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Immunopotentiator from *Pantoea agglomerans* 1 (IP-PA1) Promotes Murine Hair Growth and Human Dermal Papilla Cell Gene Expression

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Abstract. *Background/Aim: The lipopolysaccharide (LPS)-like compound derived from Pantoea agglomerans (immunopotentiator from Pantoea agglomerans 1 (IP-PA1)) has been used not only as dietary supplement or cosmetic for humans, but also by Japanese veterinarians as an anti-tumor, anti-allergy, "keep a fine coat of fur" and hair growth-promoting functional food for dogs and cats. In the present study, we focused on the hair growth-promoting effects of IP-PA1 on a hair-shaved animal model and its mechanism of action. We also investigated its potential on gene expression after stimulating human dermal papilla cells with IP-PA1. Materials and Methods: The hair on the back of a C3H/HeN mouse was shaved and IP-PA1 was orally administered or applied to the skin. The status of hair growth was observed and recorded for 14 days. Skin was collected and histological tissue examination was performed with respect to hair growth status using hematoxylin and eosin staining. After IP-PA1 administration (2 and 10 μg/ml) to human dermal papilla cell culture system for 24 h, fibroblast growth factor-7 (FGF-7) and vascular endothelial growth factor (VEGF) mRNA expression were measured using real-time polymerase chain reaction (PCR) analysis. Results: IP-PA1, when given orally, showed a tendency to promote hair growth in mice. In addition, skin application also significantly promoted hair growth, while histopathological examinations further demonstrated hair elongation from dermal papilla cells. In the human dermal papilla cell culture system, significant FGF-7 and VEGF mRNA expressions were observed ($p<0.05$). Conclusion: An underlying mechanism of gene expression by which IP-PA1 promotes hair growth was suggested to be different from that of medicine and traditional hair tonics, such as minoxidil and adenosine.*

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Key Words: Pantoea agglomerans, hair loss, dermal papilla cell, FGF-7, VEGF.

Immunopotentiator from *Pantoea agglomerans* 1 (IP-PA1) is a lipopolysaccharide (LPS)-like compound derived from *Pantoea agglomerans*, a Gram-negative microbe. It has been known to occur naturally in our surroundings and is a food component, such as wheat and brown rice (1). LPS is known to bind to Toll-like receptor 4 (TLR4) located on surfaces of cell membranes, such as those of macrophages, and activate the natural immune system; for this reason, IP-PA1 has been studied as a natural immunostimulant (2). It is structurally different from the widely known *Escherichia coli*-derived LPS and, recently, its safety and functionality in oral administration and skin application have been reported (3). Reports cover a wide range of applications due to its anti-tumor, anti-atopic, antihyperlipidemic, anti-diabetic and anti-Alzheimer effects (4, 5). It has also been simultaneously used as a functional food or cosmetic for humans; moreover, veterinarians also use it as an anti-tumor, anti-allergy and hair growth-promoting functional food in dogs and cats.

Many people of both sexes experience hair thinning or loss. It is considered to be one of the factors leading to decrease in the quality of life. In addition to aging, various internal and external factors can trigger hair thinning or loss; these factors include lifestyle (such as smoking) and stress in daily lives. Hair loss as a side-effect of anti-cancer agents having detrimental effects on young people and women (6, 7).

Table I. *Summary of primer sequences used for human dermal papilla cells qRT-PCR analysis.*

Gene	Primer name	Sequence (5'-3')
FGF-7	FGF7-For1	tctgtcgaacacagtggtacctgag
	FGF7-Rev1	gccactgtcctgatttccatga
VEGF	VEGFA-For2	aaagcatttgtttgtacaagatccg
	VEGFA-Rev2	cttgtcacatctgcaagtacgttcg
GAPDH	GAPDH-RT-For1	catccctgcctctactggcgctgcc
	GAPDH-RT-Rev	ccaggatgcccttgagggggccctc

Table II. *Score check sheet for evaluating the state of hair growth in mice.*

Score	Area of hair growth (%)
0	0
1	0-10
2	10-20
3	20-30
4	30-40
5	40-50
6	50-60
7	60-70
8	70-80
9	80-90
10	90-100

The mechanism underlying age-associated hair thinning or loss is different in men and women. In men, the conversion of testosterone, a male hormone, into dihydrotestosterone by 5-α reductase is considered to be the cause, whereas, in women, decrease in aging-induced estrogen, a female hormone, is considered the major factor for hair thinning or loss. The scalp is visible as a result of hair thinning. Finasteride and minoxidil, as treatments for hair thinning or falling in men, have been orally administered or applied on skin, respectively, and have demonstrated some effects. The inhibitory effect of finasteride on 5-α reductase and, in contrast, the mechanisms of minoxidil in vasodilation and induction effect of vascular endothelial growth factor (VEGF) in dermal papilla cells have been reported (8, 9).

