Silybum marianum extract, Manganese PCA and *Lespedeza capitata* extract are active on hair growth and anchorage in human hair follicle dermal papilla cells

D. Bacqueville[1*], C. Mas[1], M. Lévêque[1], M.J. Haure[1], A. Noustens[1], V. Mengeaud[2], S. Carrère[1], S. Bessou-Touya[1] and H. Duplan[1]

[1] R&D department, Pierre Fabre Dermo-Cosmétique, Toulouse, France

[2] Laboratoire dermatologique Ducray, Lavaur, France

* D. Bacqueville, Centre R&D Pierre Fabre BP 13562, 3 Avenue Hubert Curien 31035 TOULOUSE Cedex 1, Tel 33 5 34 50 64 27, daniel.bacqueville@pierre-fabre.com

Abstract

Background: Hair loss is linked to a dysfunction of the hair follicle (HF) that undergoes cycles of growth (anagen), regression (catagen) and resting (telogen). HF cycle is highly regulated and involved complex molecular communications among which the signals emitted by the dermal papilla play a central role. The aim of this study was to evaluate the efficacy of three active ingredients on hair growth and anchorage in human dermal papilla cells: a new patented extract from *Silybum marianum* (SME), Manganese PCA (MnPCA) and a recently patented *Lespedeza capitata* extract (LCE).

Methods: Antibody array and gene reporter assays allowed to analyse receptor tyrosine kinase phosphorylation and Wnt/β catenin pathways activation, respectively. ELISA quantified the release of Versican, VEGF and DKK1 and activity of 5α-reductase (5αR) was also measured.

Results: SME 30 µg/mL activated growth factor receptor signaling pathways (EGFR x1.9 and PDGFR x2.8) and their downstream effectors (ERK, GSK3, Akt, STAT x1.2 to 2) after 1h exposure. MnPCA 0.009% stimulated the Wnt/β catenin pathway (+80%) and induced both Versican (x33 at 0.01%) and VEGF secretion (x3.3 at 0.009%). LCE 0.01% reduced DKK1 release (-72%) and 5αR activity (-59.6%).

Conclusion: SME modulated cell growth by acting on EGFR/PDGFR signaling pathway. MnPCA and LCE enhanced anagen phase *via* the Wnt/β catenin pathway. MnPCA also improved HF anchorage and microcirculation by stimulating Versican and VEGF. LCE favored dihydrotestosterone decrease. Thus, a combination of SME + MnPCA + LCE may be useful to improve hair loss treatment by specific action on hair growth and anchorage.

Keywords: Hair loss; growth; anchorage; dermal papilla; active ingredients.

Introduction

Hair loss is a major cause of dermatological consultations and greatly impacts the quality of life of patients [1]. Over the last decades, there have been great progress in understanding the molecular mechanisms of hair morphogenesis and biology, but also the pathologic basis of hair loss [2-4]. Research have shown that the hair follicle (HF) is a "mini-organ" containing several types of cells (*dermal papilla cells, hair shaft keratinocytes, melanocytes, inner/outer root sheath cells, stem cells…*) and that alopecia is associated with defects of HF cycle. The data evidenced that hair growth could be stimulated by anagen promotion, telogen inhibition, stimulation of microcirculation, nutrients supply and more recently micro-environment improvement of stem cell niche. All these processes are fine-tuned by specific intra and extra follicle signals (*growth factors, cytokines, hormones, enzymes*) that regulate downstream signaling pathways. As for example, Wnt/β catenin signaling pathway is well known to promote hair growth whereas 5αreductase (5αR) and TGFβ signals inhibit it [5-7].
In this context, a major goal remains the identification and the characterization of innovative active ingredients able to specifically target theses signaling pathways, especially medicinal herbs or natural/phytochemical compounds [8-10]. Finally, these new therapeutic tools could be used in synergistic interaction in topical formulations or nutricosmetics and may be complementary with the different medical/physical strategies recommended by the physicians to improve alopecia.

The HF cycle is highly dynamic [11] and involves complex molecular communications among which the signals emitted by the dermal papilla cells play a central role. The 2D

culture of these cells is very useful to elucidate the key signals which control hair growth, anchorage and differentiation [12, 13]. They are also in the forefront of hair research as screening platforms by providing insights into mechanistic action at cellular level, which may further direct the development of novel hair growth modulators especially for cosmetic testing.

