Vitamins and their derivatives synergistically promote hair shaft elongation ex vivo via PlGF/VEGFR-1 signalling activation

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Abstract

Background: Although vitamins or their derivatives (Vits), such as panthenyl ethyl ether, tocopherol acetate, and pyridoxine, have been widely used in topical hair care products, their efficacy and mode of action have been insufficiently studied.

Objective: To elucidate the biological influence of Vits on hair follicles and determine the underlying mechanisms.

Methods: A mouse vibrissa hair follicle organ culture model was utilized to evaluate the effects of Vits on hair shaft elongation. Gene and protein expression analyses and histological investigations were conducted to elucidate the responsible mechanisms. A human hair follicle cell culture was used to assess the clinical relevance.

Results: In organ culture models, the combination of panthenyl ethyl ether, tocopherol acetate, and pyridoxine (namely, PPT) supplementation significantly promoted hair shaft elongation. PPT treatment enhanced hair matrix cell proliferation by 1.9-fold compared to controls, as demonstrated by Ki67-positive immunoreactivity. PPT-treated mouse dermal papilla exhibited upregulated Placental growth factor (Plgf) by 1.6-fold compared to controls. Importantly, the addition of PlGF neutralizing antibodies to the ex vivo culture diminished the promotive effect on hair growth and increase in VEGFR-1 phosphorylation achieved by 1.6-fold compared to controls. Importantly, the addition of PlGF neutralizing antibodies to the ex vivo culture diminished the promotive effect on hair growth and increase in VEGFR-1 phosphorylation achieved by PPT. A VEGFR-1 inhibitor also inhibited the promotion of hair growth. Microarray analysis suggested synergistic summation of individual Vits’ bioactivity, putatively explaining the effect of PPT. Moreover, PPT increased PlGF secretion in cultured human dermal papilla cells.

Conclusion: Our findings suggested that PPT promoted hair shaft elongation by activating PlGF/VEGFR-1 signalling. The current study can shed light on the previously underrepresented advantage of utilizing Vits in hair care products.

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1. Introduction

Male and female pattern hair loss (MPHL and FPHL) is a common complaint found in middle-aged patients in dermatology clinics [1,2], which potentially causes low self-esteem and leads to depression, anxiety, and social distress [3,4]. Topical application of minoxidil has been widely used to improve MPHL/FPHL [5], which is recommended in the Japanese Dermatological guideline for the management of MPHL/FPHL [6]. However, the clinical responses to this remedy are variable and leave room for improvement. For mildly-moderately affected MPHL/FPHL patients, hair care products currently on the market are expected to exhibit some ameliorative effects, such as promotion of hair growth and prevention of hair loss.
Vitamins and their derivatives (Vits) are representative active ingredients present in topically applied manufactured hair products. Among Vits, panthenyl ethyl ether (PEE) is a water-soluble form of pantothenic acid, also known as vitamin B5, which is frequently used as an ingredient in hair, skin, and nail care products. PEE is metabolized into pantothenic acid to synthesize coenzyme A and has been reported to promote cell proliferation in hair follicles of American mink [8]. In MPHL, sebum secretion has been reported to increase with androgen [9], possibly leading to seborrheic dermatitis/alopoeia. The addition of pyridoxine hydrochloride (PH), also named Vit B6, has been reported to suppress sebum production in vitro and would be beneficial to improve the scalp environment for hair restoration [10]. Tocopherol acetate (TA) is the active form of Vit E, which has been shown to possess antioxidative effects and promote hair growth in C57BL/6 mice [11]. Despite these scientific insights and wide ranges of use in commercial formulations, investigations to confirm their efficacy, let alone determining the underlying mechanisms that support their functionality, have been insufficiently studied.

In this study, a mouse vibrissae ex vivo culture model [12,13] was utilized to evaluate the effects of Vits on hair shaft elongation. PEE, PH, and TA were selected as representative Vits and were investigated individually or in combination to assess their bioactivities for hair growthamelioration. The factors responsible, modes of action, and signalling pathways involved were also assessed by adopting the aforementioned organ culture model and cultured human dermal papilla cells (hDPCs).

