

A Short Peptide GPIGS Promotes Proliferation of Hair Bulb Keratinocytes and Accelerates Hair Regrowth in Mice

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The aim of this study was to discover a novel agent that promotes hair growth. We carried out a screening test in 298 types of conditioned medium (CM) from cultures of bacteria by using a hair bulb keratinocyte (HBK) growth assay. As a result, we found a HBK growth factor in the CM of *Bacillus* sp. M18. This HBK growth factor was purified by collecting biologically active fractions in three steps, including HP-20 batch processing, LH-20 chromatography and C₁₈ reverse-phase high-pressure liquid chromatography, and identified as a short peptide GPIGS. GPIGS increased Akt phosphorylation in HBKs. Moreover, the GPIGS-stimulated HBK growth was inhibited by the treatment with LY294002, an inhibitor of phosphatidylinositol 3-kinase (PI-3K). These results suggest that GPIGS promotes HBK growth via the PI-3K/Akt pathway. In addition to *in vitro* tests, GPIGS was found to accelerate hair regrowth in telogen mice. Our results indicate that GPIGS is a potential agent to promote hair growth.

Key words hair growth promotion; peptide; hair bulb keratinocyte; phosphatidylinositol 3-kinase (PI-3K)/Akt pathway; mouse

Hair follicle (HF) is a miniorgan that repeats the course of perpetual cycles through three distinct phases, anagen (growth phase), catagen (regressing phase) and telogen (resting phase).¹⁾ During anagen, hair bulb keratinocytes (HBKs) proliferate and differentiate vigorously, leading to the extension of the hair shaft.

Several factors that control HF cycle have been clarified. For example, studies using knockout or transgenic mice have indicated that sonic hedgehog^{2,3)} and keratinocyte growth factor^{4,5)} initiate the onset of anagen, and that transforming growth factor- β ,^{6,7)} fibroblast growth factor-5⁸⁾ and nerve growth factor⁹⁾ accelerate the transition from anagen to catagen by inducing HBK apoptosis. The HF cycle can be controlled finely by these factors under normal conditions. However, a variety of pathological stimuli induced by stress^{10,11)} or androgen^{12,13)} cause a disorder of the HF cycle, including the shortening of anagen duration and the prolongation of telogen duration, resulting in baldness.

Minoxidil has been used for treating patients with androgenic alopecia.^{14,15)} Moreover, the treatment with minoxidil is beneficial for alopecia areata^{16,17)} and for post-hair transplantation regrowth.¹⁸⁾ It has been reported that minoxidil promotes elongation of mouse HF in organ culture.¹⁹⁾ Minoxidil also appears to increase DNA synthesis in organ-cultured human scalp HF.²⁰⁾ In addition, minoxidil stimulates the proliferation of HBKs.²¹⁾ These results suggest that the minoxidil-stimulated HBK growth could cause the clinical beneficial effect on baldness, and indicate a possibility that an agent that stimulates HBK growth might have a potential to promote hair growth.

To find out a novel agent that promotes hair growth, in the present study, we carried out a screening test by measuring HBK growth activity. A variety of agents have been discovered from bacterial products. These agents have unique structures and have been used in the development of novel medicines. Therefore, we searched the conditioned medium (CM) of bacteria for a HBK growth factor. Our results demonstrate that a short peptide GPIGS, derived from the CM of *Bacillus*

sp. M18, promotes HBK growth via the phosphatidylinositol 3-kinase (PI-3K)/Akt pathway. Furthermore, we showed that GPIGS accelerates hair regrowth in telogen mice.

