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Anti-melanogenic activity of phytosphingosine *via* the modulation of the microphthalmia-associated transcription factor signaling pathway

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ABSTRACT

Background: Microphthalmia-associated transcription factor (MITF) suppresses the expression of enzymes controlling the production of melanin. Phytosphingosine is a well-known cosmetic agent, but its anti-melanogenic activity and mechanism of action remain unclear.
Objective: This study was designed to investigate the effects of phytosphingosine on melanin synthesis and elucidate the plausible mechanism of actions *in vitro* and *ex vivo* systems.
Methods: Melanin content, cell viability, tyrosinase activity, p-CREB DNA binding activity, and the protein gene expression levels of the enzymes and proteins involved in melanogenesis were measured with the treatment of phytosphingosine.
Results: Phytosphingosine inhibits melanin synthesis in cultured melan-a cells and a reconstructed human skin model. One possible mechanism of the anti-melanogenic activity of phytosphingosine appears to be associated with the modulation of MITF, which suppresses the expression of tyrosinase, tyrosinase-related protein-1 (TRP-1), and TRP-2. Further analysis revealed that phytosphingosine suppressed paired box 3 and SRY-related HMG-box 10, critical transcription factors of MITF. Phytosphingosine also effectively downregulated the protein levels of β-catenin and the phospho-cAMP response element binding protein, an upstream regulatory factor of MITF. These results are closely related to the suppression of MITF gene expression. In addition, treatment with phytosphingosine for over 12 h, which is a relatively long period of time, did not directly suppress these MITF transcriptional factors. Instead, phytosphingosine induced ERK activation, which led to MITF phosphorylation, followed by its degradation. Therefore, the downregulation of MITF protein levels by phytosphingosine with a long time exposure is in part associated with MITF protein degradation through the MAPK kinase activation pathway.
Conclusion: The modulation of MITF by phytosphingosine is closely related with the signaling pathways, such as the suppression of the MITF gene expression and the degradation of the MITF protein, depending on the duration of treatment time. These results suggest that phytosphingosine might serve as an effective melanogenesis inhibitor in melanocytes *via* the regulation of the MITF signaling pathways.

1. Introduction

Many environmental factors such as UV light can affect skin pigmentation. Melanin is the primary cause of skin pigmentation. Melanin in the skin is produced by melanocytes found in the basal layer of the epidermis. The progression of melanin formation is called melanogenesis. Melanin is synthesized in a melanosome, and at least three enzymes are required. Those enzymes are tyrosinase (TYR), tyrosinase-related protein-1 (TRP-1), and TRP-2 [1]. TYR is the key enzyme associated with the colors of skin, eyes, and hair in animals [2]. It catalyzes the oxidation of L-tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA) and leads to DOPA quinone formation, which is the first step in melanin synthesis [3]. TRP-2 allows a quicker conversion of dopachrome to 5,6-dihydroxyindol-2-carboxylic acid (DHICA), while TRP-1 facilitates the formation of carboxyl group-containing DHICA oxidase eumelanins [4,5]. TYR is required to synthesize pheomelanin and eumelanin, which are found in mammals, but TRP-1 and TRP-2 are especially crucial for synthesizing eumelanin [6].

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Melanogenesis is known for its complex process that is balanced by various signal transduction pathways. One of the pathway involves microphthalmia-associated transcription factor (MITF), which is a key transcription regulator for melanogenesis and the expression of TYR, TRP-1, and TRP-2 [7,8]. The expression of MITF is regulated by various upstream transcription regulators, including cAMP response element-binding protein (CREB), paired box 3 (PAX3), SRY-related HMG-box 10 (SOX10), and lymphoid-enhancing factor-1 (LEF-1) [9]. CREB is an important MITF promoter [10,11], and the phosphorylation of CREB at serine 133 in melanocytes increases MITF expression by binding to the CRE in melanocytes [12].

The stimulation of c-Kit receptor tyrosine kinase is also known to have a critical role in the migration and development of melanocytes. The c-Kit signaling is known to activate the mitogen-activated protein kinase (MAPK), glycogen synthase kinase 3β (GSK3β) and phosphatidylinositol 3-kinase (PI3K) pathways [13–17]. In addition, previous studies showed that MITF transcription is able to be effectively modulated by MAPKs, such as extracellular signal-regulated kinase (ERK) and AKT [18]. ERK phosphorylates MITF at serine 73, which also causes MITF ubiquitination and degradation [19]. Since melanogenesis can be affected by several signaling pathways, it is also important to find an efficient whitening agent and to understand its mechanism of action in melanogenesis.

Sphingolipids are universal constituents of the plasma membrane in plants, animals, and fungi. Although phytosphingosine is a well-known sphingolipid molecule and is commonly used in cosmetic ingredients, the mechanism of action of its biological activity is poorly elucidated. Sphingolipid molecules, such as ceramide, sphingosine-1-phosphate, FTY720, and *N*,*N*,*N*-trimethylphytosphingosine iodine, are also previously reported as anti-melanogenic ingredients [20–23]. However, the effects of phytosphingosine, which is a sphingolipid metabolite, on melanogenesis are unclear. Because some sphingolipid molecules have already been clinically studied and commercialized into cosmetic products, phytosphingosine has a strong potential to be used as a skin whitening product. In this study, the anti-melanogenic activity of phytosphingosine and its underlying molecular mechanism were investigated in cultured melan-a cells and human skin mimic a reconstructed pigmented skin models.

2. Materials and methods

2.1. Materials

Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS), antibiotics-antimycotics solution, and TRI reagent were purchased from Invitrogen (Grand Island, NY, USA). Bovine serum albumin (BSA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and all other agents, unless otherwise indicated, were purchased from Sigma-Aldrich (St. Louis, CA, USA). Mouse p-GSK3β (Tyr216), mouse anti-β-catenin, and mouse anti-GS K3β were purchased from BD Biosciences (San Diego, CA, USA). Goat polyclonal anti-MITF, anti-tyrosinase, anti-TRP-1, anti-TRP-2, mouse anti-p-ERK, mouse anti-Pax3, mouse anti-Sox10, β-actin, goat anti-rabbit IgG-HRP, goat anti-mouse IgG-HRP, rabbit anti-goat IgG-HRP, rabbit anti-mouse IgG-HRP, mouse anti-goat IgG-HRP, and mouse anti-rabbit IgG-HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-p-GSK3β (ser21/9), Rabbit anti-c-Kit, Rabbit anti-p-AKT (ser473), Rabbit anti-p-PI3K and Rabbit anti-p-CREB were purchased from Cell Signaling Technology (Beverly, MA, USA). Rabbit anti-p-MITF (ser73) was purchased from the Assay Biotechnology Company (Sunnyvale, CA, USA). Complete protease inhibitor cocktail was purchased from Roche Applied Science (Penzberg, Germany). Gene-specific primers for real-time PCR were synthesized from Bioneer (Daejon, Korea). The Trans AM ELISA Kit was purchased from Active Motif Japan (Tokyo, Japan). The Fugene and Dual Luciferase® Reporter Assay Systems were purchased from Promega (Madison, MA, USA) and New England BioLabs (Ipswich, MA, USA), respectively.

