

Phytosphingosine-1-phosphate and epidermal growth factor synergistically restore extracellular matrix in human dermal fibroblasts *in vitro* and *in vivo*

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Received December 11, 2015; Accepted December 23, 2016

DOI: 10.3892/ijmm.2017.2866

Abstract. Phytosphingosine-1-phosphate (PhS1P), which is found in plants and fungi, is generated by the phosphorylation of phytosphingosine and is structurally similar to molecules that promote cellular growth and proliferation. The aim of this study was to ascertain whether PhS1P displays synergistic effects together with epidermal growth factor (EGF), which is also critical for activating proliferation, migration and survival pathways. We utilized cultured human dermal fibroblasts (HDFs) and a number of assays, including western blotting, cell migration assays, quantitative (real-time) PCR, and viability assays. We found that PhS1P promoted the activity of EGF *in vitro*. We then conducted a clinical trial in females over 35 years of age, with visible signs of skin aging. By evaluating skin hydration, dermal density and thickness, length of fine wrinkles, and skin elasticity, we verified the clinical efficacy of a combined treatment of PhS1P and EGF *in vivo*. On the whole, our data suggest that PhS1P displays a synergistic anti-aging effect together with EGF, both *in vitro* and *in vivo*.

Introduction

The skin is composed of two layers: the epidermis and the dermis. Mesenchymal cells, known as dermal fibroblasts, reside within the dermis and generate the major components of the extracellular matrix (ECM), which organizes the dermal

layer (1-3). Dermal fibroblasts have been reported to play a key role in controlling skin physiology, and their proliferation is also crucial for skin structural homeostasis and wound healing (4-6).

The epidermal growth factor receptor (EGFR), a glycoprotein on the cell surface membrane, acts as a signaling initiator. EGFR is composed of an extracellular receptor domain, an intracellular domain, and a transmembrane region. The intracellular domain acts as a tyrosine kinase, and activated EGFR initiates signal transduction pathways that regulate cellular proliferation, differentiation and survival (7,8). EGFR is bound by epidermal growth factor (EGF), a small peptide, which stimulates EGFR to induce cell proliferation and migration (2,8,9).

As a cellular signal transduction molecule, phytosphingosine-1-phosphate (PhS1P) is not abundant in animals. However, previous studies suggest that it is highly expressed in plants and fungi (3). PhS1P is generated by sphingosine kinase through phosphorylation of phytosphingosine. PhS1P has been shown to have a high affinity for the sphingosine-1-phosphate (S1P) receptor and shows structural similarity to S1P (3).

The aim of the present study was to ascertain whether PhS1P and EGF display synergistic effects, using *in vitro* assays and an *in vivo* clinical trial. Our results demonstrated that treatment with PhS1P and EGF induced an anti-aging effect in cell culture, as well as in study subjects, further suggesting that PhS1P holds promise as a potential anti-aging cosmetic ingredient.

Materials and methods

Cell culture. Human dermal fibroblasts (HDFs; Lonza, Basel, Switzerland) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco/Life Technologies, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA) and 1% penicillin/streptomycin (Gibco/Life Technologies) at 37°C in an atmosphere of 5% CO₂. PhS1P (Avanti Polar Lipids, Alabaster, AL, USA) and EGF (R&D Systems, Minneapolis, MN, USA) were dissolved in dimethyl sulfoxide (DMSO).

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Key words: phytosphingosine-1-phosphate, fibroblasts, aging, collagen, epidermal growth factor, AKT

Cell viability assay. HDFs were seeded at a density of 3×10^3 cells/well in 96-well plates and incubated for 24 h. The cells were then either left untreated, pretreated with a range of PhS1P concentrations (0–5 μ M), or pretreated with both PhS1P (5 μ M) and EGF (10 ng/ml) for 3 h, prior to irradiation with UVB (50 mJ/cm²). UVB irradiated cells were incubated for 24 h, and cytotoxicity was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, MTT tetrazolium salt (0.5 mg/ml; Sigma-Aldrich) was added to the cells for 4 h. The medium was then replaced with DMSO, and the absorbance of each sample was measured at 595 nm using a plate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Western blotting. Western blotting was performed as described in our previous study (2). Cells were collected and washed with cold phosphate-buffered saline (PBS). The cell pellets were lysed using modified radioimmunoprecipitation assay (RIPA) buffer [50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS)] containing protease inhibitors (Complete Tablets, Mini, EDTA-free, EASYpack; Roche Applied Science, Mannheim, Germany) at 4°C for 20 min. The lysates were then centrifuged at 12,000 \times g for 30 min, and the supernatant was decanted and saved. The concentration of total protein was determined using the Bradford assay (Bio-Rad Laboratories), and 50 μ g of total cellular protein was solubilized in SDS sample buffer and resolved by SDS-PAGE. Anti-ERK (M5670), anti-p-ERK (M9692), anti-AKT (SAB4500797) and anti-p-AKT (SAB4504332) primary antibodies for immunoblotting were purchased from Sigma-Aldrich.