MATERIALS AND METHODS

Materials Dulbecco's modified Eagle's medium (DMEM), MCDB153 medium, phosphate buffered saline without calcium and magnesium (PBS (-)), bovine insulin, mouse epidermal growth factor (mEGF), human transferrin, hydrocortisone, *o*-phosphorylethanolamine and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, U.S.A.). Marine broth and skim milk were purchased from Difco (Sparks, MD, U.S.A.), Bovine pituitary extract (BPE), trypsin-ethylenediaminetetraacetate (EDTA) and penicillin/streptomycin were obtained from Invitrogen (Grand Island, NY, U.S.A.). Fetal bovine serum (FBS) was purchased from Cansera International Inc. (Ontario, Canada), and dispase from Godo Syuse (Tokyo, Japan). Collagenase (from *Streptomyces parvulus*) was purchased from Nitta Gelatin (Osaka, Japan), and trifluoroacetic acid (TFA) and ethanolamine from Wako (Osaka, Japan). A synthetic peptide GPIGS was purchased from American Peptide Company (Sunnyvale, CA, U.S.A.). LY294002 was obtained from Calbiochem (Torrance, CA, U.S.A.). Anti-Akt and anti-phospho-Akt antibodies were obtained from Cell Signaling (Beverly, MA, U.S.A.). Horseradish peroxidase-conjugated goat antibody to rabbit IgG was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The HP-20 resin was obtained from Mitsubishi Chemical (Tokyo, Japan). The alamarBlue reagent was obtained from Biosource (Camarillo, CA, U.S.A.). The ECL detection reagent, X-ray film and the LH-20 resin were obtained from Amersham Biosciences (Piscataway, NJ, U.S.A.).

Preparation of the CM from Cultures of Bacteria 298 types of bacteria, stored in National Institute of Advanced Industrial Science and Technology, were cultured with shaking in marine broth at 20 °C for 3 d. Each CM was centrifuged at

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5000×g for 30 min, and the supernatant was stored at -20 °C.

Isolation and Culture of HBKs Primary mouse HBKs were isolated and cultured according to a method reported previously.²²⁾ Briefly, the dorsal skin of 4-d-old C3H/HeN mice (Clea Japan Inc., Tokyo, Japan) was cut into small pieces and soaked in DMEM supplemented with 500 U/ml dispase, 5% FBS and 1% penicillin/streptomycin at 4 °C for 18 h. The epidermis was peeled off and discarded. The remaining dermis layer was suspended in DMEM supplemented with 0.2% collagenase and 1% penicillin/streptomycin at 37 °C for 1 h, and gently pipetted to obtain a dermal suspension containing hair bulb tissue (HBT). The suspension was centrifuged at 200×g for 5 min and the collagenase solution was discarded. The pellet was suspended in PBS (-), and then the suspension was allowed to stand for 15 min. Dermal fibroblasts that were still suspended were removed, and the precipitates containing HBT were collected. This precipitation process was repeated three times, resulting in the accumulation of only HBT. The collected HBT was treated with 0.25% trypsin and 2.65 mM EDTA at 37 °C for 5 min and gently pipetted. The isolated HBKs were suspended in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin, and precultured in collagen-coated plates, in 5% CO₂ at 37 °C for 24 h. Then, the cells were washed once with MCDB153, and cultured in MCDB153 supplemented with 5 µg/ml bovine insulin, 5 ng/ml mEGF, 35 µg protein/ml BPE, 10 µg/ml human transferrin, 0.5 µg/ml hydrocortisone, 6.1 µg/ml ethanolamine, and 14 µg/ml *o*-phosphorylethanolamine. HBKs were used as primary cells in every assay.

HBK Growth Assay HBKs were precultured in collagen-coated 96-well plates at a starting concentration of 6×10⁴ cells/well. At the same time that the culture medium was changed from DMEM to MCDB153 containing supplements, test samples were added. After 4 d, cell growth was assessed by alamarBlue assay. Briefly, the alamarBlue reagent was added in a volume of 1/10 to the culture medium and the culture was further incubated for another 2 h. Fluorescence intensity that reflects the amount of metabolic product, a reduced form of alamarBlue, was read in the spectrophotometer (Fluoroskan Ascent FL, Labsystems) using an excitation wavelength of 544 nm and an emission wavelength of 590 nm. The number of viable cells was expressed as fluorescence intensity.

In this assay, GPIGS was dissolved and diluted with MCDB153 containing supplements. LY294002 was dissolved with DMSO and diluted with PBS (-) (when cells were treated with LY294002, final concentration of DMSO was 0.1%). When LY294002 was used, cells were pretreated with LY294002 or DMSO (as control) for 20 min prior to the addition of GPIGS. And then, LY294002 and DMSO were further added three times every 24 h.