As mentioned above, various factors need to be considered for treatment in preventing hair thinning or loss. Recently, folk remedy-based treatments, including traditional Chinese medicines and health foods, have been attracting attention along with conventional pharmaceutical products. For instance, adenosine, a nucleotide, was approved in 2004 by the Ministry of Health, Labour and Welfare of Japan, as an active ingredient of a quasi-drug (hair tonic). Its action on the induction of fibroblast growth factor-7 (FGF-7) in dermal papilla cells, as well as its mechanisms of action, has been reported (10). Recently, various hair restoration components have been reported in natural products; however, most of them were developed by targeting the expression or repression of VEGF and FGF-7, as well as their surrounding genes (11).

For this reason, in the present study, we attempted to examine the hair growth-promoting effects of IP-PA1 as a new function in a mouse and human dermal papilla cell culture system.

Materials and Methods

Sample preparation. Wheat fermentation extract (immunopotentiator from *Pantoea agglomerans* 1: containing IP-PA1 6.8 mg/g; lot.130222) was purchased from Macrophi Inc. (Shikoku, Japan). A small amount of the extract (0.5 g) was dissolved in 100 ml distilled water to prepare a sample for oral administration (IP-PA1-PO). Five grams of wheat fermentation extract was dissolved in 100 ml distilled water to prepare a sample for skin application (IP-PA1-SC). For the minoxidil skin application, a commercially available product, RiUP X plus 5%; lot.055D1, was purchased from Taisho Pharmaceutical Co., Ltd. (Tokyo, Japan).

For cell experiments, an IP-PA1 purified product, lot.M2, was purchased from Macrophi Inc. and dissolved in dimethyl sulfoxide (DMSO) (Nacalai tesque Inc., Kyoto, Japan) to prepare a 500 mg/ml stock solution. Adenosine from Wako Inc. (Tokyo, Japan) was dissolved in DMSO to a final concentration of 100 mM and minoxidil from Sigma-Aldrich Japan (Tokyo, Japan) was dissolved in 50% ethanol to a final concentration of 15 mM to prepare stock solutions, which were used as positive controls.

Dermal papilla cell growth media from TOYOBO Inc. (Osaka, Japan), dermal papilla cell subculturing unit (TOYOBO Inc.) and live cell counting reagent SF from Nakalai tesque Inc. were used for cell cultures .

Forty-eight-well Cellmatrix Type I-C (Nitta-gelatin Inc., Osaka, Japan) culture plates were used. FastLane Cell® cDNA Kit (QIAGEN Co., Ltd., Venlo, Netherlands), SYBER® Premix Ex Taq 5 ml (Takara Inc., Shiga, Japan), oligonucleotides (primers; FASMAC Inc., Kanagawa, Japan) (Table I) were purchased for use in quantitative real-time PCR (qPCR).

Murine hair growth test. Seven-week-old C3H/HeN male mice were purchased from Charles River Japan, Inc. (Kanagawa, Japan). They were housed in individual cages under conventional conditions consisting of 12-h light-dark cycle, 23±1˚C temperature and 55±15% humidity.

The back of the mice were consistently shaved using an electronic shaver PHILIPS Co Ltd., (Amsterdam, Netherlands) and animals divided into four groups of eight individuals each as follows: the group allowed to freely intake IP-PA1-PO from a water bottle; two groups that received a skin application of 250 μl IP-PA1-SC or minoxidil (RiUP X plus 5%) to their backs with a glass bar every day, respectively; and a no treatment (control). These mice were either administered the sample drug or given a skin application. Photographs were taken 14 days later to digitize the state of hair growth as shown in Table II.