Isolation of total RNA and quantitative (real-time) polymerase chain reaction (qPCR). Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as previously described (10), and RNA purity and concentration were evaluated using MaestroNano[®] microspectrophotometer (Maestrogen, Las Vegas, NV, USA). Equal amounts of RNA were used for cDNA synthesis, and this was then used as a PCR template to examine the expression of EGFR, matrix metalloproteinase 1 (MMP1), and COL1A1. PCR primers were designed by Primer3 software (<http://frodo.wi.mit.edu>) (EGFR forward, 5'-CAGCGCTACCTTGTCATTCA-3' and reverse, 5'-TGCACTCAGAGACCTCAGGA-3'; MMP1 forward, 5'-GGTCTCTGAGGGTCAAGCAG-3' and reverse, 5'-AGTTCATGGCTGCAACACG-3'; β -actin forward, 5'-GGATTCCTATGTGGGCGACGA-3' and reverse, 5'-CGCTCGGTGAGGATCTTCATG-3'); and qPCR was performed using EvaGreen dye (Solis BioDyne, Tartu, Estonia) and Line-Gene K software (Bioer, Hangzhou, China). The Ct value for each gene was normalized to that of β -actin.

Subjects for clinical evaluation. The study protocol was approved by the Institutional Review Board of the Korea Institute for Skin and Clinical Sciences (KISCS) Incorporated. Forty women over the age of 35 were registered in a randomized, double-blind clinical trial (control group: 43.85 ± 5.07 years; experimental group: 46.40 ± 5.75 years). The subjects were selected based on age and the absence of skin conditions other than those determined to be age-related; none were pregnant or nursing. All subjects were informed about the objective of the study, provided signed informed consent, and agreed to use

only study-associated products for skin care during the duration of the study. Exclusion criteria included itching, erythema, or excessive drinking or smoking. Subjects were divided into a control and an experimental group consisting of 20 subjects each. All conditions were identical for both groups, other than the exposure of the experimental group to the test material. The study lasted for 6 weeks, with none of the study subjects dropping out. Clinical parameters were evaluated three times, namely, before use, and after 3 and 6 weeks of use. At each evaluation, the investigator asked subjects about the condition of their skin and performed a visual examination of their skin condition, to assess any erythema, itching, scaling, edema, tingling, and burning sensation. The cream given to the experimental group contained 30 ppm PhS1P and 2 ppm EGF, whereas the cream given to the control group was prepared using the same volume of water in place of PhS1P and EGF.

Experimental procedures. To investigate the improvement in skin barrier function, subjects were instructed to apply 2 g of the test material to the face every morning and night for 4 weeks. Subjects and investigators were blinded as to which group received the experimental and control treatments. Moisture and transepidermal water loss (TEWL) were measured on the right cheek, and skin texture was evaluated on the left side of the forehead. All subjects washed with the cleanser provided and lay quietly in a room with constant temperature ($22 \pm 1^\circ\text{C}$) and humidity ($45 \pm 5\%$) before assessments were performed, so that they all would be evaluated under the same conditions.

Evaluation of skin moisture. To evaluate improvement in skin moisture, the DermaLab USB moisture probe (Cortex Technology, Inc., Hadsund, Denmark) was applied, and data were analyzed using the associated application software, version 1.09. All subjects were measured on the same region of the right cheek, five consecutive times, and the mean, maximum, and minimum values were determined. Measurements were taken three times, namely, before application, and after 3 and 6 weeks of use. The device applies the conductance measurement principle to measure the water-binding capacity of the stratum corneum; this conductance value correlates with skin moisture and is expressed in microsiemens (μS).

Measurement of dermal density and thickness. To evaluate dermal density, a DUB skin scanner (tpm taberna pro medicum, Lueneburg, Germany) was used. Dermal density was measured 3 cm from the left eye, applying the couplant for ultrasonic examination. The analysis range was limited to the area between the dermis and upper panniculus. The measurements were taken three times, namely, before application, and after 3 and 6 weeks of use.

Measurement of length and evenness of crow's feet. Clinical images for the measurement of facial skin evenness were obtained by a PRIMOS Lite (field of view 45x30; GFMesstechnik GmbH, Teltow, Germany), and captured images were analyzed using the associated imaging software, PRIMOS Lite, version 5.6E. Three consecutive clinical images of the subject's crow's feet were captured. Facial skin roughness was assessed based on the Ra value, which is the average of all heights and depths relative to the reference plane. The Ra value is the most widely