Screening Test We carried out screening test in 298 types of CM from cultures of bacteria by using the HBK growth assay. In this assay, each CM was added in a volume of 1/100 to the culture medium. We selected candidates under the criterion that HBK growth activity was higher than 130% relative to control (= 100%).

Purification and N-Terminal Amino Acid Sequence Analysis of HBK Growth Factor HBK growth factor in

the *Bacillus* sp. M18-conditioned medium (M18-CM) was purified by collecting biologically active fractions in three steps; HP-20 batch processing→LH-20 chromatography→C₁₈ reverse-phase high-pressure liquid chromatography (C₁₈ RP-HPLC).

The HP-20 resin that was equilibrated with H₂O after soaking in acetone was suspended in M18-CM (500 ml), and then the absorbed material was eluted with 250 ml of 70% methanol (HP-20 batch processing). The eluate was evaporated to 0.5 ml, and loaded on a LH-20 column (10×85 cm) by equilibrating with H₂O at a flow rate of 0.5 ml/min (LH-20 chromatography). Fractions were collected in 1 ml aliquots, and 10 µl of each fraction per 100 µl of MCDB153 containing supplements was used for the HBK growth assay. TFA was added to the biologically active fraction from the LH-20 chromatography to a final concentration of 0.1%, and the fraction was further purified by HPLC on a C₁₈ column (4.6×150 mm; TSKgel ODS-80Ts, Tosoh) with a gradient of acetonitrile at a flow rate of 1 ml/min in the presence of 0.1% TFA (C₁₈ RP-HPLC). Fractions were collected in 1 ml aliquots, and 10 µl of each fraction per 100 µl of MCDB153 containing supplements was used for the HBK growth assay.

To analyze the N-terminal amino acid sequence of the HBK growth factor, the biologically active fraction from the C₁₈ RP-HPLC was applied to a protein sequencer (Procise 494 HT Protein Sequencing System, Applied Biosystems).

Cell Treatments, Lysis, and Western Blotting HBKs were precultured in collagen-coated 35-mm tissue culture dishes at a starting concentration of 2×10⁶ cells/dish. At the same time that the culture medium was changed from DMEM to MCDB153 containing supplements, GPIGS was added. After 4 d, cells were washed with cold PBS (-) and solubilized in Nonidet P-40 lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 0.1 mM PMSF, 1 mM Na₃VO₄, and 1% protease inhibitor cocktail).

Whole cell lysates (10 µg) were separated by 10% SDS-PAGE and then electroblotted onto polyvinylidene fluoride membranes (Immune-Blot PVDF Membrane; BIO-RAD). The membranes were blocked using 5% nonfat dried milk in PBS (-), pH 7.2, and then primary immunoreactions were performed with the anti-Akt (1 : 1000) or anti-phospho-Akt (1 : 1000) antibody for 18 h at 4 °C. The membranes were washed three times with TBST buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) and then incubated with the secondary antibody (horseradish peroxidase-conjugated goat antibody to rabbit IgG) (1 : 2000) for 1 h at room temperature. The membranes were washed three times with TBST buffer and developed using an ECL detection reagent prior to exposure to an X-ray film.

Hair Regrowth Assay *in Vivo* Seven-week-old female C3H/He mice were purchased from Japan SLC (Shizuoka, Japan). They were acclimated for a minimum of 7 d in our quarters prior to use. The room was maintained at a constant temperature of 23 °C with a humidity of 55%, and the animals were fed a standard pellet diet and water *ad libitum*.

Hair regrowth assay was performed according to a method reported previously.²³⁾ Eight-week-old mice, whose HF cycle was in the telogen phase, were anesthetized with sodium pentobarbital (70 mg/kg, i.p.). The hair on the dorsal of each mouse was shaved using an electric clipper and an electric

shaver. After 3 d, the shaved skin areas were treated with vehicle (H₂O:propylene glycol:ethanol=3:2:5), 0.03% GPIGS and 0.1 % GPIGS once a day for 13 d. The applied volume of these test agents was 100 μ l. On the 14th day after the start of administration, the mouse dorsal skin was photographed with a data correction marker. Using image analysis software (NIH image), the hair-regrown area was determined under any threshold that was corrected by the data correction marker, and the ratio of the hair-regrown area to the shaved skin area was determined.