2. Materials and Methods

2.1. Vitamin and its derivative reagents

PEE (Kyowa Pharma Chemical Co., Ltd., S., Toyama, Japan), PH (BASF Japan Ltd., Tokyo, Japan), and TA (BASF Japan Ltd, Tokyo, Japan) were dissolved in the culture medium (formula described in supplementary materials and methods). The concentration of each reagent was determined based on observations from the cytotoxicity assay using human dermal papilla (DP) cells (Supplementary Figure 1). The combination of 0.3 % (v/v) PEE, 0.015 % (w/v) PH and 0.024 % (v/v) TA was designated PPT in this study.

2.2. Mouse vibrissa isolation

Vibrissae were isolated from day 9 postnatal B6C3F1 mouse whisker pads and dissected using a micro knife (MST22, MANI, Tochigi, Japan) under a stereomicroscope (Zeiss Stemi 508, Jena, Germany) as previously reported [14]. For the hair shaft elongation test, vibrissae on the 1st to 4th rows of the whisker pads were used exclusively. All animal experiments were approved by the intramural animal ethics committee of Rohto Pharmaceutical Co., Ltd.

2.3. Mouse vibrissa organ culture

Briefly, isolated vibrissae were incubated in RPMI 1640 medium supplemented with 0.3 % (v/v) PEE, 0.015 % (w/v) PH, and 0.024 % (v/v) TA (concentrations refer to Supplementary Fig. 1) or a combination of two or three of these substances for 72 h at 37 °C in a 5 % CO₂ incubator (n > 9). The hair lengths at day 3 were measured and subtracted from those of day 0 to calculate the amounts of hair shaft elongation. Evaluations of hair shaft elongation and fold change calculations were conducted as previously described [14]. Briefly, the mean values and standard errors of the hair shaft elongation lengths of each group were calculated for statistical analysis. Fold changes were calculated based on the average hair shaft elongation lengths. For details, including the evaluation of the effect of anti-Pigf neutralizing antibody, see the supplementary materials and methods.

2.4. Cell culture

Primary-cultured hDPCs were isolated from 66-year-old Caucasian males and 68-year-old Japanese females (CA602–05a, lots 2717 and 1616, Cell Applications, Inc., San Diego, CA, USA), primary-cultured human hair follicular keratinocytes (HFKC) (Cat. 2440, lot 9304 Cell Applications, Inc.) were cultured at 37 °C in a humidified atmosphere with 95 % O₂ and 5 % CO₂. Passage 3–5 primary-cultured cells were used in this study. Details of the culture media are described in the supplementary materials and methods.

2.5. Quantitative real-time polymerase chain reaction

For the mouse vibrissa DP gene expression analyses, total mRNA was extracted from 10 microdissected DPs as one sample using RNeasy® Micro Kit (QIAGEN, Hilden, Germany). cDNA was synthesized using ReverTra Ace® qPCR RT Master Mix kit (TOYOBO Co., Ltd.). Real-time quantitative polymerase chain reaction (RT–PCR) analyses were carried out by using Thunderbird SYBR qPCR Mix (QPS201, Toyobo Co., Ltd., Osaka, Japan) and QuantStudio Real-Time PCR System (Applied Biosystems). The reaction conditions were 94 °C for 20 s followed by 40 cycles of 2 s at 95 °C for denaturation, 5 s at 60 °C for annealing and 30 s at 72 °C for elongation. The primer sets are shown in Supplementary Tables 1A and B (FASMAC, Kanagawa, Japan). For the hDPC analyses, 5 × 10⁴ cells/well were seeded in collagen-coated 24-well plates (Corning, New York, USA) and cultured with PEE, PH, TA or PPT for 24 h to extract total mRNA. All experiments were performed in triplicate, and independent batches were examined three times.

2.6. Western blot analysis

Total proteins collected from 15 mouse vibrissae were extracted using RIPA Lysis and Extraction Buffer (89901, Thermo Fisher Scientific, Rockford, IL, USA) supplemented with HalTm Protease and Phosphatase Inhibitor Cocktail (78441, Thermo Fisher Scientific, Rockford, IL, USA), Anti-phospho-VEGFR-1 (07–758, Sigma–Aldrich, St. Louis, MO, USA), anti-VEGFR-1 (ab32152, Abcam, Cambridge, MA, USA), and anti-GAPDH (ab9484, Abcam, Cambridge, MA, USA) antibodies were used to detect the extracted proteins with a Wes Simple Western system (ProteinSimple, CA, USA). Quantification analysis of protein bands was performed by ImageJ software (https://imagej.nih.gov/ij/download.html).