Phytosphingosine (chemical structure in Fig. 1) was supplied by the Sphingolipid Bank at Seoul National University (#SLB000141), and was dissolved in 100% dimethyl sulfoxide (DMSO).

2.2. Cell culture

Melan-a cells (originally established by Dr. Bennett at the University of London) were kindly provided by the Skin Research Institute, Amore-Pacific Co., Korea. Melan-a cells were grown in RPMI 1640 medium supplemented with antimyosin (penicillin 100 unit/ml, streptomycin 100 unit/ml, and amphotericin B 250 ng/ml), 10% FBS, and 200 nM TPA. The cells were incubated at 37 °C in a humidified atmosphere of 10% CO_2.

2.3. Melanin content assay

Confluent cultures of melan-a cells were rinsed with Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (PBS) and lysed with 0.25% trypsin/EDTA. The cells were plated into 6-well plastic culture plates at a density of 1×10^5 cells/well. 48 h after plating, the media was replaced with or without (control) the test sample. After an additional 72 h incubation, the adherent cells exposed to the test samples were assayed. The melanin content was determined as follows: after removing the media and washing the cells with Ca^{2+} and Mg^{2+}-free PBS, the cell pellet was dissolved in 0.1 ml of 1N NaOH in 10% DMSO, and incubated at 60 °C for 10 min. The optical density at 475 nm was measured by an ELISA reader.

2.4. Cell proliferation assay

Cell proliferation assay was performed according to the procedure of Choi et al. [24]. MTT solution (final concentration of 500 μg/ml) was added to each well and further incubated for 4 h at 37 °C. The medium was discarded, and dimethyl sulfoxide (DMSO) was added to each well to dissolve generated formazan. The absorbance was measured at 570 nm, and the survival percentage was determined by comparison with a control group.

2.5. Western blotting

Melan-a cells were incubated with a 10 μM concentration of phytosphingosine for the indicated times. After washing with DPBS, the cells were lysed in extraction buffer (250 mM Tris–HCl at a pH of 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2% β-mercaptoethanol, 2 mM sodium ortho-vanadate, and protease inhibitor cocktail) at 4 °C. The same amount of protein in each lysate was loaded and separated by SDS-polyacrylamide gel electrophoresis and then transferred to a PVDF membrane. The

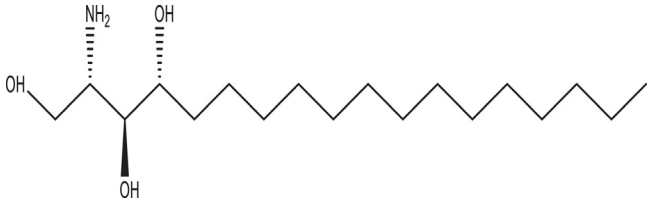

Fig. 1. The chemical structure of phytosphingosine.

membranes were blocked with 5% bovine serum albumin in Tris-buffered saline (TBS) containing 0.01% Tween-20 for 2 h at room temperature prior to an overnight incubation with the primary the antibody at 4 °C. After incubation, the membranes were rinsed three times with TBS and were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. After washing, the membranes were subjected to WestZol (iNtRON Biotechnology, Gyeonggi-do, Korea) and were visualized using the LAS-4000 imaging system.

2.6. RNA extraction and real-time PCR

Total RNA was isolated using the TRIzol reagent and were reverse transcribed at 42 °C for 60 min with 0.5 μg of oligo(dT)15 primer in a reaction volume, using the reverse transcription system. The PCR primers were as follows: 5′-CTAAGTGGTCTGCGGTGTCTC-3′ (forward) and 5′-GGTTTTCCAGGTGGGTCTG-3′ (reverse) for MITF; 5′-CACCCT-GAAAATCCTAACTTACTCA-3′ (forward) and 5′-CTCTTCTGATCTGC-TACAAATGATCT-3′ (reverse) for Tyrosinase; 5′-TGGGAACACTTTGTAACAGCA-3′ (forward) and 5′-ACTGCTGGTCTCCCTACATTTC-3′ (reverse) for TRP-1; 5′-GGCTA-CAATTACGCCGTTG-3′ (forward) and 5′-CACTGAGAGAGTTGTGGAC-CAA-3′ (reverse) for TRP-2; and 5′-AAGGCCAACCGTGAAAAGAT-3′ (forward) and 5′-GTGGTACGACCAGAGGCATAC-3′ (reverse) for β-actin.