Statistical Analysis In HBK growth assay, data were expressed as the means \pm S.D. In *in vivo* hair regrowth assay, data were expressed as the means \pm S.E. Statistical evaluation was performed one-way analysis of variance (ANOVA) followed by the two-tailed Dunnett's multiple comparison test. *p* values less than 0.05 were considered to be statistically significant.

RESULTS

Screening Test for Discovering a Novel HBK Growth Factor To discover a novel agent that promotes hair growth, we carried out screening test in 298 types of CM from cultures of bacteria by measuring the HBK growth activity. As a result, we found that the CM of *Bacillus* sp. M18 promotes HBK growth at a final concentration of 0.1% v/v or more (Fig. 1).

Purification and Identification of HBK Growth Factor We purified HBK growth factor from the M18-CM by collecting biologically active fractions. The M18-CM was concentrated about 1000-fold by evaporating the elution from HP-20 batch processing. This concentrated M18-CM was applied to the LH-20 column, and biologically active fractions with a retention time of 82—96 min were obtained (Fig. 2A). This active elution from LH-20 chromatography was loaded on the C₁₈ reverse-phase column, and the activity was eluted with 17.3% acetonitrile (Fig. 2B).

Using Edman sequence analysis, the HBK growth factor in the final fraction, corresponding to the absorbance peak indicated by an arrow in Fig. 2B, was unambiguously identified as the short peptide GPIGS (Fig. 2C). In one series of experiments, 1.04 μ mol of GPIGS was obtained from 500 ml of the M18-CM.

Effect of GPIGS on HBK Growth We verified the effect of the synthetic peptide GPIGS on HBK growth. GPIGS promoted HBK growth in a dose-dependent manner; 101 \pm 3.3, 111 \pm 2.3, 124 \pm 4.4, 141 \pm 5.1, 142 \pm 3.5 and 141 \pm 3.0% relative to control (100 \pm 2.5%) at doses of 0.3, 1, 3, 10, 30 and 100 μ M, respectively (Fig. 3).

Signaling of GPIGS-Stimulated HBK Growth The activation of the mitogen-activated protein kinase and/or PI-3K pathway is known to be involved in the growth of various cells. Therefore, we investigated whether these signaling pathways are activated after GPIGS stimulation. The exposure of HBKs to GPIGS increased Akt phosphorylation in a dose-dependent manner (Fig. 4A). In contrast, we could not observe an increase of extracellular signal-regulated protein kinase phosphorylation by the treatment with GPIGS (data not shown).

To determine whether the PI-3K pathway is involved in the GPIGS-stimulated HBK growth, LY294002, an inhibitor of

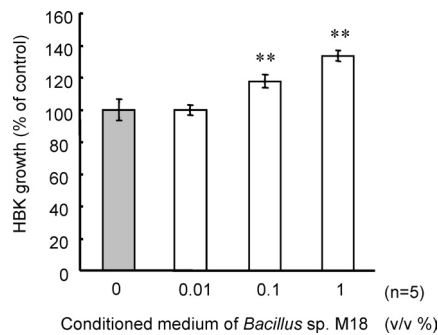


Fig. 1. Effect of Conditioned Medium from Culture of *Bacillus* sp. M18 on HBK Growth

HBKs were cultured in the presence (the indicated concentration) or absence (as control; containing equivalent volume of non-conditioned medium) of the conditioned medium from culture of *Bacillus* sp. M18 for 4 d. Cell growth was assessed by alamar-Blue assay. The values represent the means \pm S.D. of five individual cultures. ***p*<0.01 vs. control (two-tailed Dunnett's test).