Therefore, the aim of this study was to evaluate the efficacy of three active ingredients on hair growth and anchorage in dermal papilla cells isolated from human hair follicle (HFDPC). The first active ingredient was a new patented extract from *Silybum marianum* containing less than 2% silymarin (SME) (*WO/2021/023820*). This natural extract has been previously shown to stimulate the expression of the keratin 75 (K75), a specific marker of the "companion layer" of the HF which is involved in the anchorage of the hair shaft [14-15]. K75 deficit is also associated with a rare genetic alopecia: loose anagen hair syndrome [16]. Manganese PCA (MnPCA) and a *Lespedeza capitata* extract (LCE) were also tested. LCE is a phytochemical extract and was also recently patented (*WO/2020/020791A1*).

Materials and Methods

Human dermal papilla cell culture and active ingredients

All experiments were performed by using Human Follicle Dermal Papilla Cells (HFDPC) isolated from different donors and commercially available. Cells were seeded in 96-well plates or 60 mm Petri dishes in an adapted culture medium with specific complements as recommended by the supplier. HFDPC were grown 24h to 48h to reach about 80% confluency before active ingredients treatment. Active ingredients were obtained from Pierre Fabre Laboratories after specific extraction.

Growth factor receptor signaling pathways evaluation

The growth factor receptor (GFR) signaling pathways were analyzed by using human Proteome profiler™ antibody arrays (R&D Systems). These arrays allow the simultaneous and specific detection of 49 phospho-receptor tyrosine kinases and 43 downstream phospho-

kinases. Kits are a membrane-based sandwich immunoassay and were used according to the manufacturer's protocol. Capture antibodies spotted in duplicate on nitrocellulose membranes bind to specific target proteins present in the sample. Then, tyrosine phosphorylation of the captured proteins is detected with an HRP-conjugated pan phospho-tyrosine antibody and then visualized using chemiluminescent detection reagents. The signal produced is proportional to the amount phosphorylation in the bound analyte and was acquired by using Chemidoc MP imaging system and Image Lab software (Biorad). Quantification was performed with Image J software and the plugin microarray profile. HFDPC cells were stimulated for 1h with SME before harvesting and cell lysate analysis. Dimethylsulfoxide (DMSO) was used as a solvent. Experiments were performed from 2 donors in duplicate.

Wnt/β catenin signaling pathways evaluation

Wnt/β catenin pathway was studied with a gene reporter assay according to routine protocol. Briefly, HFDPC were transfected with a lentivirus expressing a luciferase gene under the control of Wnt/β catenin promoter (TCF/LEF transcriptional response element) (Cignal Lenti Reporter Qiagen). Luciferase expression was measured using Bright-Glo™ substrate and luminescence was quantified by using a microplate reader (ClarioStar). Cells were incubated 24h with MnPCA. Data were obtained from 3 independent donors and were performed in 2 independent batches.

Versican, VEGF and DKK1 release evaluation

Immunoassay tests were performed to quantify the production of Versican, VEGF and DKK1 from the HFDPC culture medium. Commercial kits were used according to supplier's protocol. Versican assay was done with the human versican ELISA kit from Cusabio whereas VEGF and DKK1 were quantified by using Milliplex® technology.

The release of versican and VEGF was analyzed following MnPCA treatment during 48h and 24h, respectively. The production of DKK1 was also measured 24h after HFDPC incubation with LCE. Data were obtained from 3-4 independent donors and were performed in triplicate.

5α–reductase activity evaluation

5α–Reductase (5αR) activity was evaluated by performing testosterone metabolism studies with [^{14}C]-testosterone and metabolites quantification on thin layer chromatography (TLC). Briefly, the steroid molecules from supernatants were extracted with a chloroform/methanol mix. The organic phase was collected and testosterone metabolites were separated by TLC by using a solvent system containing dichloromethane, ethylacetate and methanol. An autoradiography was performed on the chromatography and the transformed testosterone was estimated by densitometric analysis of the spots corresponding to DHT, 5α-androstane-3α,17β-diol, androstenedione, and 4-androstene-3,17-dione (Packard Cyclone PhosphorImager and Fujifilm Multigauge software). HFDPC were treated with LCE extract for 24h before 5αR measurement. Data were obtained from 2 independent donors and were performed in triplicate.