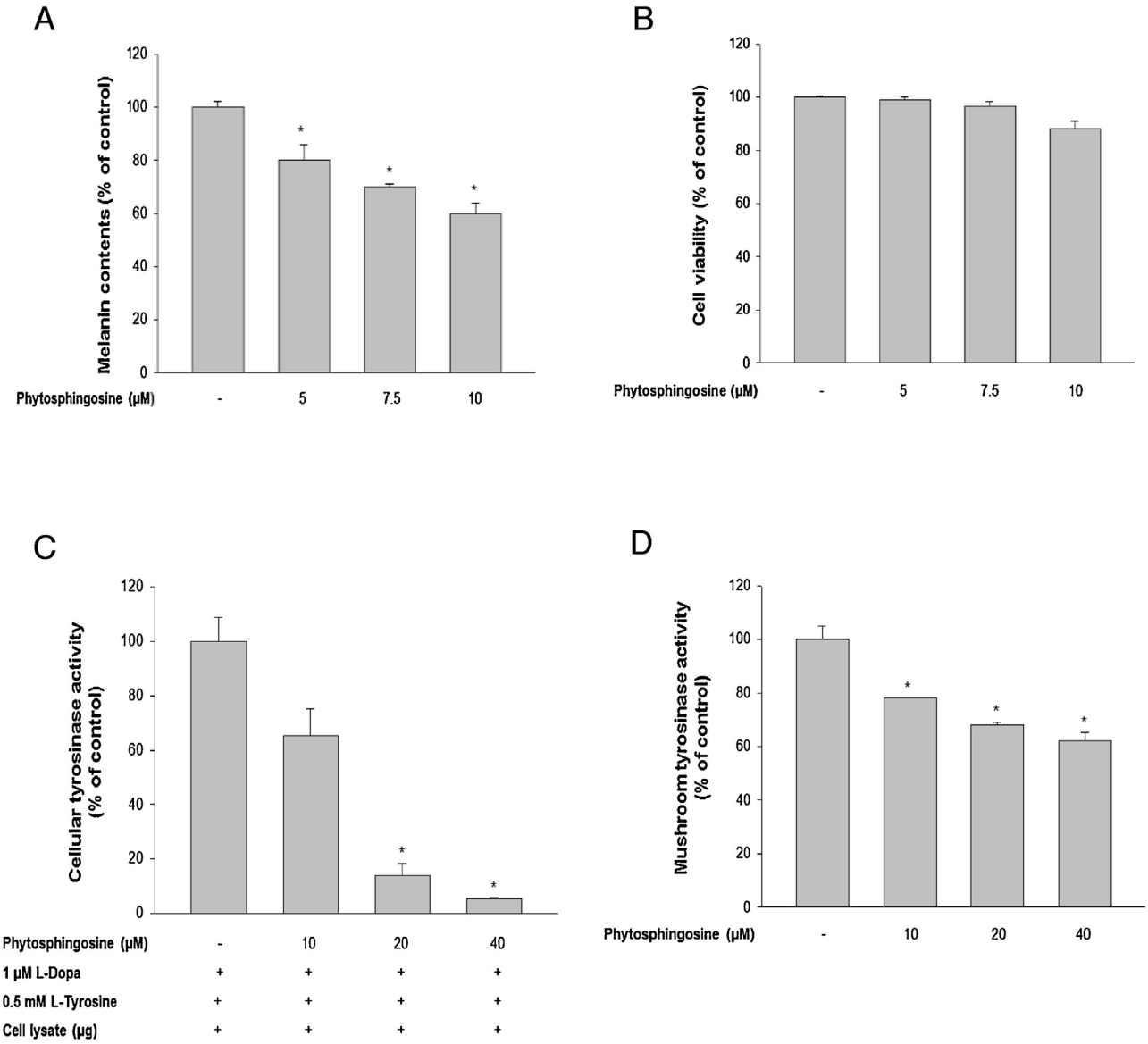

Fig. 2. Effect of phytosphingosine on melanin biosynthesis and tyrosinase activities. (A) Cells were treated with the indicated concentrations of phytosphingosine for 72 h. The melanin contents were then measured as described in Section 2. The amount of intracellular melanin contents in cultured cells were compared to the vehicle-treated control groups (% of control). (B) Cell viability was determined by a crystal violet assay with the indicated concentrations of phytosphingosine for 72 h. (C) Effect of phytosphingosine on tyrosinase activity in melan-a cells. The tyrosinase activity was measured with the indicated concentrations of phytosphingosine as described in Section 2. (D) Effect of phytosphingosine on cell-free mushroom tyrosinase activity. Data are shown as the mean ± SD. $^{*}p < 0.05$ is considered statistically significant compared to the control group.

2.7. Nuclear extraction

Nuclear extracts were prepared using the method described by Beg et al. [25]. Briefly, the cells were washed inphosphate-buffered saline, pelleted, and resuspended in lysis buffer (10 mM Tris–HCl, pH 8.0, 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 100 μM PMSF, and 1.0% NP-40). After 5 min on ice, the lysates were spun at 2500 rpm in a microcentrifuge at 4 °C for 4 min. The pelleted nuclei were briefly washed in lysis buffer without NP-40. The nuclear pellet was then resuspended in an equal-volume of nuclear extract buffer (20 mM Tris–HCl, pH 8.0, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 25% glycerol). After a 10 min incubation at 4 °C, the nuclei were briefly vortexed and spun at 14,000 rpm for 5 min. The supernatant was then removed and used as a nuclear extract. Protein concentrations were determined using the Bradford assay [26].

2.8. p-CREB-DNA binding assay

p-CREB-DNA binding activity was measured using the Trans AM ELISA kit (Active Motif Japan) according to the manufacturer's instructions. Nuclear extracts (2 μg) were incubated with a plate-coated with a double-stranded oligonucleotide containing the consensus CRE site (TGACGTCA). The plates were washed, and anti-Ser133-pCREB rabbit antibody was added to the well plates. Antibody binding was detected with the incubation of an HRP-conjugated secondary antibody and was developed with tetramethylbenzidine (TMB) substrate. The reaction intensity was measured by the absorbance at 450 nm.

2.9. Cellular tyrosinase activity assay

Melan-a cells were cultured in 100 mm dishes and treated with or without for 72 h. The cells were washed with ice-cold PBS and lysed with 1% (v/v) Triton-X/phosphate-buffered saline (pH 6.8). The cells were disrupted by freezing and thawing, and the lysates were clarified and centrifuged at 12000 rpm for 5 min at 4 °C. After quantifying the protein levels and adjusting concentrations with double distilled water (DDW), 5 μl of each lysate, containing the same amount of protein, were placed in a 96-well plate with 20 μl of lysis buffer, 20 μl of 100 mM L-DOPA and 20 μl of 0.5 mM L-tyrosine. Following incubation overnight at 37 °C, the absorbance was measured at 490 nm using an ELISA reader.

2.10. Mushroom tyrosinase activity assay

L-DOPA was used as a substrate and tyrosinase (EC 1.14.18.1) activity was monitored by dopachrome formation at 475 nm for the appropriate time (usually not longer than 10 min). The assay was performed according to the procedure of Masatomo et al. [27] with minor modifications. Briefly, the preincubation mixture consisted of 1.8 ml of 0.1 M phosphate buffer (pH 6.8), 0.6 ml of water, 0.1 ml of the sample solution, and 0.1 ml of the aqueous solution with mushroom tyrosinase (130 units). After preincubation for 5 min at room temperature, L-DOPA solution (0.4 ml of 6.3 mM) was added, the reaction was monitored at 475 nm for 5 min.

2.11. Transient transfection and dual luciferase assay

Melan-a cells were plated in 24 wells and incubated at 37 °C. The pMITF-Gluc reporter system harboring the promoter region (494 bp) of MITF and the pTyrosinase-Gluc reporter system were kindly provided by the Amore-Pacific R&D Center (Seoul, Korea). pGL3-FL was obtained from S. Oh (Inje University, Busan, Korea). At 40–50% confluency, the cells were washed with PBS and the Gaussian luciferase reporter construct pMITF-Gluc and the control Firefly luciferase vector (pGL3-FL) were transfected for 24 h using Fugene (Promega). After a 24 h incubation, the cell lysates were prepared, and the determination of Gaussia and firefly luciferase

