,
impairing the proliferation and differentiation of hair follicle stem/progenitor cells, thus resulting
in progressive HF miniaturization, a major characteristic of androgenetic alopecia [15, 18-20].
Our results demonstrate that VB-1 decreases the expression of dkk1 in cultured hDPCs.
The results of the present study show that VB-1 promotes hair shaft elongation in cultured
human hair follicles in a concentration-dependent manner within a certain concentration range.
Thus, VB-1 may be an effective therapy for the treatment of alopecia. However, further basic
and clinical studies are required to verify the results presented in this study, and more practical
dosing of VB-1 in the management of hair loss must be determined.

Conclusions
Our findings strongly suggest that VB-1 augments Wnt/β-catenin signaling in human dermal
papilla cells and significantly promotes the proliferation of hDPCs. Furthermore, VB-1 showed
hair growth-promoting effects, indicating its potential as a new therapy for alopecia treatment.

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References
[1]. Greco V, Chen T, Rendl M, Schober M, Pasolli HA, Stokes N, dela Cruz-Racelis J, and Fuchs E. 2009. A two-step mechanism for stem cell activation during hair regeneration. Cell Stem Cell 4:155-169. 10.1016/j.stem.2008.12.009
[2]. Kandyba E, Leung Y, Chen YB, Widellitz R, Chuong CM, and Kobielak K. 2013. Competitive balanc
e of intrabulge BMP/Wnt signaling reveals a robust gene network ruling stem cell homeostasis and cyclic
activation. Proc Natl Acad Sci U S A 110:1351-1356. 10.1073/pnas.1121312110
[3] Tumbar T, Guasch G, Greco V, Blanpain C, Lowry WE, Rendl M, and Fuchs E. 2004. Defining the epithelial stem cell niche in skin. *Science* 303:359-363. 10.1126/science.1092436

[4] Morgan BA. 2014. The dermal papilla: an instructive niche for epithelial stem and progenitor cells in development and regeneration of the hair follicle. *Cold Spring Harb Perspect Med* 4:a015180. 10.1101/cshperspect.a015180

[5] Kobielak K, Stokes N, de la Cruz J, Polak L, and Fuchs E. 2007. Loss of a quiescent niche but not follicle stem cells in the absence of bone morphogenetic protein signaling. *Proc Natl Acad Sci U S A* 104:10063-10068. 10.1073/pnas.0703004104

[6] Tang Y, Luo BP, Deng ZL, Wang B, Liu FF, Li JM, Shi W, Xie HF, Hu XW, and Li J. 2016. Mitochondrial aerobic respiration is activated during hair follicle stem cell differentiation, and its dysfunction retards hair regeneration. *PeerJ* 4:e1821. 10.7717/peerj.1821

[7] Enshel-Seijffers D, Lindon C, Kashiwagi M, and Morgan BA. 2010. β-catenin activity in the dermal papilla regulates morphogenesis and regeneration of hair. *Dev Cell* 18:633-642. 10.1016/j.devcel.2010.01.016

[8] Aoi N, Inoue K, Chikanishi T, Fuji k i R, Yamamoto H, Kato H, Eto H, Doi K, Itami S, Kato S, and Yoshimura K. 2012. 1α, 25-dihydroxyvitamin D3 modulates the hair-inductive capacity of dermal papilla cells: therapeu tic potential for hair regeneration. *Stem Cells Transl Med* 1:615-626. 10.5966/sctm.2012-0032

[9] Higgins CA, Chen JC, Cerise JE, Jahoda CA, and Christiano AM. 2013. Microenvironmental reprogramming by three-dimensional culture enables dermal papilla cells to induce de novo human hair-follicle growth. *Proc Natl Acad Sci U S A* 110:19679-19688. 10.1073/pnas.1309970110

[10] Ohyama M, Kobayashi T, Sasaki T, Shimizu A, and Amagai M. 2012. Restoration of the intrinsic properties of human dermal papilla in vitro. *J Cell Sci* 125:4114-4125. 10.1242/jcs.105700

[11] Chu EY, Hens J, Andl T, Kai ro A, Yamaguchi TP, Brisken C, Glick A, Wysolmerski JJ, and Millar SE. 2004. Canonical WNT signaling promotes mammary placode development and is essential for initiation of mammary gland morphogenesis. *Development* 131:4819-4829. 10.1242/dev.01347