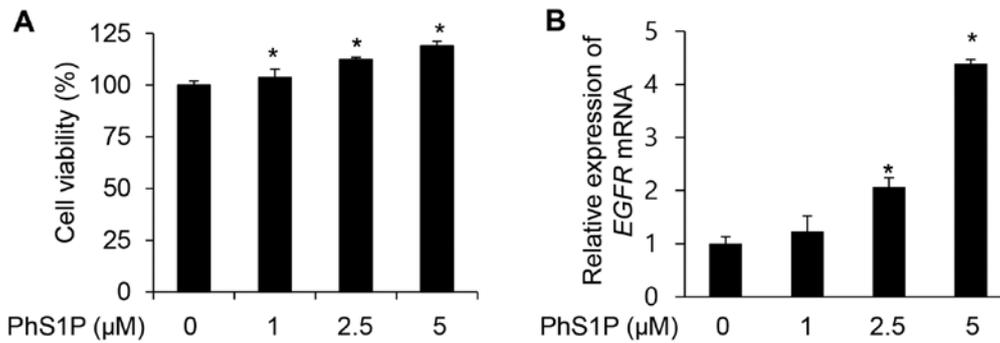


Figure 1. Cell viability and expression of epidermal growth factor receptor (EGFR) mRNA in human dermal fibroblasts (HDFs) treated with phytosphingosine-1-phosphate (PhS1P). (A) Cell viability of HDFs treated with increasing amounts of PhS1P. (B) The relative mRNA expression of EGFR in PhS1P-treated HDFs. *P<0.01 compared with non-treated HDFs, as determined by the Student's t-test.

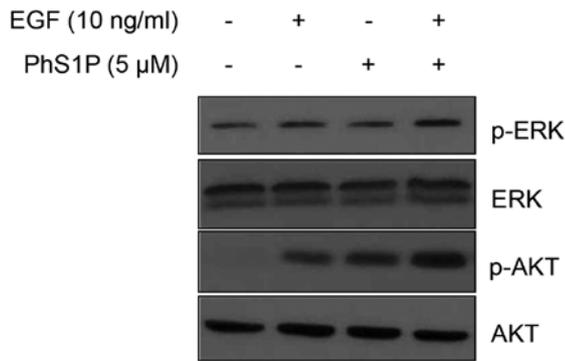


Figure 2. Effect of phytosphingosine-1-phosphate (PhS1P) on epidermal growth factor (EGF)-induced p-ERK, ERK, p-AKT and AKT protein expression in human dermal fibroblasts (HDFs).

used parameter of facial skin roughness and is the arithmetic mean of the maximum values of all measurements. To evaluate improvement in wrinkles, especially at the eye rim, a Robo skin analyzer CS50 (Inforward Inc., Tokyo, Japan) was used. The facial images were captured from each subject under identical positions, and with equal lighting, on the front, left, and right sides of the face. To evaluate improvement, measurements were taken three times, namely, before application, and after 3 and 6 weeks of use. We analyzed the captured images, matching the facial feature points accurately, and the unit of measurement for crow's feet length was mm.

Measurement of skin elasticity. To evaluate improvement in skin elasticity, a DermaLab USB elasticity probe (Cortex Technology, Inc.) was applied, and the results were analyzed using the associated application software, version 1.09. Measurements were obtained using a fixed elasticity probe on the left cheek of each subject. To analyze the elasticity measurements, Young's modulus (E) was calculated as a dose-dependent representation of skin elasticity. The measurements of elasticity were taken three times, namely, before application, and after 3 and 6 weeks of use. The unit of measurement was MPa.

Statistical and mathematical analysis. In the cellular efficacy tests, all results are presented as the mean percentage ± standard deviation (SD) of three independent experiments.

Differences with a P-value <0.05 or 0.001, as determined by Student's t-test, were considered statistically significant. In clinical efficacy tests, statistical analyses were conducted using SPSS software (SPSS, version 17.0 for Windows; IBM, Armonk, NY, USA). Paired t-tests were performed in cases of repeated measurements on the same subject. To analyze subject questionnaires, the mean values, standard deviation, and percentages were used. The formula used to measure the percent change for each skin parameter was 'Percent change = [(A - B)/B] x100', where A is defined as the individual value of any parameter at the 3- and 6-week visits, and B represents the zero hour of the assessed parameter.

Results and Discussion

PhS1P induces HDF proliferation and acts as a cognate ligand for EGFR. The PhS1P molecule is structurally similar to S1P, suggesting they perform similar functions. S1P is known as a signal transduction molecule that induces cellular proliferation (11). We therefore, aimed to determine whether PhS1P would function similarly to induce the proliferation of HDFs. We first assessed the cytotoxicity of this molecule by treating HDFs with various PhS1P concentrations, ranging from 0 to 5 μM, for 24 h. Cell viability was then measured using the MTT assay. Importantly, we observed that cell viability increased gradually with the increased dosage of PhS1P, up to a 23% increase at 5 μM PhS1P (Fig. 1A), suggesting that PhS1P induced HDF proliferation. Subsequently, in order to determine whether PhS1P affects EGFR expression, EGFR mRNA levels were measured in cells treated with PhS1P using qPCR. We observed that the relative expression of EGFR mRNA was increased 2-fold when cells were treated with 2.5 μM PhS1P and 4.5-fold when they were treated with 5 μM PhS1P (Fig. 1B). These results revealed that PhS1P induced EGFR mRNA expression in HDFs, and demonstrated that this molecule has properties that suggest it may act as a ligand for EGFR.