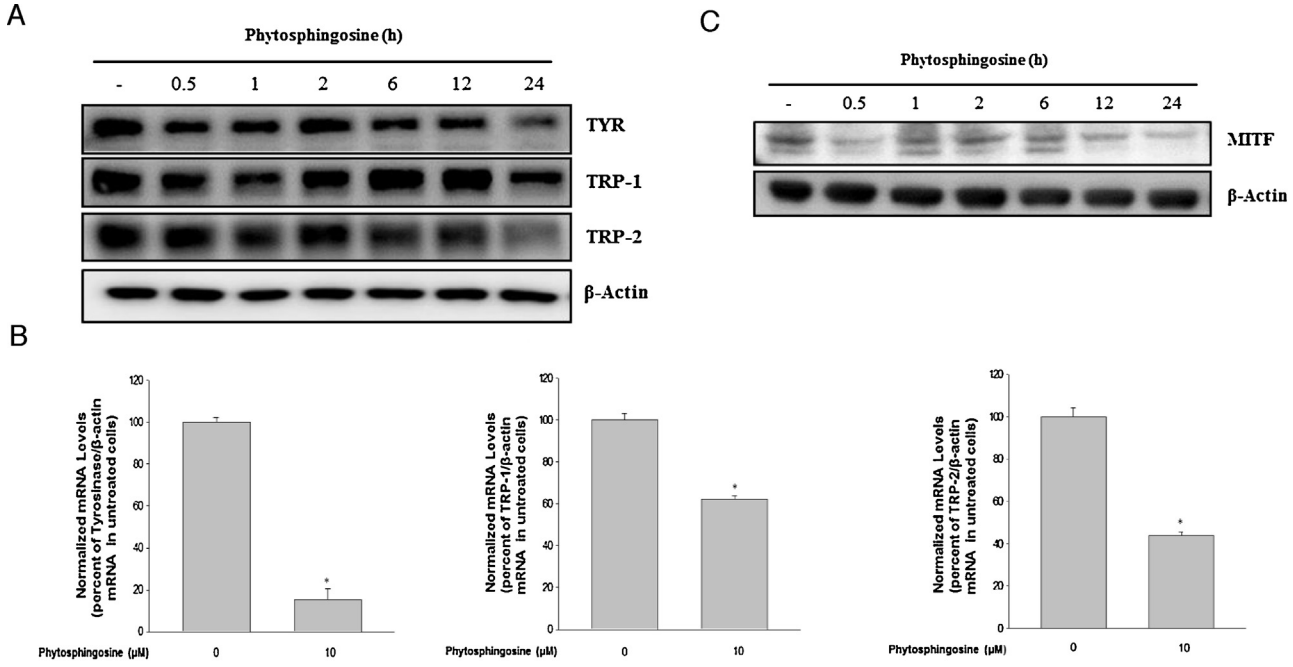

Fig. 3. Effect of phytosphingosine on the expression of melanogenesis-related proteins and genes. (A) Cells were treated with 10 μM phytosphingosine for the indicated times, and then the expression of TYR, TRP-1 and TRP-2 proteins were determined by Western blotting. (B) Cells were treated with 10 μM phytosphingosine for 24 h, and then the levels of TYR, TRP-1 and TRP-2 genes were determined by real-time RT-PCR. (C) Cells were treated with 10 μM phytosphingosine for the indicated times. The expressions of MITF proteins were examined by Western blotting. β-actin was used as an internal standard. Data shown represent the mean ± SD ($n = 3$) and are representative of three separate experiments.

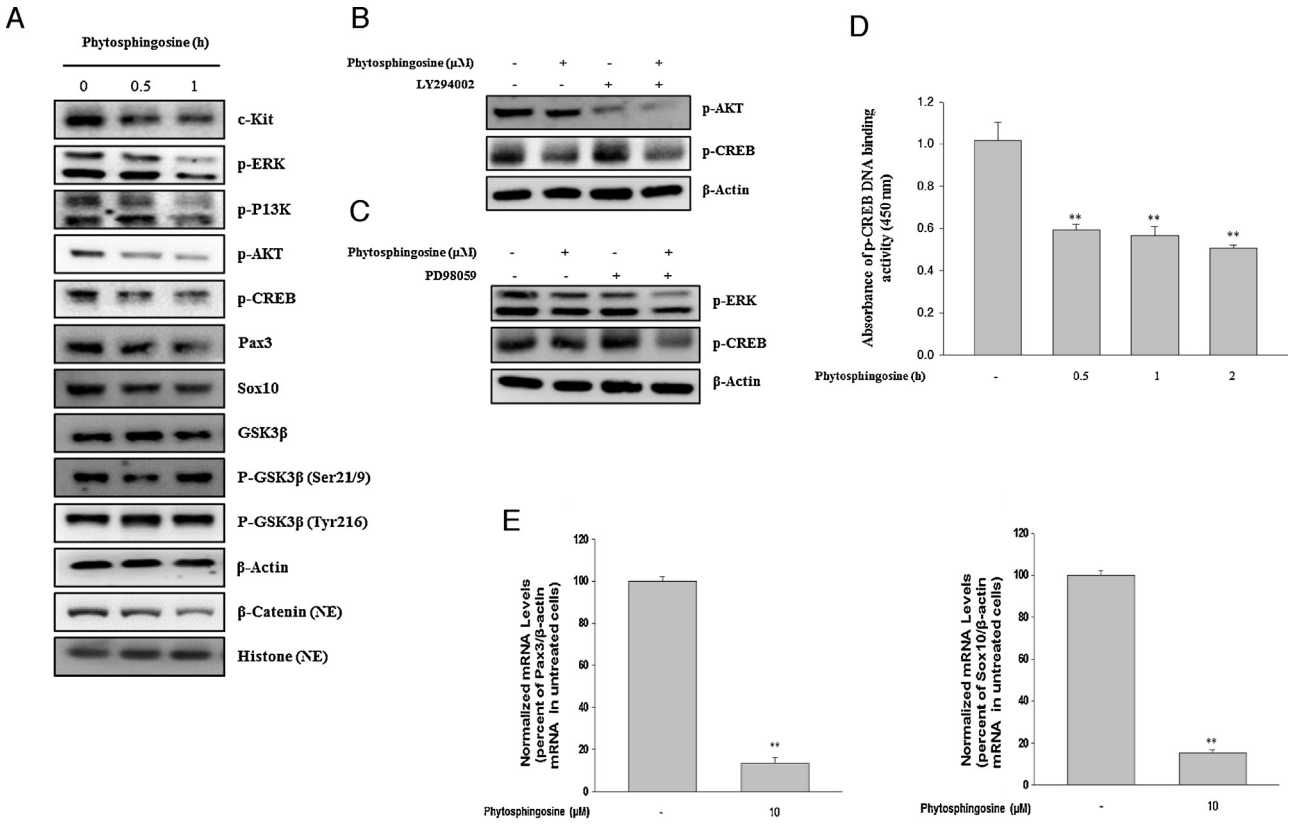

Fig. 4. Effect of phytosphingosine on MITF transcriptional factors. (A) Cells were treated with 10 μM phytosphingosine for the indicated times. The expression level of MITF related signaling molecules were examined by Western blotting. (B) Cells were treated with 10 μM of phytosphingosine in the absence or presence of LY294002 and PD98059 for 1 h. The protein levels were determined by Western blotting. (C) Cells were treated with 10 μM of phytosphingosine for the indicated times. The phospho-CREB DNA binding activity in phytosphingosine-treated cells was measured as described in Section 2. (D) Cells were treated with 10 μM of phytosphingosine for 1 h, and then the expression of Pax3 and Sox 10 genes were examined by real-time RT-PCR. β-actin was used as an internal standard. The data shown represent the mean ± SD. $^{*}p < 0.05$ is considered statistically significant compared to the control group.

activities was conducted using the Gaussia luciferase assay kit (New England BioLabs) and the luciferase reporter assay system (Promega), respectively, according to the manufacturers' protocols, and were measured using a luminometer (MicroLumat Plus, Berthold Technologies, Dortmund, Germany).