At the end of the experiment, animals were sacrificed and skin was obtained. The skin tissues were fixed in 10% neutral-buffered formalin, samples were embedded in paraffin and thin sections (5

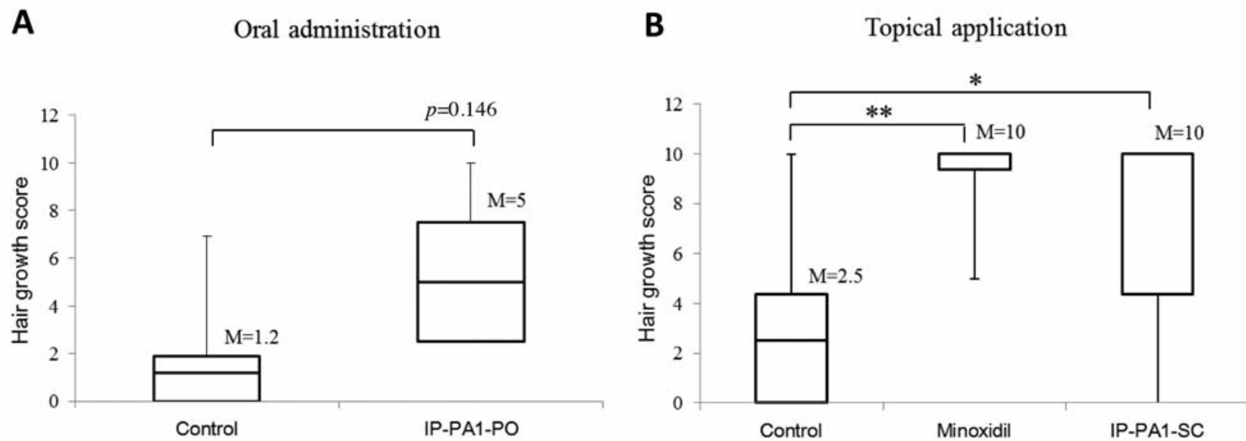

Figure 1. *Changing hair growth score in oral or topical application of IP-PA1. The back hair of mice was shaved and, after 14 days, hair growth efficiency was evaluated. A) Oral administration of IP-PA1-PO sample (0.5% free access). B) Topical application of minoxidil (RiUP X plus 5%) or IP-PA1-SC (5%) samples. Data are shown as means±SD. Significant differences (*p<0.05, **p<0.01) were obtained between control and samples on topical application examination. M=Median.*

µm) were cut. Sections were stained with hematoxylin and eosin (H&E) solution. Skin lesions were histologically examined using a light microscope (Olympus AX70; Olympus, Tokyo, Japan) with 40× and 100× objective lenses.

The present investigation conforms to the Guiding Principles for the Care and Use of Experimental Animal of Hokkaido Pharmaceutical University (published 1998, revised in 2001 and 2007). The protocol approval number was H27-009.

In vitro cell viability test. Human dermal papilla cells were obtained from the calvaria of a 63-year-old Caucasian male (Code No. CA602t05a; TOYOBO Inc.). Cells were cultivated using designated culture media and plates.

For the cytotoxicity test, a control (final concentration of 0.1% DMSO) and IP-PA1 (final concentration of 0.1% DMSO) were adjusted to seven different concentrations. These samples were added to cells that had reached confluence (n=3). Cells were further incubated for 48 h and viability was measured using SF reagent (OD 450 nm).

cDNA preparation. FastLane Cell® cDNA Kit was used to extract total RNA and 2 µl gDNA Wipeout Buffer, 1 µl Fast Lane Lysate, as well as 1 µl RNase-Free Water were added to a PCR tube, followed by incubation at 42˚C for 5 min. Then, 6 µl reverse transcription master mix solution (Quantiscript Reverse Transcriptase 1 µl, Quantiscript RT Buffer 4 µl, RT Primer Mix 1 µl) was added and incubated at 42˚C for 30 min. Finally, the mixture was incubated at 95˚C for 3 min to inactivate the reverse transcriptase. The product was used as synthesized cDNA for the qPCR analysis.

Quantitative real-time polymerase chain reaction. qPCR was performed using SYBER® Premix Ex Taq. Previously reported primers were used (Table I). PCR conditions were set at 95˚C for 10 s and 60˚C for 30 s. Relative quantification was performed by normalizing target expression to the housekeeping gene *GAPDH*. Data were expressed as change (n-fold) in *FGF-7* and *VEGF* mRNA expression compared with human dermal papilla cells incubated without samples before co-culture.

Statistical analysis. Results are expressed as means±S.E. One-way analysis of variance followed by Tukey's honestly significant difference test was used for comparing differences among multiple groups. Differences were considered significant at $^{**}p<0.01$ and $^{*}p<0.05$.