Co-treatment with PhS1P and EGF induces phosphorylation of ERK and AKT in HDFs. The binding of EGF to EGFR activates downstream signaling proteins, such as ERK and AKT, by phosphorylation (12). Furthermore, S1P, a molecule that has structural and functional similarity to PhS1P, also induces cellular proliferation and growth through the ERK

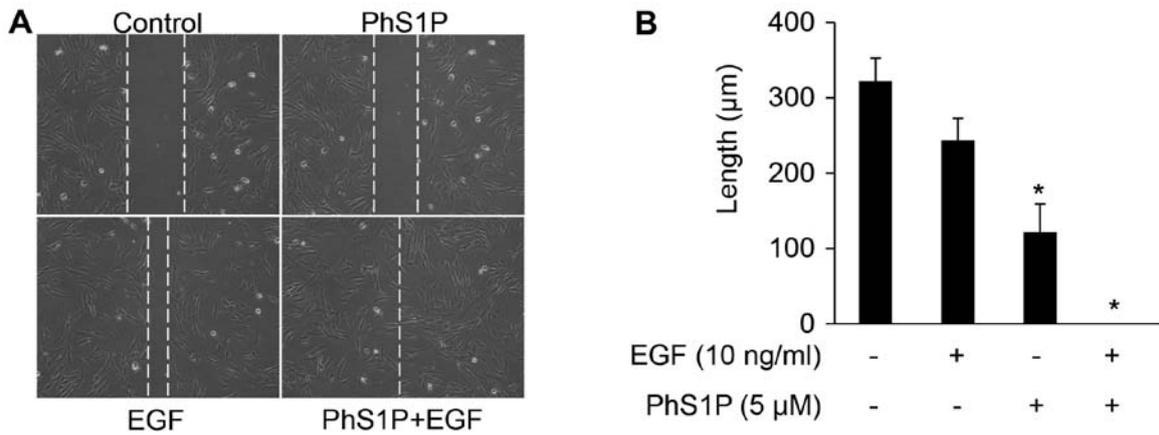


Figure 3. Cell migration of human dermal fibroblasts (HDFs) in a scratch wound healing assay after treatment with phytosphingosine-1-phosphate (PhS1P) and epidermal growth factor (EGF). (A) The microscopic images illustrate the migration of HDFs after treatment with PhS1P and/or EGF. (B) Average wound distance of scratched HDFs from quantitative analysis of the microscopic images. * $P < 0.01$ compared with non-treated HDFs, as determined by the Student's *t*-test.

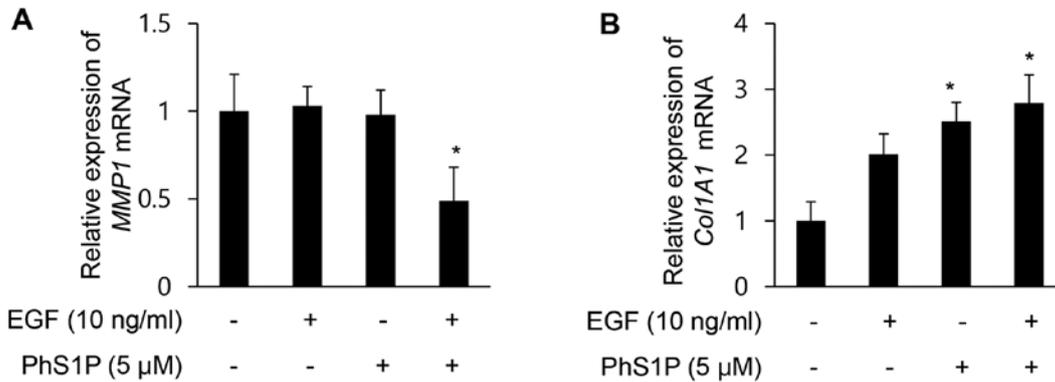


Figure 4. Effects of phytosphingosine-1-phosphate (PhS1P) and epidermal growth factor (EGF) on extracellular matrix (ECM)-regulated mRNA expression in human dermal fibroblasts (HDFs). (A) The relative levels of MMP1 mRNA in HDFs after treatment with EGF and PhS1P. (B) The relative levels of COL1A1 mRNA in HDFs after treatment with EGF and PhS1P. * $P < 0.01$ compared with non-treated HDFs, as determined by the Student's *t*-test.

and AKT pathways (13), suggesting that PhS1P may have a similar function. To test this, HDFs were treated with PhS1P (5 μM) and EGF (10 ng/ml), alone and in combination, and the protein levels of ERK, phospho-ERK (p-ERK), AKT, and phospho-AKT (p-AKT) were measured using western blotting. Treatment with 10 ng/ml EGF or 5 μM PhS1P induced phosphorylation of ERK and AKT (Fig. 2). Importantly, in cells co-treated with PhS1P and EGF, the p-AKT and p-ERK levels were significantly higher than these levels in the cells treated with either molecule alone. These results suggest that PhS1P exerts a synergistic effect on EGF-induced expression of p-ERK and p-AKT, and may function to induce cellular proliferation through these pathways.