2.12. Immunocytochemistry

For immunocytochemistry, the cells were grown on cover slips in dishes. After treatment, the cells were fixed with 4% paraformaldehyde (in PBS) for 15 min, and the fixed cells were permeabilized with 0.1% Triton X-100 (PBS) for 5 min. After blocking with 1% BSA (in PBS) for 30 min at room temperature, the cells were incubated with the primary antibody at 4 °C overnight. Following the overnight incubation, the cells were incubated with FITC-conjugated secondary antibody for 2 h at room temperature. DAPI (0.5 μg/ml) was used to counterstain the nuclei. The images were acquired using a Zeiss ApoTome microscope (Carl Zeiss, Jena, Germany).

2.13. Reconstructed human skin model

The reconstructed human skin model, Neoderm-ME (Tego-science, Seoul, Korea), was purchased from Tego Science (Seoul, Korea). Neoderm-ME, a 3-dimensionally cultured human skin model, consists of human primary keratinocytes and melanocytes, which shows a similar morphology and physiology of human skin. The Neoderm-ME was irradiated with UVB doses of 40 mJ/cm^2. After irradiation of UVB, Neoderm-ME was incubated for 3 days and then various concentrations of phytosphingosine were treated for 72 h. The Neoderm-ME was dissolved in 1 N NaOH and sonicated to extract melanin, and the absorbance of the supernatants was measured at 405 nm.

2.14. Human skin primary irritation test

31 healthy subjects without skin problems participated in the skin primary irritation test. The age range of the subjects were from 36 years to 50 tears and the average age was 43.8 ± 4.3. Phytosphingosine (1%) formulated with squalene was patched on to the skin for 48 h. After the patch removed, the condition of the skin attached with the patch was evaluated according to the modified Frosch&Kligman and CTFA guidelines. Skin reaction intensity was measured twice at 30 min and 24 h after the patch removal. This test was performed by Dermapro Skin Science Research Center (Seoul, Korea) and conducted according to the human test guidelines regulations based on the Declaration of Helsinki.

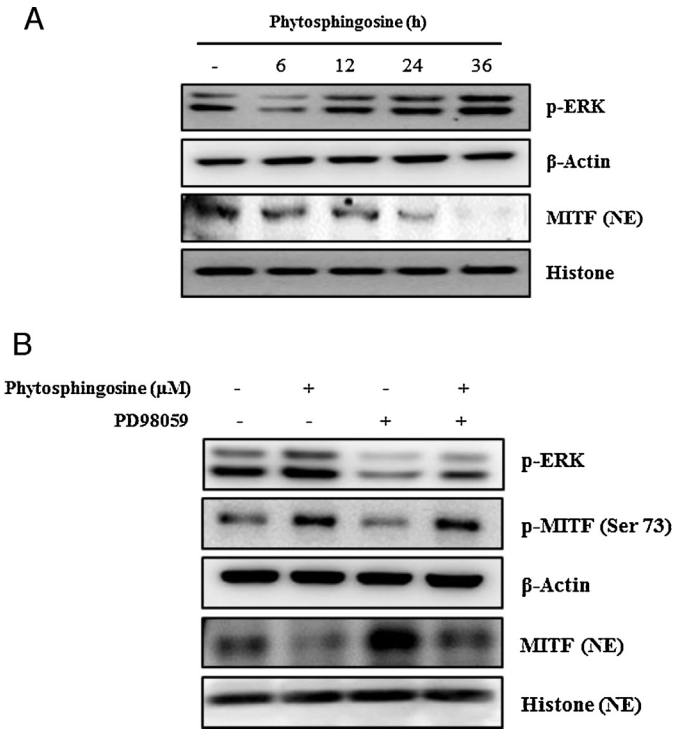

Fig. 5. Effect of phytosphingosine on MITF phosphorylation. (A) Cells were treated with 10 μM phytosphingosine in the presence or absence of PD98059 for 36 h. (B) Cells were treated with 10 μM of phytosphingosine for the indicated times. The expressions of MITF and p-ERK were examined by Western blotting. β-actin was used as an internal standard.

3. Results

3.1. Phytosphingosine inhibits melanin content and tyrosinase activity in melan-a cells

To determine the effect of phytosphingosine on melanin synthesis, the melanin content was measured. The melan-a cells were treated with different concentrations (5, 7.5, and 10 μM) of phytosphingosine for 72 h, and the results showed that the melanin content of phytosphingosine-treated cells was more significantly decreased in a concentration-dependent manner with the inhibition of 21.3%, 39.5%, and 46.1%, respectively (Fig. 2A). These results exhibited that phytosphingosine is able to inhibit melanogenesis in melan-a cells.

MTT assay was also performed to examine the cytotoxicity of phytosphingosine in melan-a cells. Melan-a cells were treated with different concentrations (5, 7.5, and 10 μM) of phytosphingosine for 72 h. Phytosphingosine did not show a significant cytotoxic effect in cultured melan-a cells (Fig. 2B). To further investigate the effect of phytosphingosine on pigmentation, the tyrosinase (TYR), a rate-limiting enzyme for melanogenesis, activity was determined, and the TYR activity was significantly inhibited by phytosphingosine over the test concentrations of 10 μM (Fig. 2C). In addition, to further analyze whether phytosphingosine is able to directly inhibit TYR activity, the effect of phytosphingosine on TYR activity in a cell-free system was also evaluated. This was done by using mushroom TYR. As shown in Fig. 2D, phytosphingosine also inhibited the mushroom TYR in a concentration-dependent manner, suggesting that phytosphingosine is also able to possibly direct inhibit the TYR activity in a cell-free system.

3.2. Phytosphingosine suppresses the expression of melanogenesis-associated proteins and genes

To better understand the anti-melanogenic molecular mechanism of phytosphingosine in melan-a cells, primarily, the effects of phytosphingosine on the protein expressions of the major melanogenesis-associated enzymes, including TYR, TRP-1, and TRP-2, were examined by western blotting. The expressions of those enzymes were started to decrease after 30 min, and the extent of the suppression was the most significant after the 24 h treatment of 10 μM phytosphingosine (Fig. 3A). In addition, the gene expression levels were also determined by real time RT-PCR analysis. The mRNA levels of TYR, TRP-1 and TRP-2 were also significantly suppressed when treated with 10 μM phytosphingosine for 24 h (Fig. 3B). These data suggest that phytosphingosine is able to regulate the gene and protein expressions of TYR, TRP-1, and TRP-2 in cultured melanocyets.