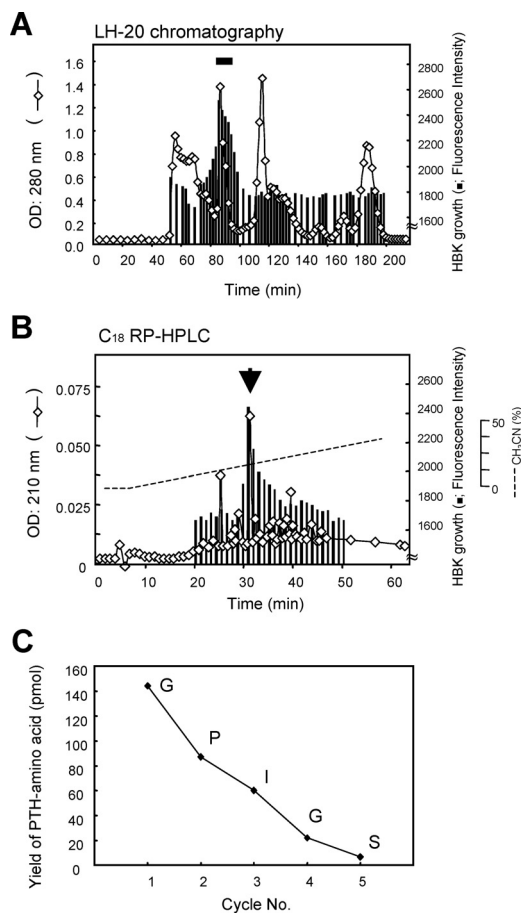


Fig. 2. Purification and Structure of HBK Growth Factor

(A) LH-20 chromatogram. The concentrated eluate from HP-20 batch processing was loaded on a LH-20 column (10 \times 85 cm) equilibrated with H₂O at a flow rate of 0.5 ml/min. The eluate absorbance at 280 nm was recorded and the biologically active fraction designated by a solid bar was collected. (B) C₁₈ RP-HPLC. TFA was added to the biologically active fraction from LH-20 chromatography to a final concentration of 0.1%, and applied on a C₁₈ column (4.6 \times 150 mm; TSKgel ODS-80Ts, Tosoh) equilibrated with 0.1% TFA. The absorbed materials were eluted with a gradient of acetonitrile at a flow rate of 1 ml/min, and the eluate absorbance at 210 nm was recorded. HBK growth activity was coeluted with the absorbance peak indicated by an arrow. (C) Peptide sequence analysis. The yield of phenylthiohydantoin (PTH)-amino acids at each cycle of Edman degradation is shown with the one-letter amino acid notation.

PI-3K, was used. The GPIGS-stimulated HBK growth was attenuated following the treatment with LY294002 (Fig. 4B). These results suggest that GPIGS promotes HBK growth *via*

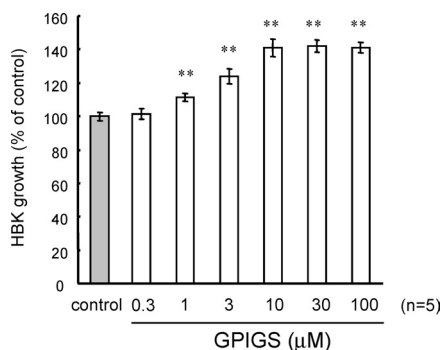


Fig. 3. Effect of GPIGS on HBK Growth

HBKs were treated with GPIGS (at indicated concentrations) for 4 d. Cell growth was assessed by alamarBlue assay. The values represent the means±S.D. of five individual cultures. ***p*<0.01 vs. control (two-tailed Dunnett's test).