2.7. Human Pigf ELISA

The hDPCs were seeded in 24-well plates and treated with PPT for 3 days. The Pigf concentrations in the supernatants were measured using a Human PLGF ELISA kit (ab1000629, Abcam, Cambridge, MA, USA), and the absorbance values at 450 nm were measured by VersaMax™ ELISA (Molecular devices, Tokyo, Japan) following the manufacturer’s instructions.

2.8. Immunofluorescence staining

Immunofluorescence staining was performed using anti-Ki67 antibody (ab16667, Abcam, Cambridge, MA, USA), anti-VEGF receptor 1 antibody (ab32152, Abcam), Hoechst 33342 solution (346–07951, Dojindo, Kumamoto, Japan), and goat anti-rabbit Alexa Fluor 594 (A-11005, Thermo Fisher Scientific, Rockford, IL, USA) secondary antibodies.

2.9. Microarray

Isolated total RNA was amplified and biotin-labelled using a T7 promoter primer-based Low Input Quick Amp Labelling kit
compared to the control (Agilent Microarray Scanner (G2600D) according to the manufacturer's protocol.

2.10. Bioinformatic analysis of microarray data

R (version 4.1.2) and RStudio (version 1.2.5033) platforms were used for data analysis and visualization of the microarray data. Details of the bioinformatic analysis are provided in the supplementary materials and methods. The microarray data are deposited in GSE202865.

2.11. Statistical analysis

Statistical data are expressed as the mean ± standard deviation (SD). Comparisons of more than two groups were analysed using one-way analysis of Dunnett’s test, while comparisons of two groups were evaluated by Student's t-test. P < 0.05 were considered statistically significant.

3. Results

3.1. Vits combination synergistically enhanced hair shaft elongation in the ex vivo mouse vibrissa organ culture

In the ex vivo organ culture [14], mouse vibrissa continued to grow hair shafts for 72 h (Supplementary Fig. 2), and this period was considered to be sufficient to evaluate the effect of Vits on hair shaft elongation. PEE, PH, or TA were added to anagen mouse vibrissa cultures; however, hair shaft elongation was not affected (Fig. 1A). Neither of the two vits combinations promoted hair shaft elongation (Fig. 1B). In contrast, treatment with PPT (PEE, PH, and TA mixture) significantly increased hair shaft elongation by 2.8 ± 1.4-fold when compared to the control (P < 0.01) (Fig. 1C). This promotive effect was evident, as it was readily noticeable by macroscopic observations (Fig. 1D).

These data suggested that each component in PPT might have an undetectable biological influence on vibrissae, but synergistically promoted hair shaft elongation ex vivo.

3.2. PPT enhanced the proliferation of hair follicular matrix keratinocytes

Next, Ki67 staining of the hair bulbs of PPT- and nontreated vibrissae was performed to quantify the number of proliferative cells in the hair matrix (Fig. 1E). The percentage of Ki67-positive cells per total Hoechst-counterstained hair matrix keratinocytes below Auber’s line was calculated. The analysis determined that 62.8 ± 4.9 % and 37.7 ± 16.5 % of keratinocytes located around the DP showed Ki67-positive immunoreactivity in the PPT-treated and control samples, respectively (Fig. 1F). Thus, PPT treatment caused 1.6 ± 0.1-fold increase in the number of proliferating hair matrix keratinocytes (P < 0.01).

These findings suggested that PPT accelerated hair shaft growth by promoting hair matrix keratinocyte proliferation ex vivo.