Treatment with PhS1P and EGF promotes wound healing in a scratch plate assay. Both the proliferation and migration of HDFs are essential for cutaneous wound healing (14), and treatment with a combination of EGF and EGFR activates the AKT and ERK signaling pathways, inducing cellular migration (12,15). Here, we observed that co-treatment with EGF and PhS1P also significantly increased AKT and ERK phosphorylation in HDFs (Fig. 2). In order to determine whether the enhanced phosphorylation of AKT and ERK observed in cells treated with EGF and PhS1P affects HDF migra-

tion, we utilized a scratch wound healing assay. As shown in Fig. 3A and B, the wound distance was decreased in the HDFs treated with either PhS1P or EGF. Additionally, when cells were co-treated with PhS1P and EGF, the wound distance decreased further, as compared to the HDFs treated with either molecule alone (Fig. 3A and B). These results demonstrated that PhS1P induced cellular migration in HDFs, and did so more efficiently in combination with EGF.

PhS1P promotes EGF-induced ECM-related gene expression. Fibroblasts can alter the ECM by degrading fibrin and promoting the production of collagen (16,17) and components of the ECM, particularly collagen I and III, are essential for wound healing (17,18). Collagen is the major structural component of dermal tissue, and therefore, structural changes in this molecule result in typical signs of aging, such as wrinkling (19,20). One member of the MMP family, MMP1 (collagenase-1), is generated in fibroblasts and is also capable of degrading type I and III fibrillar collagen (21). Members of the MMP family have also been shown to be capable of degrading all components of the ECM, including collagen, elastin, laminin, proteoglycans, and fibronectin (21,22). To investigate the effects of PhS1P on the expression of MMP1 and COL1A1, qPCR was performed on HDFs treated with

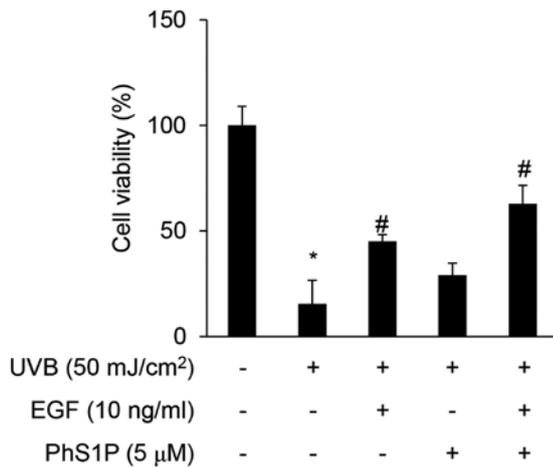


Figure 5. Phytosphingosine-1-phosphate (PhS1P) and epidermal growth factor (EGF) protect against UVB-induced cell death in irradiated human dermal fibroblasts (HDFs). *P<0.01 compared with non-treated HDFs and #P<0.01 compared with UVB-irradiated HDFs, as determined by the Student's t-test.

EGF (10 ng/ml) and/or PhS1P (5 µM). We observed that the relative expression of MMP1 decreased 0.49-fold in the HDFs treated with both PhS1P and EGF (Fig. 4A). Conversely, treatment with both molecules increased COL1A1 mRNA levels 2.79-fold (Fig. 4B). These results demonstrated that PhS1P promoted EGF-induced COL1A1 mRNA expression, and together, these molecules suppressed MMP1 expression, two genes that regulate the ECM and influence skin elasticity.

PhS1P protects against UVB-induced decreased cell viability and stimulates EGF-induced cell viability. Ultraviolet (UV) radiation is a well-characterized mutagen that induces both skin aging and cancer. Here, we showed that PhS1P stimulated cell migration and promoted the expression of cellular proliferation markers. We, therefore, aimed to determine whether PhS1P can protect HDFs from UVB-induced damage. HDFs were irradiated with UVB at 50 mJ/cm², and under these conditions, only 15.37% of the cells remained viable. However, the cells treated with either EGF or PhS1P prior to UVB irradiation retained a viability of 45.12 and 28.73%, respectively. Notably, HDFs co-treated with both PhS1P and EGF before UVB irradiation maintained a cell viability of 62.78% (Fig. 5). These data suggest that PhS1P protects against UVB-induced decreased cell viability, and further indicate that its protective ability is increased in the presence of EGF.