[12] Lim X and Nusse R. 2013. Wnt signaling in skin development, homeostasis, and disease. *Cold Spring Harb Perspect Biol* 5:a008029. 10.1101/cshperspect.a008029

[13] Plikus MV. 2012. New activators and inhibitors in the hair cycle clock: targeting stem cells’ state of competence. *J Invest Dermatol* 132:1321-1324. 10.1038/jid.2012.38

[14] Kwack MH, Kim MK, Kim JC, and Sung YK. 2012. Dickkopf 1 promotes regression of hair follicle s. *J Invest Dermatol* 132:1554-60. 10.1038/jid.2012.24

[15] Kwack MH, Sung YK, Chung Ej, Im SU, Ahn JS, Kim MK, and Kim JC. 2008. Dihydrotestosterone e-inducible dickkopf 1 from balding dermal papilla cells causes apoptosis in follicular keratinocytes. *J Invest Dermatol* 128:262-269. 10.1038/sj.jid.5700999

[16] Oshimori N and Fuchs E. 2012. Paracrine TGF-β signaling counterbalances BMP-mediated repression in hair follicle stem cell activation. *Cell Stem Cell* 10:63-75. 10.1016/j.stem.2011.11.005

[17] Deng ZL, Lei XH, Zhang XD, Zhang HS, Liu S, Chen Q, Hu HM, Wang XY, Ning LN, Cao YJ, Zhao TB, Zhou JX, Chen T, and Duan EK. 2015. mTOR signaling promotes stem cell activation via count erbalancing BMP-mediated suppression during hair regeneration. *J Mol Cell Biol* 7:62-72. 10.1093/jmcb/mjv005
[18]. Inui S and Itami S. 2011. Molecular basis of androgenetic alopecia: from androgen to paracrine mediators through dermal papilla. J Dermatol Sci 61:1-6. 10.1016/j.jdermsci.2010.10.015

[19]. Shin H, Yoo HG, Inui S, Itami S, Kim IG, Cho AR, Lee DH, Park WS, Kwon O, Cho KH, and Won CH. 2013. Induction of transforming growth factor-beta 1 by androgen is mediated by reactive oxygen species in hair follicle dermal papilla cells. BMB Rep 46:460-464. 10.5483/BMBRep.2013.46.9.228

[20]. Ceruti JM, Leirós GJ, and Balañá ME. 2017. Androgens and androgen receptor action in skin and hair follicles. Mol Cell Endocrinol 465:122-133. 10.1016/j.mce.2017.09.009

[21]. Hu HM, Zhang SB, Lei XH, Deng ZL, Guo WX, Qiu ZF, Liu S, Wang XY, Zhang H, and Duan EK. 2012. Estrogen leads to reversible hair cycle retardation through inducing premature catagen and maintaining telogen. PLoS One 7:e40124. 10.1371/journal.pone.0040124

[22]. Varothai S and Bergfeld WF. 2014. Androgenetic alopecia: an evidence-based treatment update. Am J Clin Dermatol 15:217-230. 10.1007/s40257-014-0077-5

[23]. Adlercreutz H. 2007. Lignans and human health. Crit Rev Clin Lab Sci 44:483-525. 10.1080/10408360701612942

[24]. Adlercreutz H. 2002. Phyto-oestrogens and cancer. Lancet Oncol 3:364-373. 10.1016/S1470-2045(02)00777-5

[25]. Zhou YJ, Liu YE, Cao JG, Zeng GY, Shen C, Li YL, Zhou MC, Chen YD, Pu WP, Potters L, and Shi YE. 2009. Vitexins, nature-derived lignan compounds, induce apoptosis and suppress tumor growth. Clin Cancer Res 15:5161-5169. 10.1158/1078-0432.CCR-09-0661

[26]. Xin H, Kong Y, Wang Y, Zhou YJ, Zhu YZ, Li DP, and Tan WF. 2013. Lignans extracted from Vitex negundo possess cytotoxic activity by G2/M phase cell cycle arrest and apoptosis induction. Phytomedicine 20:640-647. 10.1016/j.phymed.2013.02.002

[27]. Yang ZB, Tan B, Li TB, Lou Z, Jiang JL, Zhou YJ, Yang J, Luo XJ, and Peng J. 2014. Protective effect of vitexin compound B-1 against hypoxia/reoxygenation-induced injury in differentiated PC12 cells via NADPH oxidase inhibition. Naunyn Schmiedebergs Arch Pharmacol 387:861-871. 10.1007/s00210-014-1006-0