3.3. PPT upregulated Plgf gene expression in mouse vibrissa DPs

DP plays key roles in HF morphogenesis and regeneration [15]. To further determine the mode of action of PPT, DPs were collected from mouse vibrissa ex vivo organ culture to extract total RNA for gene expression analyses of the representative growth factors that putatively regulate hair matrix cell proliferation [16], namely, placental growth factor (Prgf) [17], insulin-like growth factor 1 (Igf1) [18], hepatocyte growth factor (Hgf) [19], fibroblast growth Factor 7 (Fgf7) [20], vascular endothelial growth Factor A (Vegfa) [21], and plateleter-derived growth factor subunit A (Pdgfa) [22]. Igf1, Fgf7, and Vegfa were expressed in analogous manners in PPT-treated and nontreated mouse DP cells, while Hgf tended to be downregulated in PPT-treated DP cells (Fig. 2). Notably, Plgf was significantly upregulated in PPT-treated DP cells compared to nontreated controls by 1.6 ± 0.17-fold (P < 0.05) (Fig. 2).

These results highlighted Plgf as a potential factor responsible for promoting hair shaft elongation by PPT.

3.4. Involvement of Plgf in enhanced hair matrix keratinocyte proliferation by PPT

When mouse vibrissae were cultured with recombinant Plgf, the average length of hair shaft elongation after 3 days of treatment was 1010 ± 345 μm, 447 ± 23 μm longer than that in nontreated samples. Thus, Plgf promoted hair shaft elongation by 1.8 ± 0.6-fold vs. controls (P < 0.01) (Fig. 3A, B). This effect of Plgf was cancelled by adding anti-mouse Plgf neutralizing antibodies (Fig. 3A, B). The magnitude of the promotive effect of PPT on hair shaft elongation was moderate compared to that of Plgf. Importantly, anti-mouse Plgf neutralizing antibodies inhibited the effect of PPT treatment in an analogous manner, as observed in PPT-treated mouse vibrissae (P < 0.01) (Fig. 3A, B). The number of Ki67-positive hair matrix cells below Auber’s line, which was 27.6 ± 13.4 % on average in the controls, increased to 44.4 ± 12.7 % and 43.3 ± 5.3 % in Plgf- and PPT-treated vibrissae, respectively (P < 0.05) (Fig. 3C). Anti-mouse Plgf neutralizing antibodies individually decreased the aforementioned increases to 32.0 ± 13.7 % and 17.3 ± 9.9 % in Plgf- and PPT-treated vibrissae, respectively, (Fig. 3C).

These findings suggested the involvement of Plgf in enhanced hair matrix keratinocyte proliferation by PPT.

3.5. Acceleration of hair shaft elongation by PPT was Plgf/VEGFR-1 signalling pathway-dependent

Plgf has been reported to bind to vascular endothelial growth factor receptor 1 (VEGFR-1) to facilitate downstream signalling [23]. VEGFR-1 is expressed in follicular matrix cells of normal mouse hair vibrissae. (Fig. 3D). Upon Plgf binding, VEGFR-1 is phosphorylated to be activated [24]. Both Plgf and PPT increased VEGFR-1 phosphorylation compared to the controls by 1.6 ± 1.0- and 1.9 ± 0.4-fold, respectively, as detected by the WES simple western system (Fig. 3E, F). In agreement with the observations obtained in ex vivo vibrissa culture experiments, anti-Plgf neutralizing antibodies suppressed VEGFR-1 phosphorylation in Plgf- and PPT-treated samples (Fig. 3E, F).

To further investigate the involvement of VEGFR-1 signalling in hair shaft elongation, a VEGFR-1 inhibitor, ZM306416, was added to the ex vivo mouse vibrissa culture. Hair shaft elongation was inhibited in a dose-dependent manner by ZM306416 regardless of the presence of PPT (P < 0.05 or 0.01) (Fig. 3G). Importantly, ZM306416 more strongly inhibited hair shaft elongation in PPT-treated vibrissae vs. controls, as demonstrated by the ratios of hair shaft elongation inhibition between PPT- and nontreated samples (P < 0.05) (Fig. 3H).