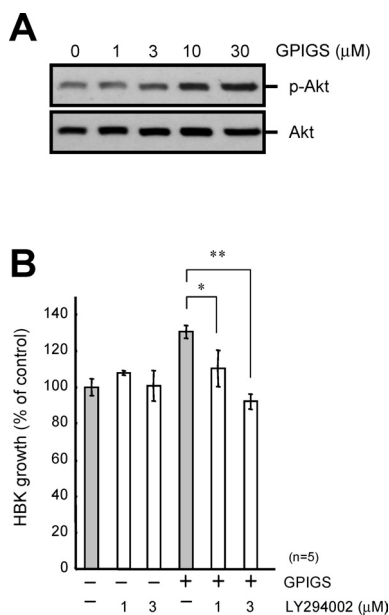


Fig. 4. Involvement of the PI-3K/Akt Pathway in GPIGS-Stimulated HBK Growth

(A) The dose response of GPIGS-induced phosphorylation of Akt. Cells were cultured in the presence of GPIGS at the indicated concentrations for 4 d. Activation of Akt was analyzed by Western blotting using the anti-phospho-Akt antibody. After stripping, Akt expression was verified by reprobing the same blots with the anti-Akt antibody (lower panels). (B) The inhibition of GPIGS-stimulated HBK growth by blocking the PI-3K activity. HBKs were cultured in the presence of LY294002 alone (the indicated concentration) or LY294002 plus 10 μM GPIGS for 4 d as described in Materials and Methods. Cell growth was assessed by alamarBlue assay. The values represent the means±S.D. of five individual cultures. **p*<0.05, ***p*<0.01 vs. GPIGS alone (two-tailed Dunnett's test).

the PI-3K/Akt pathway.

Effect of GPIGS on Hair Regrowth in Telogen Mice

We examined the effect of GPIGS on hair regrowth in telogen mice. Since telogen mice have homogeneously pink dorsal skin, the first macroscopic signs of anagen development can be appreciated by a corresponding change in skin color from pink to gray. The administration of 0.1% GPIGS resulted in the change of skin color from pink to gray uniformly within 5 to 6 d. On the other hand, in the vehicle-treated group the skin color changed partially from pink to gray within 8 to 9 d.

As shown in Fig. 5B, the ratio of the hair-regrown area to the shaved skin area in the vehicle-treated group was 29.3±8.3%. Compared with the vehicle-treated group, the

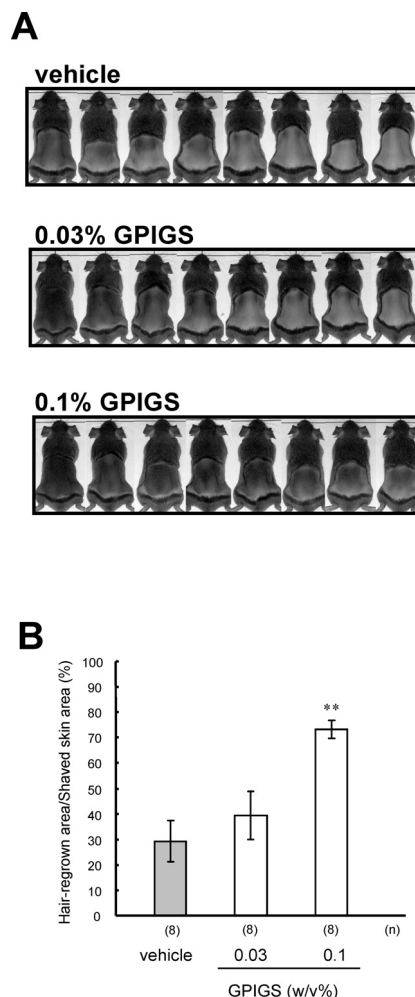


Fig. 5. Effect of GPIGS on Hair Regrowth in Telogen Mice

Test agents (100 μl/body) were applied to C3H/He telogen mice once a day. On the 14th day after the start of topical application of the test agents, photographs were taken (A) and the ratio of the hair-regrown area to the shaved skin area was measured (B). The values represent the means±S.E. of eight mice. ***p*<0.01 vs. vehicle (two-tailed Dunnett's test).

ratio in the GPIGS-treated group was increased in a dose dependent manner; 39.4±9.6 and 73.5±3.6% at doses of 0.03 and 0.1%, respectively (*p*<0.01 for 0.1% GPIGS). This result indicates that GPIGS promotes hair regrowth in telogen mice.

DISCUSSION

We demonstrated that the short peptide GPIGS, derived from the CM of *Bacillus* sp. M18, promoted HBK growth. The HBK growth-promoting effect of GPIGS requires the activation of the PI-3K/Akt pathway. In addition to *in vitro* tests, the administration of GPIGS accelerated hair regrowth in telogen mice. These results indicate a potential of GPIGS as an agent for hair growth promotion.