Because phytosphingosine downregulated the expressions of melanogenesis-related proteins and genes, it was further hypothesized that phytosphingosine may affect the MITF expression, which plays a significant role in melanin synthesis. To prove this hypothesis, changes in the protein levels of the MITF were evaluated in a time course experiment after treatment of phytosphingosine. The MITF protein level was suppressed after the 30-min phytosphingosine treatment and significantly decreased after the treatment of phytosphingosine for 24 h (Fig. 3C). The changes in the protein level of the MITF by phytosphingosine were similar to the changes in the protein levels of the TYR, TRP-1, and TRP-2, suggesting that the downregulation of TYR, TRP-1, and TRP-2 expressions might contribute to the suppression of MITF expression.

3.3. Phytosphingosine suppresses MITF-associated transcriptional factors after 30 min treatment

Many studies have suggested that the c-Kit and Wnt signaling pathways play critical roles in the regulation of melanin synthesis. Furthermore, MITF expression is stimulated by CREB, Pax3 and Sox10 [9]. Therefore, further studies were investigated whether phytosphingosine affects to the expressions of the MITF-associated transcriptional factors.

As the MITF expression was suppressed after the 30 min treatment of phytosphingosine, the MITF upstream transcription regulators and the c-Kit and Wnt pathway-related proteins were also changed. The 1 h treatment of phytosphingosine clearly suppressed the protein expressions of c-Kit, p-PI3K, p-AKT, p-ERK, p-CREB, Pax3, Sox10, and β-catenin. In addition, GSK3β (inactive form) was decreased and GSK3β (active form) was increased after 30 min, but they increased again after 1 h treatment (Fig. 4A).

Although the AKT/CREB pathway plays critical roles in many areas [28,29], the role of the pathway in melanogenesis is still to be elucidated. Since the expressions of phosphorylated AKT and phosphorylated CREB were shown to have similar patterns when treated with phytosphingosine (Fig. 4B), melan-a cells were pretreated with 20 μM of LY294002, an AKT inhibitor, to examine whether phytosphingosine is able to degrade the level of CREB by dephosphorylating AKT. LY294002 was found to facilitate the phytosphingosine-induced suppression of p-AKT and p-CREB expressions (Fig. 4B). Moreover, c-kit/SCF stimulation is known to induce CREB phosphorylation through ERK phosphorylation [30]. Therefore, to further analyze whether the regulation of CREB expression by phytosphingosine is also associated with the ERK pathway, the melan-a cells were pretreated with 20 μM of PD98059, an ERK inhibitor, and then the expressions of CREB and ERK levels were detected after treatment of phytosphingosine for 30 min. PD98059 was found to facilitate the phytosphingosine-

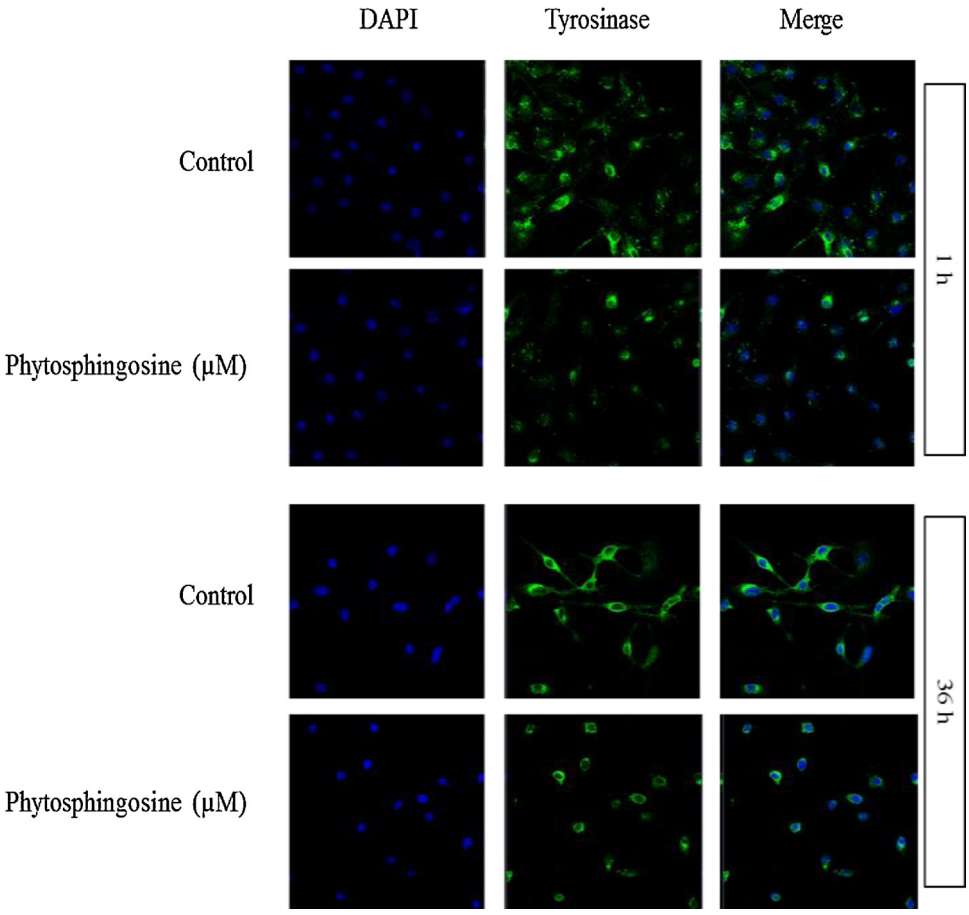

Fig. 6. Effect of phytosphingosine on tyrosinase expression determined by immunocytochemistry. Cells were treated with 10 μM of phytosphingosine for 1 h or 36 h, and then stained with tyrosinase and DAPI antibodies. The merged images are shown in the right panels.

induced suppression of ERK level and the phytosphingosine-dependent suppression of CREB level, suggesting phytosphingosine is able to degrade the level of CREB by dephosphorylating ERK (Fig. 4C).

Since phytosphingosine regulated p-CREB, the role of phytosphingosine in mediating the DNA-binding activity of phosphorylated CREB was further examined. Using the ELISA-based Trans AM method and specific p-CREB primary Ab, phytosphingosine (10 μM) significantly inhibited the DNA-binding activity of phosphorylated CREB in a time-dependent manner. These findings indicate that phytosphingosine downregulated CREB activation and its related DNA-binding activity (Fig. 4D). Phytosphingosine was also effectively found to downregulate the expressions of Pax3 and Sox10 proteins and mRNA levels (Fig. 4E). Therefore, the suppression of the mRNA and protein levels of these MITF upstream regulators might eventually affect to the downregulation of the MITF protein level at the same time point.