These findings revealed a previously less-recognized role of the Plgf/VEGFR-1 signalling pathway in hair shaft elongation and suggested that the aforementioned promotive effect of PPT was pathway dependent.
Fig. 1. PPT synergistically enhanced mouse vibrissa hair shaft elongation compared to single and double vitamin treatments in ex vivo hair organ culture. (A) The changes in hair shaft elongation in mouse vibrissae treated with 0.03% (v/v) panthenyl ethyl ether, 0.015% (w/v) pyridoxine or 0.024% (v/v) tocopherol acetate were unremarkable (n=9). (B) Hair shaft elongation in mouse vibrissae was minimally affected by treatment with the two-reagent combination (n=13). (C) The combination of 0.03% (v/v) panthenyl ethyl ether, 0.015% (w/v) pyridoxine, and 0.024% (v/v) tocopherol acetate (PPT) significantly improved hair shaft elongation (n=9). ** P < 0.01, Student's t-test. (D) Representative pre- and posttreatment images of mock- or PPT-treated vibrissae. The results are expressed as the mean ± SD. Scale bars = 500 μm. For (A)-(D), evaluations were conducted 3 days after cultivation. (E) Increases in the number of proliferative cells (Ki67-positive, red fluorescence) in PPT-treated vibrissae compared to the nontreated control. Scale bars: 200 μm for low magnification and 50 μm for high magnification. The dashed area represents the area examined. (F) Quantitative analyses of the number of Ki67-positive cells. (n=6). ** P < 0.01, Student's t-test.
3.6. Changes in the hair follicle transcriptome illustrated the synergistic effects of Vits combinations as exhibited by PPT

To elucidate in more detail the molecular events explaining the effect of PPT on vibrissa hair follicles, global gene expression analyses were conducted. Microarrays were generated from vibrissa treated with single, double, or triple mixtures of PEE, PH, and TA to conduct principal component analysis (PCA) (Fig. 4A-E).

Gene ontology (GO) enrichment analysis was performed using the top 200 positively contributing gene sets for each of PC1, PC2, PC3, PC4, and PC5 to elucidate the biological meaning of each component. As a result, the gene sets from PC1, 2, and 5 were annotated with several GO terms, while none were annotated for PC3 and PC4. The heatmap visualized the top five GO terms annotated in the PC1, PC2, and PC5 gene sets, which were ranked by their adjusted p-values calculated by R following Benjamini and Hochberg procedure (Fig. 4A). The PC1 gene set was related to skin development (adjusted p-value: $5.3 \times 10^{-6}$), hair follicle development (adjusted p-value: $4.0 \times 10^{-5}$) and hair cycle process (adjusted p-value: $4.3 \times 10^{-5}$), which were annotated with the contributing genes, Shh, Tgfβ2, Lgr5, and Foxe1. The PC2 gene set was annotated with the glutathione metabolic process (adjusted p-value: $6.3 \times 10^{-7}$), cholesterol biosynthetic process (adjusted p-value: $3.1 \times 10^{-6}$), while the PC5 gene set was related to the regulation of epidermal cell differentiation (adjusted p-value: $4.9 \times 10^{-5}$) and epidermis development (adjusted p-value: $5.0 \times 10^{-5}$). The PC5 gene set included Notch1 and Atoh1, suggesting the involvement of Notch signalling in this group (Table 1).

Two- and three-dimensional plots of the PCA data were constructed to determine the contributions of individual Vits to each PC (Fig. 4B-E). The PC1 vs. PC2 plot indicated that PH and TA were involved in PC1, while PEE was associated with PC2 (Fig. 4B). The PC1 vs. PC5 and PC2 vs. PC5 plots suggested that PH and TA were involved in PC5 but contributed antagonistically (Fig. 4C and D). The three-dimensional plot indicated that the biological effect of PPT on hair elongation could be attributed to the synergistic summation of bioreactivities exhibited by PEE, PH, and TA, which was putatively mediated via characteristic transcriptome regulation of Shh signalling, glutathione cholesterol metabolism, and Notch signalling (Fig. 4E).