PhS1P improves skin moisture content. Collectively, our *in vitro* data suggest that PhS1P, in combination with EGF, can promote pathways that are protective against aging-associated skin damage. We therefore, designed a double-blind clinical trial to test the efficacy of a cream containing PhS1P and EGF in women >35 years of age. Skin dryness is one of the hallmarks of skin aging (23), and therefore, we analyzed skin moisture content in the study subjects using the DermaLab USB moisture probe. We observed that in the control group, which used a cream that did not contain either PhS1P or EGF, the skin conductance (which directly reflects moisture content) was 523.93 µS at the beginning of the study, and 527.45 µS and 538.09 µS after 3 and 6 weeks of applica-

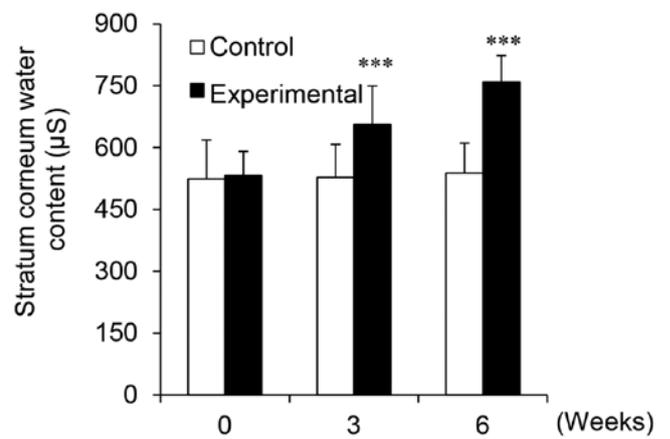


Figure 6. Effects of a phytosphingosine-1-phosphate (PhS1P) and epidermal growth factor (EGF)-containing cream on skin moisture content in study subjects. Mean stratum corneum water content measurements in experimental and control subjects at 0, 3 and 6 weeks. ***P<0.001 compared with control group, as determined by the Student's t-test.

tion, respectively (Fig. 6). We then calculated the degree of improvement (Fig. 6) and found that the skin conductance of the control group increased by 0.67 and 2.70% after 3 and 6 weeks, respectively. These changes were not statistically significant (P>0.05), indicating that the control cream had no measurable effect on moisture content. Conversely, the skin conductance in the experimental group, which used the PhS1P and EGF-containing cream, was 532.37 µS before use, 655.95 µS after 3 weeks, and 758.88 µS after 6 weeks (Fig. 6). Notably, the use of the PhS1P and EGF-containing cream significantly improved skin conductance by 23.21 and 42.55% after 3 and 6 weeks, respectively (P<0.001). These experiments demonstrated that the use of the PhS1P and EGF-containing cream improved skin moisture content in the study subjects.

PhS1P improves EGF-induced dermal thickness and density. During the aging process, dermal thickness and density are reduced over time. Concurrently, MMP proteins show increased expression and degrade skin substrate proteins (24). Clinically, it has been shown that dermal thickness and density decrease with increasing concentrations of MMPs, as these proteins degrade albuminoids and the collagen layer (24). Here, we showed that PhS1P and EGF downregulated MMP1 and promoted expression of the collagen-encoding gene, COL1A1. We, therefore, evaluated the synergistic efficacy of PhS1P and EGF on dermal thickness and density in aging skin *in vivo*. In the control subjects, dermal thickness measurements were 94.00 µm before use, and 96.85 and 94.75 µm after 3 and 6 weeks of use, respectively. Thickness measurements in the subjects using the PhS1P and EGF-containing cream were 115.00 µm before use, and 125.45 and 147.15 µm after 3 and 6 weeks of application, respectively; these data were statistically significant (P<0.001). We then calculated the improvement in dermal thickness as a percentage, based on the values before and after application. Notably, the dermal thickness improvement was 3.03% after 3 weeks and 0.80% after 6 weeks in the control group, whereas the experimental group showed improvements in dermal thickness of 9.09 and 27.96% after 3 and 6 weeks of use, respectively (Fig. 7A).