Data analysis and Statistics

Data were obtained from 2-4 donors and were performed in duplicate or triplicate according to the different readouts. Calculations were performed in Microsoft Excel software and results are presented as mean ± standard deviation.

The inter-group comparison was performed by a One-way Anova completed by a Dunnett's multiple comparison test on normalized data comparatively to the untreated control. Statistical analysis was performed with PRISM software: * $p<0.05$ and ** $p<0.01$.

Results and Discussion

Effect of Silybum marianum extract on GFR signaling pathway

SME has been previously described (patent *WO/2021/023820*) and is able to boost hair anchorage by stimulating K75 expression, a biomarker of "companion layer" [14-16]. The first objective of the study was to better understand how this natural extract could acts on HFDPC growth. Therefore, growth factor receptor (GFR) signaling pathways were analyzed by using Proteome profiler^{TM} antibody arrays that allow specific detection of tyrosine phosphorylation of receptor tyrosine kinases and their downstream phospho-kinases. As

presented in **Figure 1**, SME 30 µg/mL showed activation of GFR signaling pathways in HFDPC. The tyrosine phosphorylation of the EGFR and PDGFR were specifically and respectively induced by 1.9 and 2.8-fold following 1h stimulation in the presence of SME. Moreover, the downstream effectors of these GFR were also increased and they were upregulated from 1.2 to 2-fold for ERK, GSK3, Akt and STAT after SME treatment. *Thus, SME stimulates HFDPC growth by activating EGFR and PDGFR signaling.*

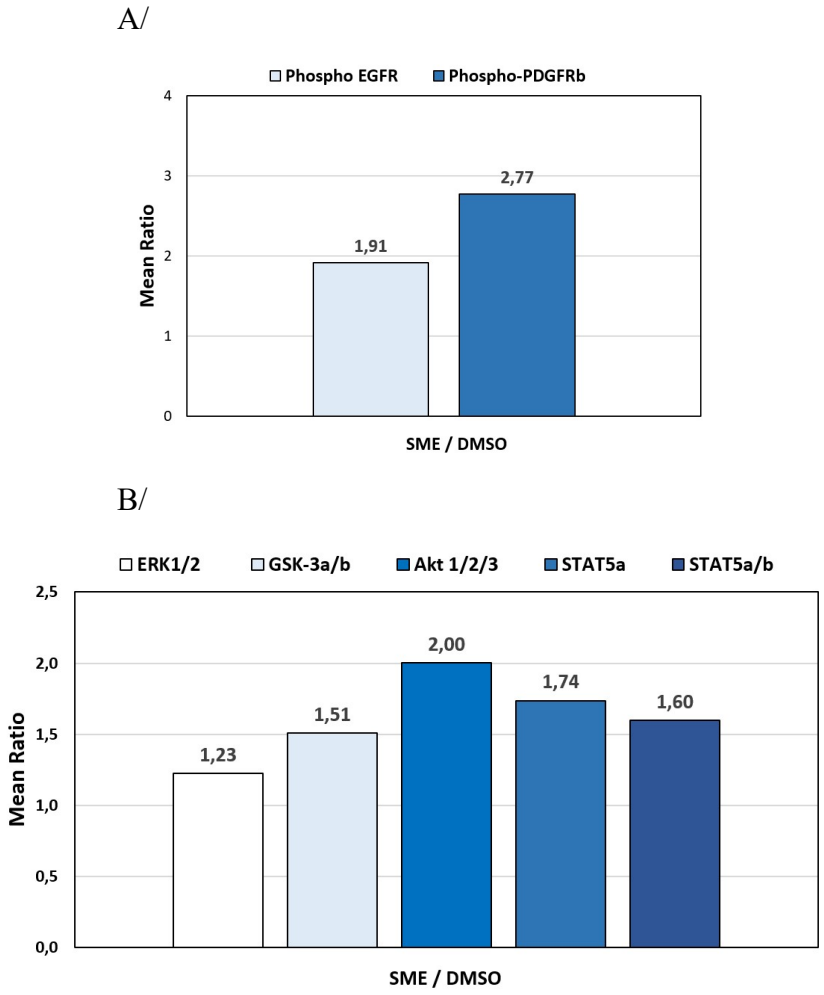


Figure 1: Induction of tyrosine phosphorylation of receptor tyrosine kinases and their downstream effectors by SME. A/ Phospho-RTK array of the EGFR and PDGFRβ**.** B/ Phospho-kinases array of the effectors.