[28]. Wang JG, Zheng XX, Zeng GY, Zhou YJ, and Yuan H. 2014. Purified vitexin compound 1 inhibits growth and angiogenesis through activation of FOXO3a by inactivation of Akt in hepatocellular carcinoma. Int J Mol Med 33: 441-448. 10.3892/ijmm.2013.1587

[29]. Thompson LU, Chen JM, Li T, Strasser-Weippl K, and Goss PE. 2005. Dietary flaxseed alters tumor biological markers in postmenopausal breast cancer. Clin Cancer Res 11:3828-3835. 10.1158/1078-0432.CCR-04-2326

[30]. Osada A, Iwabuchi T, Kishimoto J, Hamazaki TS, and Okochi H. 2007. Long-term culture of mouse vibrissal dermal papilla cells and de novo hair follicle induction. Tissue Eng 13:975-982. 10.1089/ten.2006.0304

[31]. Zhang SB, Hu HM, Zhang HS, Liu S, Liu S, Zhang Y, Lei XH, Ning L, Cao YJ, and Duan EK. 2012. Hair follicle stem cells derived from single rat vibrissa via organ culture reconstitute hair follicles in vivo. Cell Transplant 21:1075-1085. 10.3727/096368912X640538

[32]. Zhang HS, Zhang SB, Zhao HS, Qiao JQ, Liu S, Deng ZL, Lei XH, Ning LN, Cao YJ, Zhao Y, a
nd Duan EK. 2015. Ovine hair follicle stem cells derived from single vibrissae reconstitute haired skin. Int J Mol Sci 16:17779-17797. 10.3390/ijms160817779

[33]. Yang CC and Cotsarelis G. 2010. Review of hair follicle dermal cells. J Dermatol Sci 57:2-11. 10.1016/j.jdermsci.2009.11.005

[34]. Ohyama M, Zheng Y, Paus R, and Stenn KS. 2010. The mesenchymal component of hair follicle neogenesis: background, methods and molecular characterization. Exp Dermatol 19:89-99. 10.1111/j.1600-0625.2009.00935.x

[35]. Fancy SP, Harrington EP, Yuen TJ, Silbereis JC, Zhao C, Baranzini SE, Bruce CC, Otero JJ, Huang EJ, Nusse R, Franklin RJ, and Rowitch DH. 2011. Axin2 as regulatory and therapeutic target in newborn brain injury and remyelination. Nat Neurosci 14:1009-1016. 10.1038/nn.2855

[36]. Roussos DE and Kim SW. 2014. A review of medical and surgical treatment options for androgenetic alopecia. JAMA Facial Plast Surg 16:444-450. 10.1001/jamafacial.2014.316

[37]. Rogers NE and Avram MR. 2008. Medical treatments for male and female pattern hair loss. J Am Acad Dermatol 59:547-566. 10.1016/j.jaad.2008.07.001

[38]. Lien WH, Polak L, Lin M, Lay K, Zheng D, and Fuchs E. 2014. In vivo transcriptional governance of hair follicle stem cells by canonical Wnt regulators. Nat Cell Biol 16:179-190. 10.1038/ncb2903

[39]. Plikus MV. and Chuong CM. 2014. Macrouenvironmental regulation of hair cycling and collective regenerative behavior. Cold Spring Harb Perspect Med 4:a015198. 10.1101/cshperspect.a015198

[40]. Fischer TW, Hipler UC, and Elsner P. 2007. Effect of caffeine and testosterone on the proliferation of human hair follicles in vitro. Int J Dermatol 46:27-35. 10.1111/j.1365-4632.2007.03119.x

[41]. Gledhill K, Gardner A, and Jahoda CA. 2013. Isolation and establishment of hair follicle dermal papilla cell cultures. Methods Mol Biol 989:285-292. 10.1007/978-1-62703-330-5_22

[42]. Li J, Jiang TX, and Chuong CM. 2013. Many paths to alopecia via compromised regeneration of hair follicle stem cells. J Invest Dermatol 133:1450-1452. 10.1038/jid.2012.511