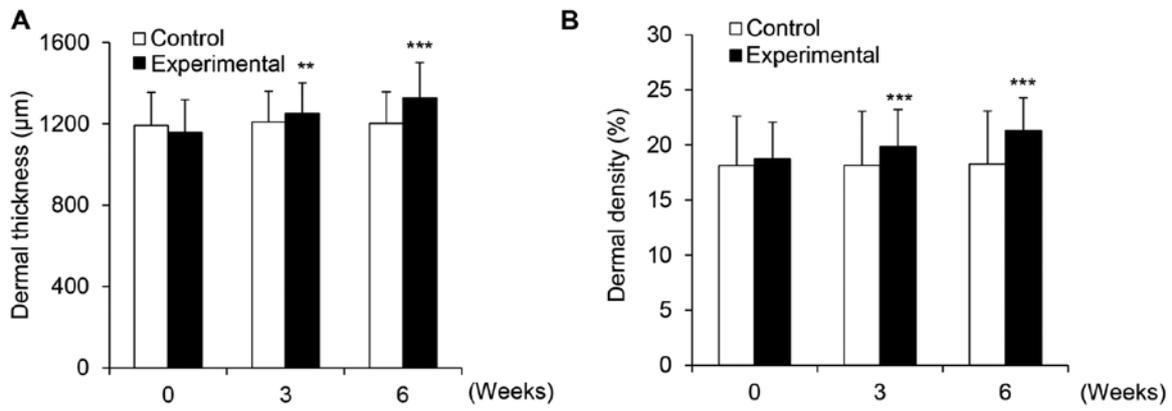


Figure 7. Effects of the PhS1P and epidermal growth factor (EGF)-containing cream on dermal thickness and density. (A) Mean dermal thickness measurements at 0, 3 and 6 weeks. (B) Mean dermal density measurements at 0, 3 and 6 weeks. **P<0.01 and ***P<0.001 compared with control group, as determined by the Student's t-test.

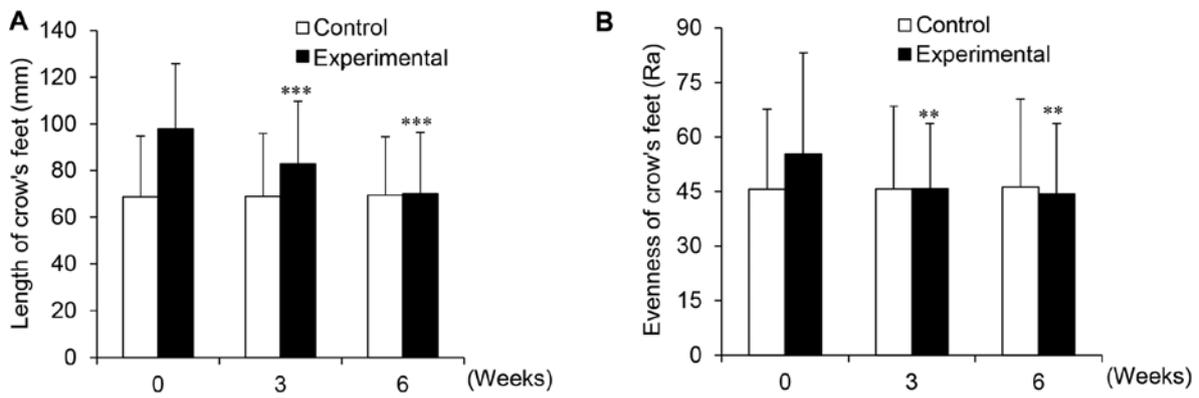


Figure 8. Effects of the PhS1P and epidermal growth factor (EGF)-containing cream on crow's feet. (A) The mean length of crow's feet at 0, 3 and 6 weeks. (B) The mean crow's feet evenness values at 0, 3 and 6 weeks. **P<0.01 and ***P<0.001 compared with control group, as determined by the Student's t-test.

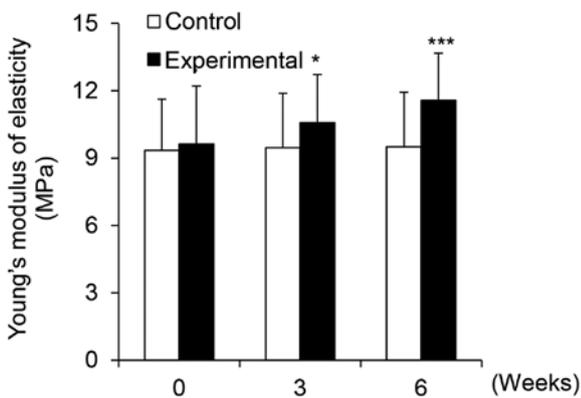


Figure 9. Effects of the PhS1P and epidermal growth factor (EGF)-containing cream on skin elasticity. *P<0.05 and ***P<0.001 compared with control group, as determined by the Student's t-test.

Dermal density measurements are represented as percentages, which are proportional to the skin density. In the subjects using the control cream, density measurements were 18.13% before application, and 18.16 and 18.28% after 3 and 6 weeks of use, respectively. Conversely, the subjects using the experimental cream displayed an average density of 18.76% before application, and densities of 19.85 and 21.30% after 3 and 6 weeks of use, respectively (Fig. 7B). The results for the

experimental group were statistically significant (P<0.001). We then calculated the improvement in dermal density and found that application of the experimental cream resulted in improvements of 5.82 and 13.57% after 3 and 6 weeks, respectively, whereas those using the control cream improved by only 0.20 and 0.84%, respectively. These results demonstrated that PhS1P, in combination with EGF, improved both dermal thickness and density.