Results

Murine hair growth test. Mice from the IP-PA1-PO group, subjected to oral administration, showed a trend toward hair growth (control median=1.2, IP-PA1-PO median=5), although it was not significant, whereas mice from the minoxidil and IP-PA1-PO groups, which received skin application, showed significant hair growth (control median=2.5, minoxidil median=10, IP-PA1-SC median=10) (Figure 1). Moreover, when skins were collected from each group and observed after H&E staining, both the minoxidil and IP-PA1-PO groups showed hair elongation from the tissues surrounding the dermal papilla cells (Figure 2).

In vitro cell viability test. Cell viability of IP-PA1 on dermal papilla cells was examined based on short- and long-term treatments. The results indicated no cytotoxicity up to 48 h even when a maximum concentration of 10 µl/mg IP-PA1 was used (Figure 3). Therefore, 0.4, 2 and 10 µl/mg of IP-PA1 were added to cells and cultured for 24 h for the mRNA expression analysis.

Quantitative real-time PCR. Figure 4 shows the analysis results of *FGF-7* and *VEGF* mRNA expression. For *VEGF* mRNA, a trend toward mRNA expression was observed with minoxidil use and a significant expression ($p<0.05$) was confirmed with IP-PA1 (2 and 10 µl/mg) administration. For *FGF-7* mRNA, a significant expression ($p<0.01$) was observed after adenosine

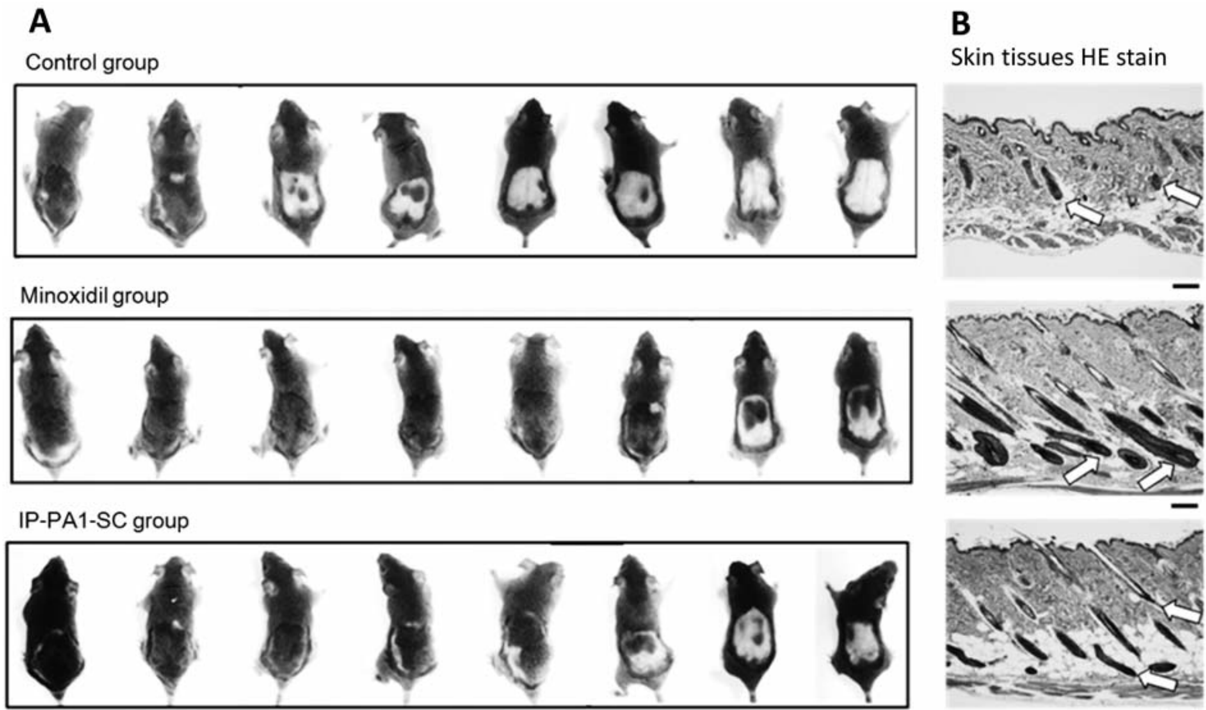

Figure 2. *Back hair growth and skin histological features. A) Photograph of back hair status in mice topically treated with control solution, minoxidil and IP-PA1-SC. These photos show both samples to enhance hair growth after shaving back hair in 14 days. B) Histological features of hair cells of the back skin lesions were stained with hematoxylin and eosin (HE). Both minoxidil and IP-PA1-SC groups showed hair elongation from the tissues surrounding the dermal papilla cells. Scale bars=100 μm.*

administration and, similarly, a significant expression (p<0.05) was confirmed with IP-PAI1 (2 and 10 μl/mg) use.