Interestingly, PDGF isoforms have been shown to induce and to maintain the anagen phase of murine hair follicles [17]. Moreover, PDGF signals produced by immature intradermal

adipocyte lineage cells were linked in controlling HF stem cell activation and hair regeneration. Analysis of phospho-PDGFR demonstrated that during anagen induction, PDGFR is activated in the dermal papilla and the lower part of the hair germ [18]. Finally, a model of PDGF-mediated hair growth was proposed: PDGF from HF and subcutaneous adipocytes binds to PDGFR in HFDPC, this ligand-receptor interaction induces epiregulin release which in turn binds to EGFR in follicular keratinocytes, and finally binding of epiregulin to EGFR enhances the proliferation and differentiation (keratin expression especially K75) of HF keratinocytes leading to hair growth [19]. Thus, we might speculate that SME could stimulate hair growth *via* a molecular mechanism that depends on both PDGFR and EGFR signaling crosstalk in the HF.

Effect of Manganese PCA on Wnt/β catenin activation and Versican/VEGF release

The second active ingredient was a manganese salt called Manganese PCA (MnPCA). It was tested first on Wnt/β catenin pathway activation. In HFDPC, this signaling network leads to the production of secreted factors (HGF, IGF1) that are essential to anagen onset and hair growth. These factors promote the proliferation and differentiation of epithelial stem cells of the bulge (anagen onset) and matrix cells (anagen maintenance) [20, 21]. In androgenetic alopecia, androgens impair dermal papilla-induced hair follicle stem cell (HFSC) differentiation inhibiting Wnt signaling [22]. Data have shown that MnPCA stimulated the Wnt/β catenin pathway up to 80% at a dose 0.009% (**Figure 2**). *Therefore, MnPCA promotes anagen onset and participates to hair growth for optimizing HF cycle.*

Versican, a large chondroitin sulfate proteoglycan molecule, shows specific gene expression in the dermal papilla during anagen phase, and decreases with catagen onset. Its expression is regulated by the β catenin signaling pathway. This specific expression of versican suggests its importance to maintain the normal growing phase of HF [23, 24]. In addition, previous work of our laboratory showed that HFDPC produce VEGF [25]. This cytokine is an endothelial cell growth factor, it promotes supply of oxygen and nutrients to the HF [26]. The expression level of this protein varies during the hair growth cycle: widely produced in anagen phase, its expression decreases when HF enters into catagen phase.