3.7. Molecular biological effects of vitamins on HFKCs and hDPCs

To assess the clinical relevance, human hair follicle keratinocytes and dermal papilla cells (HFKCs and hDPCs) were cultured with or without PPT. PPT did not promote cell proliferation of HFKCs and hDPCs, suggesting the necessity of epithelial-mesenchymal interaction to reproduce the effect of PPT (Fig. 5A, B).
Fig. 3. Increases in hair shaft elongation by PPT were PlGF/VEGFR-1 signalling pathway dependent. (A) Representative images of individually treated vibrissa and hair shaft elongation. Scale bar = 500 µm. (B) Changes in hair shaft elongation in mouse vibrissae incubated with PlGF and PPT with or without anti-PlGF neutralizing antibody (n = 10). Note that PlGF and PPT enhanced hair shaft elongation, which was cancelled by anti-PlGF neutralizing antibody. *P < 0.05, **P < 0.01, Dunnett’s test. (C) The percentages of Ki67-positive cells within matrix cells within treated mouse vibrissae (n = 5 or 6). **P < 0.01, *P < 0.05, Student’s t-test. (D) Immunohistological images showing VEGFR-1 localization patterns in hair bulbs of mouse vibrissae. (E) Phosphorylated VEGFR-1 protein expressions in vibrissae treated with PlGF or PPT with or without anti-PlGF neutralization antibody (n = 3). (F) Quantification analysis of the detected bands presented in (E). *P < 0.05, Student’s t-test. The results are expressed as the mean ± SD. Changes in hair shaft elongation lengths (G) and elongation ratios (H) in PPT-treated or nontreated mouse vibrissae with or without the VEGFR-1 inhibitor, ZM306416. Note that the inhibitory effect of ZM306416 in the presence of PPT was greater than that in the absence of PPT. *P < 0.05, **P < 0.01, Dunnett’s test.
Fig. 4. Changes in the hair follicle transcriptome due to combination vitamin treatments. (A) Heatmap showing gene set enrichment for GO (Biological Process) analysis associated with PCA. Each adjusted p-value (p.adjust value) was calculated by p.adjust function of R following Benjamini and Hochberg procedure. The colours denote the -log (p.adjust value) obtained by the Benjamini and Hochberg procedure. Green, increased -log (p.adjust value); white, decreased -log (p.adjust value); and grey, missing value. (B-D) Two-dimensional PCA plot for the combination of PC1, PC2, and PC5. The proportions of variance for each component are indicated by the percentage scores on each axis. The colours of each treatment are listed in the colour legend below. (E) Three-dimensional PCA plot for PC1, PC2, and PC5. The proportions of variance for each component are indicated by the percentage scores on each axis. The colours of each treatment are listed in the colour legend below.
When supplemented into hDPC culture, PEE increased PlGF expressions in a dose-dependent manner by 1.39 ± 0.01-fold (P < 0.05) and 1.57 ± 0.25-fold (P < 0.01) at concentrations of 0.1 % and 0.3 %, respectively (Fig. 5C). However, neither PH nor TA increased PlGF gene expression. Notably, PPT-treated hDPCs upregulated PlGF gene expressions by 2.37 ± 0.06-fold compared to their nontreated counterparts (P < 0.01) (Fig. 5D). The extent of upregulation by PPT was greater than that of 0.3 % (v/v) PEE alone (Fig. 5 C). PlGF secretion by cultured hDPC was further confirmed by ELISA. PPT-treated hDPCs produced 1.76 ± 1.28 times more PlGF than the nontreated controls (P < 0.01) (Fig. 5E). Similar findings were observed in additional experiments using hDPCs derived from a different donor (Supplementary Figure 3).

These results implied that PPT could exhibit some biological activity to improve hair growth in human HFs.

4. Discussion

Using Vits in commercial hair care products has been considered to be preferable; however, solid evidence to support their efficacy has been limited. To provide experimental evidence to rationalize the use of such ingredients, the current study was conducted. Intriguingly, PPT-, but not single- or double-Vits-treated mouse vibrissa, promoted hair shaft elongation. The nearly twofold increase in proliferating hair matrix cells in PPT-treated vibrissa suggested that enhanced hair shaft elongation could be a consequence of the growth–cumulative effect of PPT. In the current study, the evaluation was made after 3 days of incubation. As the vibrissa starts to enter catagen/telogen after this evaluation window in this ex vivo model [13], the possibility that single- or double-vitamin treatment might have exhibited some promotive hair growth effects over longer periods is less likely.

As we detected enhanced hair matrix cell proliferation in PPT-treated hair follicles, the expression levels of representative growth factors, which have been reported to be associated with anagen phase hair growth [17,22], were evaluated to determine whether PlGF was upregulated by PPT. The expression levels of Pdgfa tended to be higher in PPT-treated vibrissae, and the potential contributions of other noninvestigated growth factors cannot be ruled out. Nevertheless, it is reasonable to conclude that the promotive effect of PPT is mainly mediated by PlGF, as PlGF neutralizing antibodies cancelled hair shaft elongation and the increase in K67-positive hair matrix cells was enhanced in an analogous manner by the addition of PlGF and PPT.