Discussion

VEGF facilitates hair follicle angiogenesis from the hair-bulb containing dermal papilla cells and is considered to accelerate hair growth by facilitating entry into the bloodstream and providing cells with nutrients. Minoxidil has been reported to facilitate VEGF production in dermal papilla cells, expand sulfonyl urea receptor-mediated capillary vessels and repress apoptosis against hair matrix cells (12, 13). In the present study, a tendency toward VEGF production induced by minoxidil was observed. The lack of significant facilitation may be due to the minoxidil concentration and culture time used in the *in vitro* experiments. Highly significant promotion (p=0.004) was observed in animal experiments and, therefore, in addition to dermal papilla cells, surrounding cellular tissues and stimuli are considered to be involved *via* a complex pathway.

FGF-7 reportedly promotes hair growth by accelerating cell proliferation, in both hair matrix and hair root sheath cells from dermal papilla cells. Adenosine has been reported to facilitate FGF-7 production *via* the adenosine A2b receptor

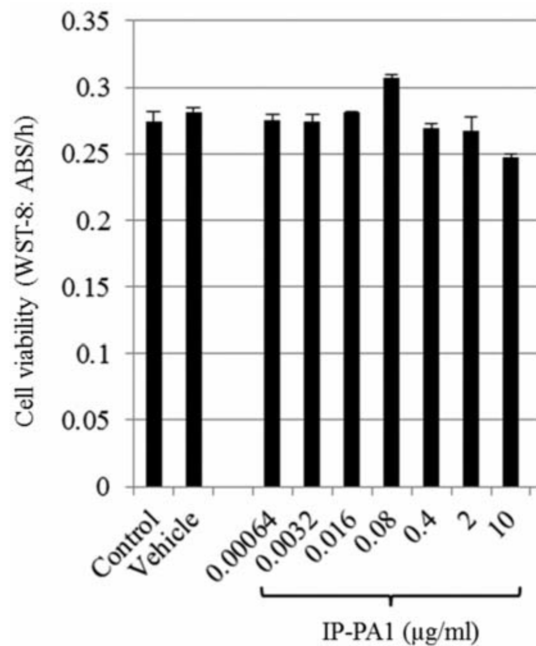

Figure 3. *Measurement of cell viability of human dermal papilla cells. The human dermal papilla cells cultured with IP-PA1 (0.00064~10 μg/ml) for 48 h. The concentrations of 0.4, 2 and 10 μg IP-PA1/ml for qRT-PCR test were determined. Vehicle=0.1% DMSO.*