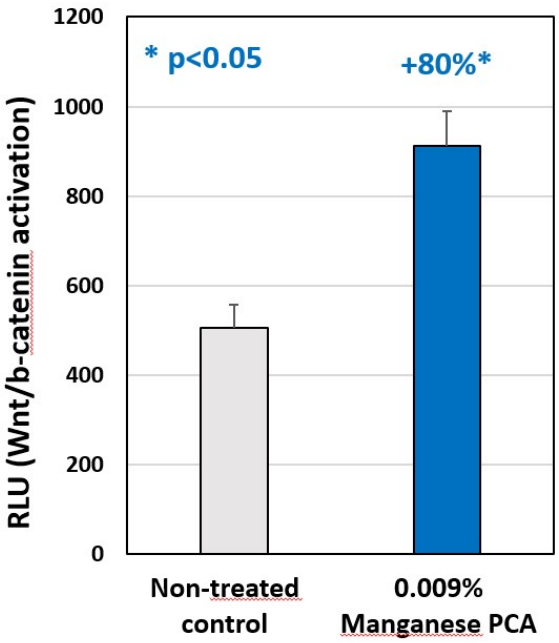
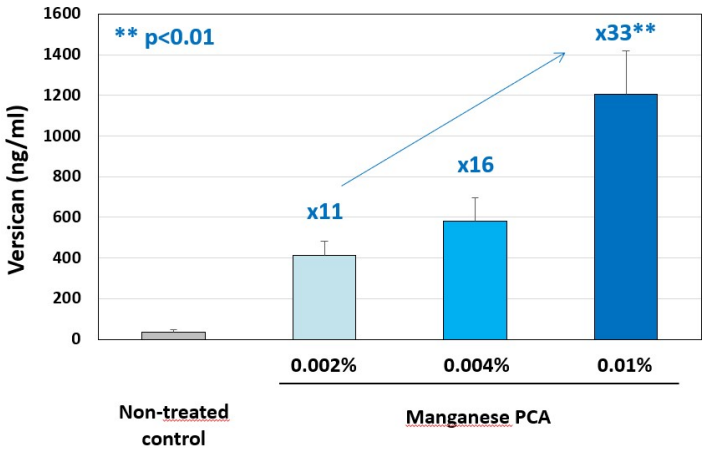


Figure 2: Stimulation of Wnt/β-catenin pathway by MnPCA.

HFDPC were transfected with a luciferase gene reporter for Wnt/β catenin promoter and the pathway activation was measured in relative luminescence unit (RLU).

As shown in **Figure 3**, MnPCA boosted the release of both versican and VEGF in the HFDPC culture medium. The production of versican was significantly induced by 33-fold at a 0.01% dose whereas the angiogenic factor VEGF was stimulated up to 3.3-fold at 0.009%. *Thus, MnPCA is able to stimulate the extracellular matrix but also the supply of nutrients necessary for hair life cycle.*

A/



B/

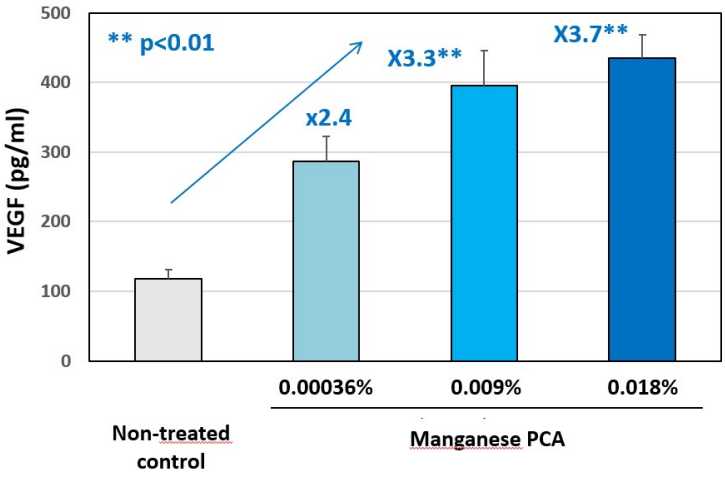


Figure 3: Induction of versican and VEGF release by MnPCA.

The culture medium of HFDPC was assayed by immunoassays to measure the level of versican (A) and VEGF (B) released following active ingredient treatment.

Effect of Lespedeza capitata extract on DKK1 production and 5α-reductase activity

The hair growth cycle is a highly regulated process during which different compartments of the hair follicle (dermal papilla, stem cells residing in the bulge, hair follicle matrix keratinocytes) maintain close relations through many molecular exchanges. Among the involved signals, Wnt/β catenin signaling is known to promote hair growth [20, 21, 27]. Androgenetic alopecia, which is the most common form of hair loss, is clearly an androgen-

dependent condition [28]. Androgens deregulate dermal papilla-secreted factors resulting in an abnormal stem cell differentiation *via* the inhibition of the canonical Wnt signaling pathway. The hair growth cycle inhibitor DKK1 (Dickkopf 1) is known to block canonical Wnt-mediated activation of β-catenin signaling and promotes catagen progression [29]. DKK1 is also described as being upregulated by androgens [30]. **Figure 4** revealed that LCE reduced the release of DKK1. The inhibition was dose-dependent and reached 72% at a dose 0.001%. *Thus, LCE modulates the Wnt/β-catenin signaling by acting on DKK1 secretion in HFDPC.*