[43]. Lei M, Schumacher LJ, Lai YC, Juan WT, Yeh CY, Wu P, Jiang TX, Baker RE, Widelitz RB, Yang L, and Chuong CM. 2017. Self-organization process in newborn skin organoid formation inspires strategy to restore hair regeneration of adult cells. Proc Natl Acad Sci U S A 114:E7101-E7110. 10.1073/pnas.170475114

[44]. Lei MX and Chuong CM. 2016. Aging, alopecia, and stem cells. Science 351:559-560. 10.1126/science.aaf1635

[45]. Lei MX, Yang L, and Chuong CM. 2017. Getting to the core of the dermal papilla. J Invest Dermatol 137:2250-2253. 10.1016/j.jid.2017.07.824

[46]. Lei MX, Guo HY, Qiu WM, Lai XD, Yang T, Widelitz RB, Chuong CM, Lian XH, and Yang L. 2014. Modulating hair follicle size with Wnt10b/DKK1 during hair regeneration. Exp Dermatol 23:407-413. 10.1111/exd.12416

[47]. Lei MX, Chuong CM, and Widelitz RB. 2013. Tuning Wnt signals for more or fewer hairs. J Invest Dermatol 133:7-9. 10.1038/jid.2012.446

[48]. He L, Lei MX, Xing YZ, Li YH, Hu CY, Chen PX, Lian XH, Yang T, Liu WQ, and Yang L. 2017. Gsdma3 regulates hair follicle differentiation via Wnt5a-mediated non-canonical Wnt signaling pathway. O
Figure 1

VB-1 facilitates the proliferation of human DPCs

(A) Morphology of human DPCs treated with VB-1 (0-10 μM) at indicated days. Arrow indicates colony growth of DPCs. (B) Human DPCs (1×10⁴ cells) were plated in 24-well dishes and cultured in the presence of different concentrations of VB-1 (0-10 μM) for 8 days. Growth curves indicate the mean of three independent experiments (±SEM). (C) Culture days per passage of human DPCs treated with VB-1 (0-10 μM). Experiments were carried out in triplicates. (D) OD value of human DPCs (4×10³ cells) were plated in 96-well dishes and cultured in the presence of different concentrations of VB-1 (0-10 μM) for 3 days. Data are reported as mean+SEM. Student’s t-test was used to compare data. *P < 0.05, **P < 0.01.
Figure 2

VB-1 increases the expression of the signature genes of human DPCs

(A-F) Dose-dependent effects (0-10 μM) of VB-1 on *lef1*, *wnt5a*, *bmp2*, *bmp4*, *alpl* and *vcan* mRNA expression, in human DPCs cultured for 24 hours. Data are shown as the ratio of the respective gene expression to gapdh mRNA expression. Experiments were carried out in triplicates. Data are reported as mean+SEM. Student’s t-test was used to compare data. *P < 0.05, **P < 0.01.
Figure 3

VB-1 promotes Wnt/β-catenin signaling in human DPCs

(A) Concentration-dependent effects (0-10 μM) of VB-1 on dkk1 mRNA expression in human DPCs cultured for 24 hours. (B) Concentration-dependent effects (0-10 μM) of VB-1 on tgf-β mRNA expression in human DPCs cultured for 24 hours. (C) Immunoblotting analysis of active β-Catenin expression in hDPCs treated with VB-1 (0-10 μM) for 24 h. (D) Quantification of active β-Catenin protein expression. (E) Real-time PCR analysis for gene expression of axin2 in hDPCs treated with VB-1 (0-10 μM) for 24 h. (F) Immunoblotting analysis of AXIN2 expression in hDPCs treated with VB-1 (0-10 μM) for 24 h. (G) Quantification of AXIN2 protein expression. Experiments were carried out in triplicates. The typical blot was presented and quantification of three independent experiments is shown for C and F. Data are reported as mean+SEM. Student’s t-test was used to compare data. *P < 0.05, **P < 0.01, “ns” indicates no significant difference.
Figure 4

VB-1 promotes the elongation of hair shafts in cultured hair follicles

Isolated human scalp hair follicles were cultured for 14 days in the presence of different doses of VB-1. (A) Typical pictures of the hairs at day 0, 2, 4, 6, 8, 10, 12, 14. (B) Data are presented as the elongated length of the hair follicles treated with VB-1. Data are reported as mean +SEM. Student’s t-test was used to compare data. *P < 0.05, **P < 0.01.