The HBK growth activity was discovered in the CM of *Bacillus* sp. M18 (Fig. 1). This HBK growth factor was purified (Figs. 2A, B) and identified as a short peptide GPIGS (Fig. 2C). Such a short peptide is likely to be a degradation product of any peptide although it is unclear whether *Bacillus* sp. M18 itself produces a precursor peptide of GPIGS. If not so, *Bacillus* sp. M18 might produce an enzyme that cat-

alyzes the formation of GPIGS. Further studies are necessary to clarify how GPIGS appears in the M18-CM.

We verified that the synthetic peptide GPIGS promotes HBK growth in a dose-dependent manner (Fig. 3). GPIGS increased Akt phosphorylation in HBKs (Fig. 4A). Moreover, the GPIGS-stimulated HBK growth was inhibited by the treatment with LY294002 (Fig. 4B). These results suggest that GPIGS promotes HBK growth *via* the PI-3K/Akt pathway. There is evidence to indicate that Akt plays an important role in cell growth. Diehl *et al.*²⁴ demonstrated that Akt inhibits glycogen synthase kinase-3 β and stabilized cyclin D1 in the nucleus to stimulate cell growth. Besides, Zhou *et al.*²⁵ showed that Akt can phosphorylates p21Cip1/WAF1 and cause it to localize to cytoplasm, thereby suppressing its growth-inhibiting activity. In this study, it remains to be determined how GPIGS stimulates the PI-3K/Akt pathway in HBKs. The PI-3K/Akt pathway has been reported to be a downstream signaling of various cell surface receptors, including receptor tyrosine kinases,²⁶ G protein-coupled receptors²⁷ and integrins.²⁸ Therefore, we suppose a possibility that GPIGS may activate either cell surface receptor and in turn stimulate the PI-3K/Akt pathway in HBKs. Alternatively, GPIGS might stimulate directly an upstream signaling molecule leading to the activation of the PI-3K/Akt pathway. We are now investigating the underlying mechanism by which GPIGS activates the PI-3K/Akt pathway.

The administration of GPIGS increased the ratio of the hair-regrown area to the shaved skin area in telogen mice (Figs. 5A, B). In our *in vivo* test, the transition from telogen to anagen was occurred in the non-treated group (data not shown). This result suggests that the mechanical stimuli induced by shaving initiated the onset of anagen. Therefore, we suppose that GPIGS can promote hair growth during anagen.

In a preliminary toxicity test, when we continuously administered GPIGS to mice for 120 d, no inflammation of the skin was macroscopically observed throughout, suggesting that GPIGS may be a nonirritant and nonantigenic. In conclusion, our results indicate that GPIGS is a potential agent to promote hair growth. Several agents that facilitate the proliferation of HBKs, such as minoxidil, procyanidin²³ and KF19418,²⁹ have been reported to accelerate hair regrowth in telogen mice. Minoxidil^{14,15} and procyanidin^{30,31} are useful for the treatment of androgenic alopecia. Moreover, minoxidil shows the clinical beneficial effect on alopecia areata^{16,17} and post-hair transplantation regrowth.¹⁸ Therefore, we suppose that GPIGS may be a candidate therapeutic agent for androgenic alopecia, alopecia areata and post-hair transplantation regrowth. We are presently preparing to perform a human clinical study.