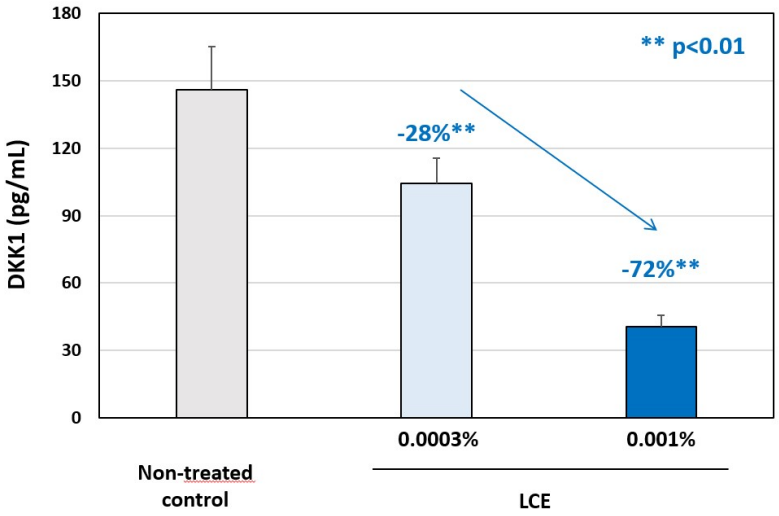


Figure 4: Blockade of DDK1 production by LCE.

The culture medium of HFDPC was assayed by immunoassays to measure the level of DDK1 released following LCE treatment.

As noted previously, androgenetic alopecia, is an androgen-dependent hair loss [28]. The primary and most well-known androgen is testosterone. Testosterone is metabolized to dihydrotestosterone (DHT) by the enzyme 5αR. DHT binds to a specific androgen receptor to form a complex that can regulate gene expression, especially in the HF in activating the genes responsible for hair follicle regression [31]. Thus, testosterone metabolism was studied to analyze 5αR activity. As shown in **Figure 5**, the assay was validated by using finasteride as a positive drug control for 5αR inhibitor with an efficiency of about 94% at a dose 10 µM. LCE was also able to decrease the 5αR activity with a 59.6% inhibition at 0.01% dose. *Thus,*

LCE blocked the 5αR activity and this natural extract is efficient to block testosterone metabolism.

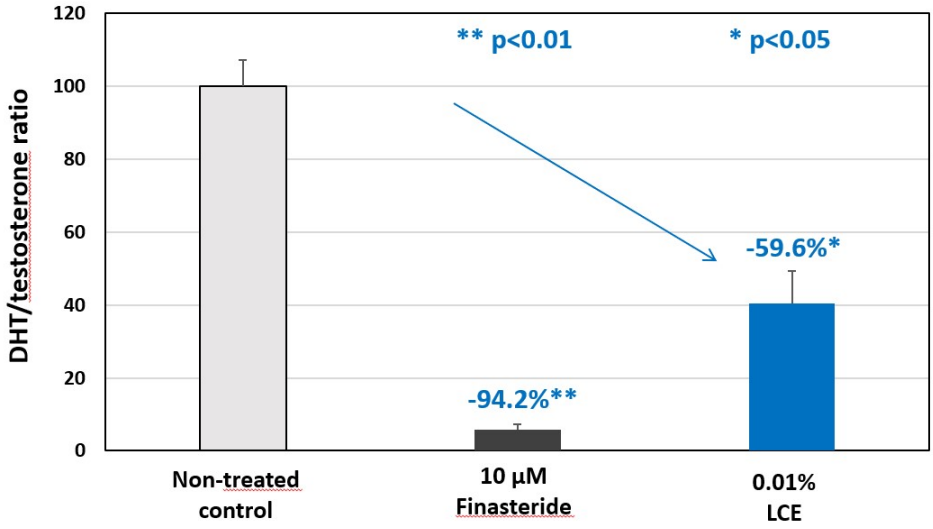


Figure 5: Inhibition of 5α-reductase activity by LCE.