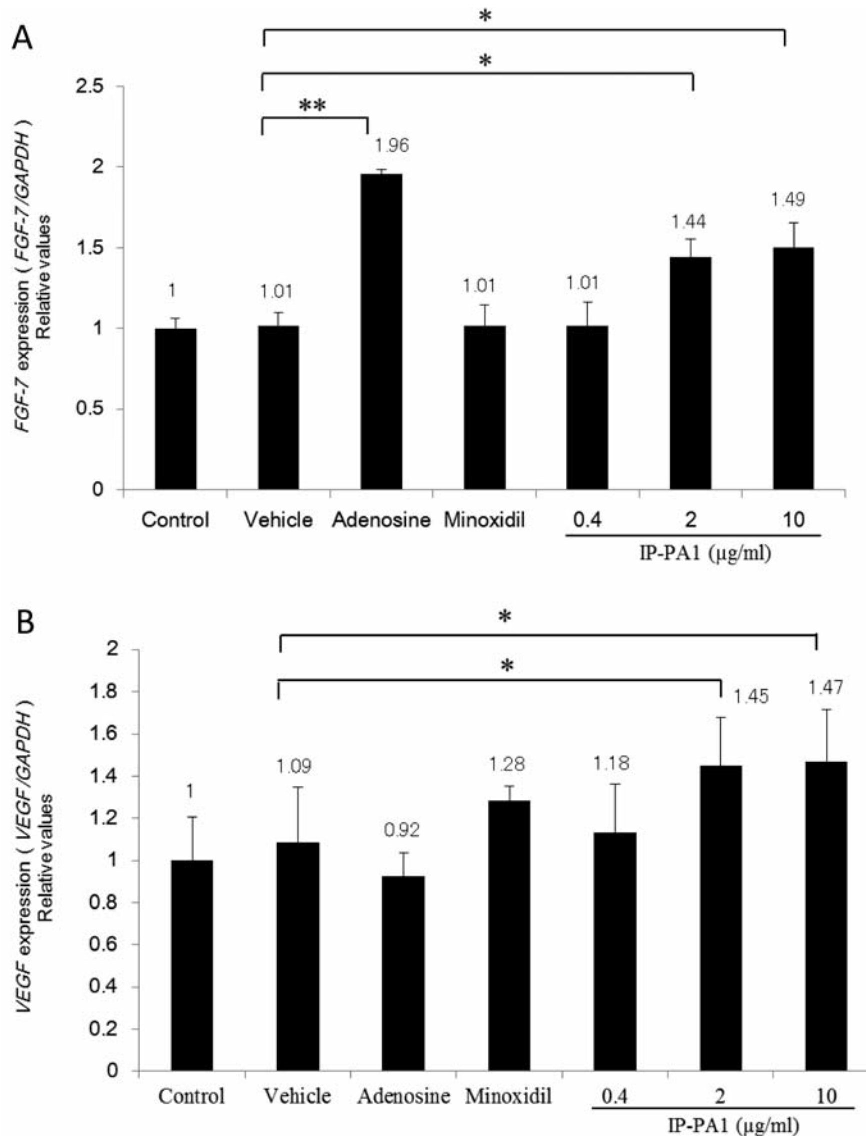

Figure 4. *Effect of FGF-7 and VEGF mRNA expression in human dermal papilla cells treated with IP-PA1. Gene expression level in human dermal papilla cells cultured with adenosine (100 μM), minoxidil (30 μM) and IP-PA1 (0.4, 2 and 10 μg/ml). Expression of FGF-7 (A) and VEGF (B) were measured by qRT-PCR. Significant differences (*p<0.05, **p<0.01) were obtained between vehicle and adenosine and IP-PA1 (2 and 10 μg/ml).*

in dermal papilla cells. Immunohistochemical staining has confirmed that the adenosine A2b receptor protein was expressed in the surrounding dermal papilla cells and outer root sheath in the hair follicles of healthy humans (14). In the present study, it was found that adenosine significantly facilitated the production of *FGF-7* mRNA in human dermal papilla cells. Indeed, the effect of skin application of adenosine has already been reported in humans (15).

In contrast, IP-PA1 showed a significant increase in the production of *VEGF* and *FGF-7* mRNA on dermal papilla cells, although the *p*-value was below 0.05. This suggests that the mechanism of IP-PA1-mediated hair growth is different from that of minoxidil or adenosine. In fact, hair growth was also promoted by oral administration or skin application in animal experiments, suggesting that hair growth is promoted by some complex biological reactions in addition to the direct involvement of IP-PA1 in dermal papilla cells.

Both *VEGF*- and *FGF-7*-encoding genes need to actively function during the growth phase (hair growth) of the hair growth cycle; however, these genes also need to be repressed during the recession phase (hair falling) of the cycle. For a rapid transition between the recession phase and the

following growth phase (hair-bulb regeneration), repression of *VEGF*- and *FGF-7*-encoding genes is considered important. Thus, the development of hair tonic is required to consider various factors, including the cause of hair loss and the hair growth cycle.

In addition to differences in sex and aging, several factors may cause hair loss: a) inflammatory damage to the hair follicle; b) increased hair shedding due to an underlying medical problem; c) side-effect of chemotherapy; d) a number of autoimmune conditions; and e) scarring from underlying conditions. IP-PA1 not only appears to stimulate macrophages but also regulates the immune system. In fact, its contributions to several effects have been reported: i) improvement of inflammatory symptoms in human and mouse; ii) regulatory function of Th1/Th2 balance in an allergic model mouse; and iii) facilitation of *IL-10* mRNA production in THP-1 cells (16-20). As shown in these reports, IP-PA1 potentially promoted hair growth *via* immune regulation or anti-inflammatory effects in addition to acting on *FGF-7* and *VEGF* genes; therefore, detailed studies in mice and humans are warranted in the future.

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