3.4. Phytosphingosine induces ERK activation after 12 h treatment, causing MITF phosphorylation

We also found that phytosphingosine induces the phosphorylation of ERK after 12 h, which was coincided with the reduction of the MITF protein level (Fig. 5A). However, the MITF upstream transcription regulators and the Wnt pathway-related proteins did not change after the 36-h treatment (data not shown).

It is known that MITF exhibits different patterns of subcellular localization depending on types of cells [31]. The levels of MITF in nucleus are important as a transcriptional factor. Therefore, we determined the MITF protein level in the nuclear extract after treatment of phytosphingosine in melan-a cells. Phytosphingosine (10 μM) clearly decreased the MITF levels in a time-dependent manner up to 36 h (Fig. 5A). The levels of MITF were also found to be reversely correlated with the levels of p-ERK (Fig. 5A). To further clarify whether the activation of ERK affects to the degradation of MITF in nucleus, the ERK inhibitor PD98059 was pretreated, and then p-ERK and MITF, and p-MITF levels by phytosphingosine were determined by western blotting. The treatment of PD98059 effectively downregulated p-ERK and p-MITF, but MITF level was increased in nucleus. However, the co-treatment of phytosphingosine increased the levels of p-ERK and p-MITF, but the MITF level was decreased in nucleus (Fig. 5B).

3.5. The suppression of the TYR levels by phytosphingosine is correlated with the MITF levels

To further determine whether the TYR levels by phytosphingosine is affected by the MITF levels, the expression levels of TYR were examined using immunocytochemistry after treatment of phytosphingosine for 1 h and 36 h. Phytosphingosine effectively decreased the TYR levels both time points compared to the vehicle-treated control cells, indicating that the suppression of MITF levels is able to affect to the TYR levels (Fig. 6).

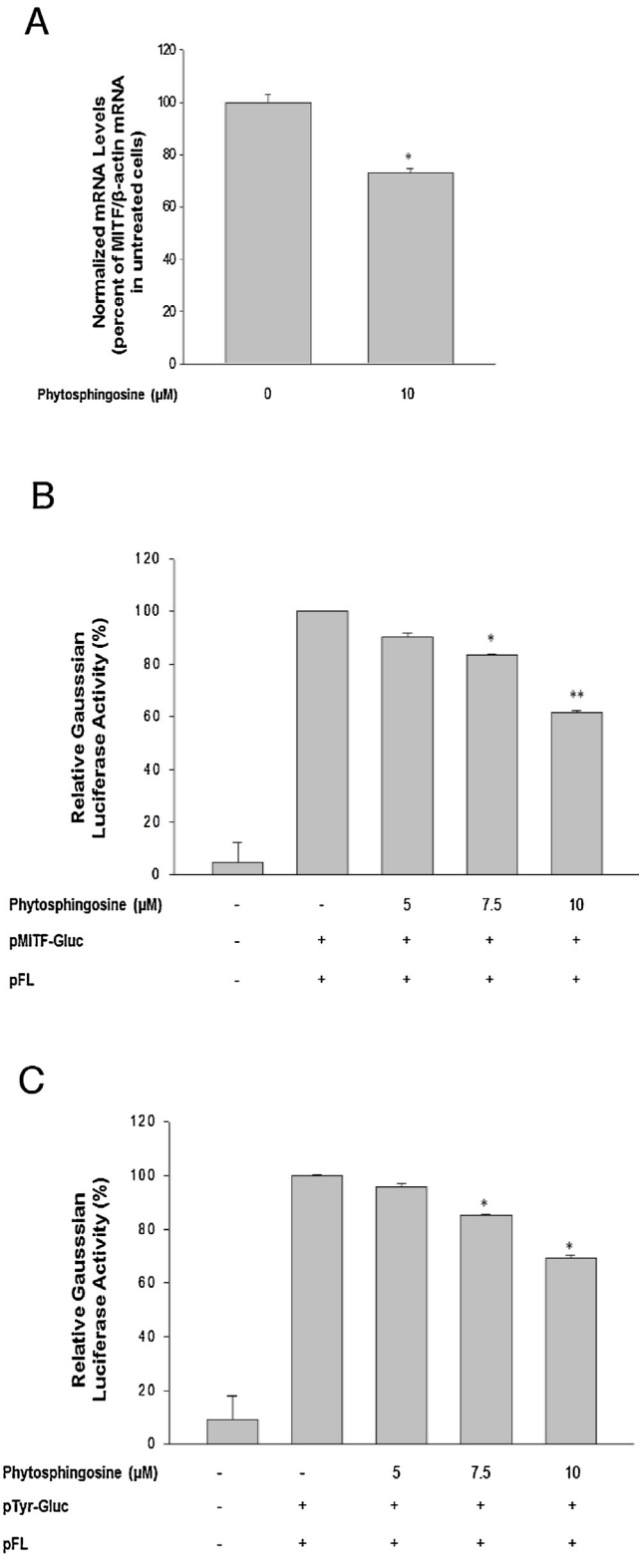

Fig. 7. Effects of phytosphingosine on gene expression and transactivation of MITF and tyrosinase. (A) Cells were treated with 10 μM phytosphingosine for 30 min, and then the levels of MITF were determined by real-time RT-PCR. (B) Cells were transfected with Gaussia luciferase reporter constructs, pMITF-Gluc and pTyr-Gluc plasmid, and then treated with the indicated concentrations of phytosphingosine for 24 h. Cells were also co-transfected with the Firefly luciferase control vector (pGL3-FL) to normalize transfection rates. Data shown represent the mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.005$ are considered statistically significant compared to the control group.

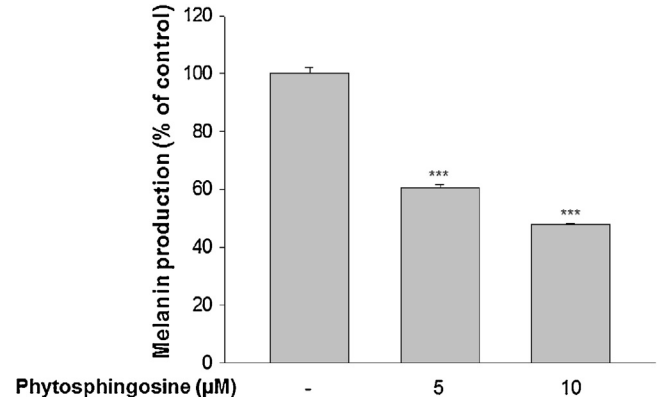

Fig. 8. Effect of phytosphingosine on melanin biosynthesis in a reconstructed human skin model, Neoderm-ME. UVB-induced Neoderm ME was treated with the indicated concentrations of phytosphingosine, and then melanin production was measured as described in Section 2. Data shown represent the mean ± SD ($n = 3$) and are representative of three separate determinations. $^{*}p < 0.05$ or $^{**}p < 0.01$ is considered statistically significant compared to the control group.