5αR activity was evaluated by performing testosterone metabolism studies with [^{14}C]-testosterone and metabolites quantification on TLC. DHT/testosterone ratio was calculated following the treatment of HFDPC by both finasteride 10 µM and LCE 0.01% treatments.

Conclusions

In this study, the efficacy of three active ingredients was evaluated on hair growth and anchorage by using HFDPC as a pre-clinical model. Results have shown that SME modulates the cell growth by acting on EGFR/PDGFR signaling pathways. In addition, MnPCA and LCE enhanced the anagen phase via the Wnt/β catenin pathway. MnPCA also improved HF anchorage and microcirculation by stimulating Versican and VEGF production, respectively. Finally, LCE favored DHT decrease and inhibited androgen metabolism. Altogether, the data suggest that a combination of SME + MnPCA + LCE may be useful to improve hair loss treatment by a specific action on both hair growth and anchorage.

Conflict of Interest Statement. NONE.

References

1. Urban J, Qi L, Zhao H, et al. Comparison of hair manifestations in cardio-facio-cutaneous and Costello syndromes highlights the influence of the RAS pathway on hair growth. J Eur Acad Dermatol Venereol 2020;34(3): 601-07.
2. Jeffrey E Plowman, Duane P Harland. The Follicle Cycle in Brief. Review Adv Exp Med Biol. 2018;1054: 15-17.
3. Gentile P, Garcovich S. Advances in Regenerative Stem Cell Therapy in Androgenic Alopecia and Hair Loss: Wnt pathway, Growth-Factor, and Mesenchymal Stem Cell Signaling Impact Analysis on Cell Growth and Hair Follicle Development. Cells. 2019 May 16;8(5): 466.
4. Chen CC, Plikus MV, Tang PC, Widelitz RB, Chuong CM. The Modulatable Stem Cell Niche: Tissue Interactions during Hair and Feather Follicle Regeneration. J Mol Biol. 2016 Apr 10;428(7): 1423-40.
5. Choi BY. Targeting Wnt/β-Catenin Pathway for Developing Therapies for Hair Loss. Int J Mol Sci. 2020 Jul 12;21(14): E4915.
6. Jungyoon Ohn, Kyu Han Kim, Ohsang Kwon. Evaluating Hair Growth Promoting Effects of Candidate Substance: A Review of Research Methods. Review J Dermatol Sci. 2019 Mar;93(3): 144-149.
7. Jörn Michael Völker, Nadine Koch, Maike Becker, Adolf Klenk. Caffeine and Its Pharmacological Benefits in the Management of Androgenetic Alopecia: A Review. Review Skin Pharmacol Physiol. 2020 Jun 29;1-17.
8. Anna Herman, Andrzej P Herman. Topically Used Herbal Products for the Treatment of Hair Loss: Preclinical and Clinical Studies. Review Arch Dermatol Res. 2017 Oct;309(8): 595-610.
9. Herman A, Herman AP. Mechanism of action of herbs and their active constituents used in hair loss treatment. Fitoterapia. 2016 Oct;114: 18-25.
10. Daniels G, Akram S, Westgate GE, Tamburic S. Can plant-derived phytochemicals provide symptom relief for hair loss? A critical review. Int J Cosmet Sci. 2019 Aug;41(4): 332-345.