REFERENCES

- 1) Paus R., Cotsarelis G., *N. Engl. J. Med.*, **341**, 491—497 (1999).
- 2) Sato N., Leopold P. L., Crystal R. G., *J. Clin. Invest.*, **104**, 855—864 (1999).
- 3) Chiang C., Swan R. Z., Grachtchouk M., Bolinger M., Litingtung Y., Robertson E. K., Cooper M. K., Gaffield W., Westphal H., Beachy P. A., Dlugosz A. A., *Dev. Biol.*, **205**, 1—9 (1999).
- 4) Danilenko D. M., Ring B. D., Yanagihara D., Benson W., Wiemann B., Starnes C. O., Pierce G. F., *Am. J. Pathol.*, **147**, 145—154 (1995).
- 5) Guo L., Degenstein L., Fuchs E., *Genes Dev.*, **10**, 165—175 (1996).
- 6) Foitzik K., Lindner G., Mueller-Roeber S., Maurer M., Botchkareva N., Botchkarev V., Handjiski B., Metz M., Hibino T., Soma T., Dotto G. P., Paus R., *FASEB J.*, **14**, 752—760 (2000).
- 7) Soma T., Tsuji Y., Hibino T., *J. Invest. Dermatol.*, **118**, 993—997 (2002).
- 8) Hebert J. M., Rosenquist T., Gotz J., Martin G. R., *Cell*, **78**, 1017—1025 (1994).
- 9) Botchkarev V. A., Botchkareva N. V., Albers K. M., Chen L. H., Welker P., Paus R., *FASEB J.*, **14**, 1931—1942 (2000).
- 10) Aoki E., Shibasaki T., Kawana S., *Exp. Dermatol.*, **12**, 371—377 (2003).
- 11) Arck P. C., Handjiski B., Peters E. M., Peter A. S., Hagen E., Fischer A., Klapp B. F., Paus R., *Am. J. Pathol.*, **162**, 803—814 (2003).
- 12) Matias J. R., Malloy V., Orentreich N., *Arch. Dermatol. Res.*, **281**, 247—253 (1989).
- 13) Sundberg J. P., Beamer W. G., Uno H., Van Neste D., King L. E., *Exp. Mol. Pathol.*, **67**, 118—130 (1999).
- 14) De Villez R. L., *Arch. Dermatol.*, **121**, 197—202 (1985).
- 15) Olsen E. A., Weiner M. S., Delong E. R., Pinnell S. R., *J. Am. Acad. Dermatol.*, **13**, 185—192 (1985).
- 16) Fenton D. A., Wilkinson J. D., *Br. Med. J.*, **287**, 1015—1017 (1983).
- 17) Weiss V. C., West D. P., Fu T. S., Robinson L. A., Cook B., Cohen R. L., Chambers D. A., *Arch. Dermatol.*, **120**, 457—463 (1984).
- 18) Avram M. R., Cole J. P., Gandelman M., Haber R., Knudsen R., Leavitt M. T., Leonard R. T. Jr., Puig C. J., Rose P. T., Vogel J. E., Ziering C. L., *Dermatol. Surg.*, **28**, 894—900 (2002).
- 19) Kamiya T., Shirai A., Kawashima S., Sato S., Tamaoki T., *J. Dermatol. Sci.*, **17**, 54—60 (1998).
- 20) Imai R., Jindo T., Miura Y., Mochida K., Takamori K., Ogawa H., *Arch. Dermatol. Res.*, **284**, 466—471 (1993).
- 21) Tanigaki-Obana N., Ito M., *Arch. Dermatol. Res.*, **284**, 290—296 (1992).
- 22) Tanigaki N., Ando H., Ito M., Hashimoto A., Kitano Y., *Arch. Dermatol. Res.*, **282**, 402—407 (1990).
- 23) Takahashi T., Kamiya T., Hasegawa A., Yokoo Y., *J. Invest. Dermatol.*, **112**, 310—316 (1999).
- 24) Diehl J. A., Cheng M., Roussel M. F., Sherr C. J., *Genes Dev.*, **12**, 3499—3511 (1998).
- 25) Zhou B. P., Liao Y., Xia W., Spohn B., Lee M. H., Hung M. C., *Nat. Cell Biol.*, **3**, 245—252 (2001).
- 26) Porter A. C., Vaillancourt R. R., *Oncogene*, **17**, 1343—1352 (1998).
- 27) Radeff-Huang J., Seasholtz T. M., Matteo R. G., Brown J. H., *J. Cell. Biochem.*, **92**, 949—966 (2004).
- 28) Kumar C. C., *Oncogene*, **17**, 1365—1373 (1998).
- 29) Shirai A., Ikeda J., Kawashima S., Tamaoki T., Kamiya T., *J. Dermatol. Sci.*, **25**, 213—218 (2001).
- 30) Kamimura A., Takahashi T., Watanabe Y., *Phytomedicine*, **7**, 529—536 (2000).
- 31) Takahashi T., Kamimura A., Yokoo Y., Honda S., Watanabe Y., *Phytother. Res.*, **15**, 331—336 (2001).