PlGF is a member of the vascular endothelial growth factor (VEGF) subfamily and functions in angiogenesis, as well as the survival, proliferation, and migration of endothelial cells and smooth muscle cells by binding to VEGFR-1 (Flt-1) [25,26]. Activation of the PlGF/VEGFR-1 signalling pathway in follicular matrix cells was found to be necessary for PPT-induced hair shaft elongation. Indeed, increases in phosphorylated VEGFR-1 were observed in PlGF– and PPT-treated vibrissa, which were cancelled by PlGF neutralizing antibodies. In addition, VEGFR-1 inhibitors markedly reduced the rate of hair shaft elongation that was increased by PPT. Although PIGF was shown to be expressed in outer root sheath keratinocytes during the anagen phase of the hair follicle cycle [17,27], DP could be the main target of PPT and a predominant source of PlGF secretion. This is supported by the observation that modulation of PlGF expression by PPT in whole vibrissae paralleled that in hDPCs.

PCA and GO enrichment analyses suggested that PEE, PH, and TA differentially influenced vibrissa hair follicles. The genes that contributed to PC1 and PC5, in which PH and TA were involved, included Shh, Lgr5, and Notch. Lgr5 is a marker of hair follicle stem cells and is associated with hair follicle regeneration [28]. Shh signalling has been shown to play a key role in anagen induction and control. PIGF production in the cerebellar stroma in medulloblastoma has been reported to be Shh pathway dependent. [29]. Notch signalling is important for hair follicle development [30]. Metabolism-related genes such as cholesterol and glutathione were annotated in PC2, in which PEE was preferentially influenced, reported to be Shh pathway-dependent. [30]. Notch signalling is important for hair follicle development [30]. Metabolism-related genes such as cholesterol and glutathione were annotated in PC2, in which PEE was preferentially influenced, reported to be Shh pathway-dependent. [29]. Notch signalling is important for hair follicle development [30]. Metabolism-related genes such as cholesterol and glutathione were annotated in PC2, in which PEE was preferentially influenced, reported to be Shh pathway-dependent. [29]. Notch signalling is important for hair follicle development [30].
We are aware of the limitations of this study. The long-term effect of PPT was not assessed because of the nature of the ex vivo assay [12], although the assay has been widely adopted as a reliable indicator to assess the effects of reagents on hair follicles. The data could be influenced by various factors, represented by the viability of the vibrissa after surgical isolation. In human cell experiments, the influence of PPT on epithelial-mesenchymal interactions has not been assessed. The clinical efficacy of PPT, especially for MPHL and FPHL, was not evaluated, which can be theoretically assessed by a coculture system adopting HF KCs and hDPCs collected from affected individuals, but such materials are scarce. A clinical study could be conducted to more convincingly demonstrate the advantage of PPT. Theoretically, PPT can be used in combination with minoxidil, finasteride or dutasteride to improve the therapeutic outcome of PPT for MPHL; future clinical trials are indispensable for verification.

Despite these limitations, the findings in this study can shed light on the previously underrepresented advantage of utilizing Vits to ameliorate hair care products.

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**Conflict of interest**

All authors except M.O. are employees of Rohto Pharmaceutical Co. M.O. receives advisory fees from Rohto Pharmaceutical Co., Eli Lilly Japan K.K., Pfizer Japan Inc., Janssen Pharmaceutical KK., and Taisho Pharmaceutical Co., and research grants not related to the current work from Maruho Co., Sun Pharma Japan Ltd. and Shiseido Co.
CRediT authorship contribution statement

Liuying Hu: Conceptualization, Investigation, Formal analysis, Writing – original draft, Visualization, Validation. Shun Kimura: Conceptualization, Methodology, Investigation, Resources, Data curation, Visualization, Writing – review & editing. Takashi Shimizu: Project administration. Tsu Yoshi Ishii: Project administration, Funding acquisition, Writing – review & editing. Sayo Kashiwagi: Investigation. Kyo ko Takagi: Project administration.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jdermsci.2022.09.003.

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