11. Jeffrey E Plowman , Duane P Harland . The Follicle Cycle in Brief. Review Adv Exp Med Biol. 2018;1054: 15-17.
12. Topouzi H, Logan NJ, Williams G, Higgins CA. Methods for the isolation and 3D culture of dermal papilla cells from human hair follicles. Exp Dermatol. 2017 Jun;26(6): 491-496.
13. Madaan A, Verma R, Singh AT, Jaggi M. Review of Hair Follicle Dermal Papilla cells as in vitro screening model for hair growth. Int J Cosmet Sci. 2018 Oct;40(5): 429-450.
14. Winter H, Langbein L, Praetzel S, Jacobs M, Rogers MA, Leigh IM, Tidman N, Schweizer J. A novel human type II cytokeratin, K6hf, specifically expressed in the companion layer of the hair follicle. J Invest Dermatol. 1998 Dec;111(6):955-62.
15. Mesler AL, Veniaminova NA, Lull MV, Wong SY. Hair Follicle Terminal Differentiation Is Orchestrated by Distinct Early and Late Matrix Progenitors. Cell Rep. 2017 Apr 25;19(4):809-821.
16. Chapalain V, Winter H, Langbein L, Le Roy JM, Labrèze C, Nikolic M, Schweizer J, Taïeb A. Is the loose anagen hair syndrome a keratin disorder? A clinical and molecular study. Arch Dermatol. 2002 Apr;138(4):501-6.
17. Tomita Y, Akiyama M, Shimizu H. PDGF isoforms induce and maintain anagen phase of murine hair follicles. J Dermatol Sci. 2006 Aug;43(2):105-15.
18. Festa E, Fretz J, Berry R, Schmidt B, Rodeheffer M, Horowitz M, Horsley V. Adipocyte lineage cells contribute to the skin stem cell niche to drive hair cycling. Cell. 2011 Sep 2;146(5):761-71.
19. Jeong HS, Kwack MH, Joo HW, Kim MK, Kim JC, Sung YK. Platelet-derived growth factor-AA-inducible epiregulin promotes elongation of human hair shafts by enhancing proliferation and differentiation of follicular keratinocytes. J Dermatol Sci. 2020 Feb;97(2):168-170.
20. Kishimoto J, Burgeson RE, B A Morgan BA. Wnt signaling maintains the hair-inducing activity of the dermal papilla. Genes Dev. 2000 May 15;14(10):1181-5.
21. Wang X, Tredget EE, Wu Y. Dynamic signals for hair follicle development and regeneration. Stem Cells Dev. 2012 Jan;21(1):7-18.

22. Leirós GJ, Ceruti JM, Castellanos ML, Kusinsky AG, Balañá ME. Androgens modify Wnt agonists/antagonists expression balance in dermal papilla cells preventing hair follicle stem cell differentiation in androgenetic alopecia. Mol Cell Endocrinol . 2017 Jan 5;439:26-34.
23. Soma T, Tajima M, Kishimoto J. Hair cycle-specific expression of versican in human hair follicles. J Dermatol Sci. 2005 Sep;39(3):147-54.
24. Yang Y, Li Y, Wang Y, Wu J, Yang G, Yang T, Gao Y, Lu Y. Versican gene: Regulation by the β-catenin signaling pathway plays a significant role in dermal papilla cell aggregative growth. J Dermatol Sci. 2012 68:157-163.
25. Lachgar S, Moukadiri H, Jonca F, Charveron M, Bouhaddioui N, Gall Y, Bonafe JL, Plouët J. Vascular endothelial growth factor is an autocrine growth factor for hair dermal papilla cells. J Invest Dermatol. 1996 Jan;106(1):17-23.
26. Kozlowska U, Blume-Peytavi U, Kodelja V, Sommer C, Goerdt S, Majewski S, Jablonska S, Orfanos CE. Expression of vascular endothelial growth factor (VEGF) in various compartments of the human hair follicle. Arch Dermatol Res. 1998 Dec;290(12):661-8.
27. Leirós GJ, Attorresi AI, Balañá ME. Hair follicle stem cell differentiation is inhibited through cross-talk between Wnt/β-catenin and androgen signalling in dermal papilla cells from patients with androgenetic alopecia. Br J Dermatol. 2012 May;166(5):1035-42.
28. Kelly Y., Blanco A, Tosti A. Androgenetic Alopecia: An Update of Treatment Options. Drugs. 2016 Sep;76(14):1349-64.
29. Kwack MHK, Kim MK, Kim JC, Sung YK. Dickkopf 1 promotes regression of hair follicles. J Invest Dermatol. 2012 Jun;132(6):1554-60.
30. Kwack MH, Sung YK, Chung EJ, Im SU, Ahn JS, Kim MK, Kim JC. Dihydrotestosterone-inducible dickkopf 1 from balding dermal papilla cells causes apoptosis in follicular keratinocytes. J Invest Dermatol. 2008 Feb;128(2):262-9.
31. Randall VA. Role of 5 alpha-reductase in health and disease. Baillieres Clin Endocrinol Metab. 1994 Apr;8(2):405-31.