Cream containing PhS1P and EGF improves the length and evenness of crow's feet. Wrinkles occur in response to structural changes in cells and tissues, and arise due to intrinsic aging factors, including decreases in collagen and elasticity, denaturalization of elastic fibers and the stratum corneum, and loss of skin moisture (25). Wrinkles can also form as a result of extrinsic aging factors, such as reactive oxygen species (ROS), which damage lipids and proteins in the skin via inflammatory cytokine production (26,27). We, therefore, determined whether the PhS1P and EGF-containing cream can relieve wrinkles through clinical efficacy experiments. As shown in Fig. 8A, in the control group, the mean length of crow's feet was 68.90 mm before application, and 68.90 and 69.45 mm after 3 and 6 weeks, respectively. Conversely, for the experimental group, the mean length was 97.75 mm before application, and 82.80 and 70.20 mm after 3 and 6 weeks application, respectively; these values were statistically significant (P<0.001). When improve-

ment over time was calculated, for the experimental group, the length of crow's feet improved markedly by 15.29 and 28.18% after 3 and 6 weeks, respectively. In the control group, improvement was only 0.29% after 3 weeks and 1.09% after 6 weeks. Based on these results, we concluded that the cream containing both PhSIP and EGF decreased wrinkle length *in vivo*.

When we evaluated the evenness of crow's feet, we found that in the control group, this was 45.57 Ra before application, and 45.66 and 46.27 Ra after 3 and 6 weeks, respectively. However, in the experimental group, the evenness was 55.37 Ra before application, and 45.81 and 44.39 Ra after 3 and 6 weeks of application, respectively (Fig. 8B); these values were statistically significant ($P < 0.001$). To compare improvement in crow's feet evenness between the control and experimental group, we calculated the percentage of improvement using the values before application, and after 3 and 6 weeks of use. The control group showed an improvement of 0.20% after 3 weeks and 1.54% after 6 weeks application, whereas for the experimental group, improvement was 17.27% after 3 weeks and 19.83% after 6 weeks of use. Based on these data, we concluded that the cream containing PhSIP and EGF improved facial wrinkles, particularly in regards to the length and evenness of the crow's feet.

PhSIP and EGF-containing cream improves skin elasticity.

The dermis is composed of dermal fibroblasts that exist within the extracellular matrix consisting of fibrous proteins, and it is largely involved in the regulation of skin elasticity. Various factors cause wrinkle formation and decrease skin elasticity through the denaturation of dermal structural components, including collagen and elastin (28). Therefore, in this experiment, we investigated the effects of the PhSIP and EGF-containing cream on skin elasticity. Using a DermaLab USB, we measured elasticity in our study subjects and found that before application, the mean elasticity was 9.35, whereas it was 9.46 and 9.51 after 3 and 6 weeks, respectively. For the experimental group, mean elasticity was 9.63 before application, and 10.58 and 11.57 after 3 and 6 weeks of application, respectively (Fig. 9); these values were statistically significant ($P < 0.001$). When assessing improvement in skin elasticity over time, the control group improved 1.18 and 1.71% after 3 and 6 weeks application, respectively. Conversely, the experimental group showed an improvement of 9.81 and 20.15% after 3 and 6 weeks, respectively. From these data, we concluded that cream containing PhSIP and EGF significantly improved skin elasticity.

Analysis of adverse effects of the PhSIP and EGF-containing cream. In the present study, investigators asked the subjects individually regarding the condition of their skin and performed a visual evaluation to assess any possible skin reactions, such as erythema, itching, scaling, tingling, tightness, prickling and burning sensation at every visit. No unusual reactions were reported, based on either the visual evaluation or the questionnaire.

In the present study, we showed that PhSIP can act as a stimulator of EGFR, inducing signal transduction to promote cell proliferation and migration. Our *in vitro* experiments demonstrated that PhSIP upregulated EGFR mRNA expression and displayed a synergistic effects with EGF at the cellular level. Co-treatment of HDFs with PhSIP and EGF

enhanced cellular migration, protected against UVB-induced decreased cell viability, and influenced both the phosphorylation and expression of molecules critical for regulating cellular proliferation and the ECM. Furthermore, our *in vivo* experiments demonstrated that application of the cream containing PhSIP and EGF improved skin hydration, dermal density and thickness, evenness of crow's feet, and skin elasticity. EGF is a well-known and highly valued cosmetic ingredient that has shown efficacy as an anti-aging agent (29). This study suggests that PhSIP may represent a new efficacious anti-aging cosmetic ingredient, which displays synergistic effects with EGF in aged skin efficacy trials.

Acknowledgements

This study was supported by the KU Research Professor (H.-J.C.) Program of Konkuk University. Funding was also provided by a grant from the Ministry of Science, ICT and Future Planning (no. 20110028646) of the Republic of Korea and a grant from the Korean Health Technology R&D Project, Ministry of Health and Welfare, Republic of Korea (grant no. HN13C0075).

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