3.6. Phytosphingosine suppresses promoter activities of MITF and TYR

To further elucidate the suppression of MITF protein levels by phytosphingosine is related with the decrease of MITF gene expression, the mRNA expression of the MITF was investigated by real time RT-PCR after treatment of phytosphingosine for 30 min. Phytosphingosine effectively suppressed the MITF mRNA level, indicating that the level of MITF was transcriptionally regulated by phytosphingosine (Fig. 7A). In addition, to further determine whether phytosphingosine affects to the MITF and TYR promoter activity, dual luciferase assay was performed. Phytosphingosine effectively downregulated the promoter activities of MITF and TYR in a concentration-dependent manner (Fig. 7B and C).

3.7. Phytosphingosine inhibits melanin synthesis in a reconstructed human skin model

To examine the effect of phytosphingosine on the melanin synthesis in human skin, a reconstructed 3D human skin epidermis model (Neoderm-ME) was employed. Neoderm-ME was irradiated with UVB (40 mJ/cm^2), and then incubated for 3 days to induce melanin formation. After incubation, the irradiated human skins were treated with phytosphingosine for 72 h, and then determined the melanin contents as described in Methods. Phytosphingosine effectively inhibited the UVB-induced melanin synthesis in a reconstructed human skin model (Fig. 8).

3.8. Evaluation of phytosphingosine on human primary skin irritation

Skin irritation is also one of the major considerable issues for cosmetic ingredients. To further determine whether phytosphingosine is an applicable ingredient for cosmetic components, primarily, the effect of phytosphingosine on human skin irritation was performed by employing a patch test in human arm skins. As shown in Table 1, phytosphingosine did not demonstrate any special skin irritation detected at 30 min and 24 h after removal of patches. This finding suggests that phytosphingosine is considerable to be a relatively safe for application as a cosmetic ingredient.

4. Discussion

Sphingolipids are universal constituents of the plasma membrane in plants, animals, and fungi [32]. Of the structural analogs of

Table 1
Human skin primary irritation test.

No.	Test material	48 h					72 h					Reaction grade		
		±	1+	2+	3+	4+	±	1+	2+	3+	4+	48 h	72 h	Mean
1	Control	–	–	–	–	–	–	–	–	–	–	0	0	
2	Phytosphingosine (1%)	–	–	–	–	–	–	–	–	–	–	0	0	

No reaction; Reaction grade = ∑[{Grade × No. of Responders}/{4(Maximum grade) × 30(Total subject)}] × (1/2).

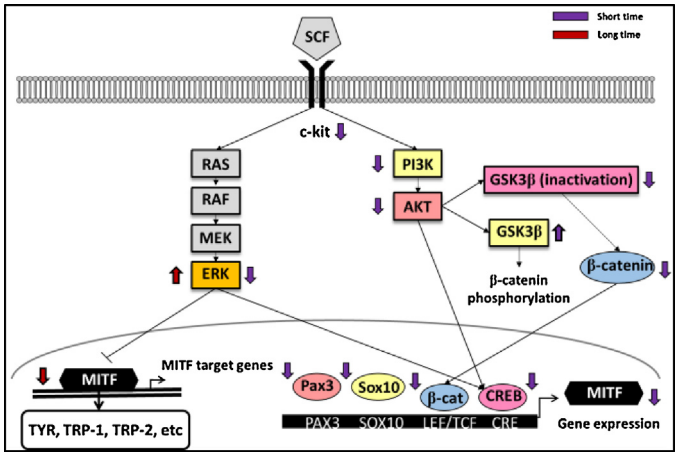

Fig. 9. The proposed mechanisms for the anti-melanogenic activity of phytosphingosine.

sphingolipids, phytosphingosine is well-known to be involved in many significant cellular responses including apoptosis, differentiation, and migration [32–34]. Moreover, phytosphingosine is commonly used as acne treatments because of its anti-bacterial and anti-inflammatory activities [35–37]. Therefore, phytosphingosine and its derivatives are applicable for maintaining healthy human skin.

To our knowledge, this is the first study demonstrating the anti-melanogenic activity of phytosphingosine. Previous study reported that the representative sphingolipid molecule, sphingosine-1-phosphate (S1P), is also known as an anti-melanogenic ingredient. The plausible mechanism was suggested by the induction of MITF phosphorylation and subsequently proteasome-mediated degradation of MITF by S1P [21]. Another study reported by Sprong et al. demonstrated that glycosphingolipids are also required for sorting melanosomal proteins from the Golgi complex to the melanosome [38]. Therefore, the biological functions of sphingolipids on melanogenesis are still unclear. In the present study, we found that the anti-melanogenic activity of phytosphingosine is in part associated with both the suppression of MITF gene expression and degradation of MITF. As confirmed by MTT assay, phytosphingosine was not cytotoxic up to 10 μM, and the level of melanin production at 10 μM was inhibited by 46.1%. To further understand the mechanisms of actions in detail, we firstly examined the effects of phytosphingosine on the enzymatic activity in melanogenesis. The TYR activity was effectively inhibited by phytosphingosine in both the cell-based and cell-free systems. Phytosphingosine also significantly suppressed the gene and protein expressions of the TYR, TRP-1, and TRP-2, which are main enzymes in melanogenesis, suggesting that the anti-melanogenic activity of phytosphingosine is in part associated with the direct inhibition of the enzymatic activity and transcriptional and translational regulation of main target enzymes in melanin biosynthesis.

In addition, the MITF, a major transcription factor in melanin biosynthesis, protein level was found to be dramatically decreased two times shortly after the 30 min and 12–24 h treatment of phytosphingosine. This dual shift similarly occurred in TYR, TRP-1 and TRP-2, suggesting that the downregulation of TYR, TRP-1, and TRP-2 by phytosphingosine can be caused by the MITF suppression.

Many evidences suggested that the Wnt signaling pathway is also involved in the MITF expression. When the canonical Wnt pathway is activated, which leads to the GSK3β inactivation (phosphorylated at ser21/9) and β-catenin accumulation [39]. The accumulated β-catenin in cytoplasm subsequently triggers to translocate into the nucleus and enhance the expression of MITF [40]. Therefore, the melanogenesis can be promoted by inhibiting GSK3β. Moreover, activated GSK3β (phosphorylated at Tyr216) is able to induce the phosphorylation of β-catenin, which causes the ubiquitination and degradation of β-catenin [41]. As a result, the present findings showed that the inhibition of MITF levels by phytosphingosine is in part due to the decrease of GSK3β inactive form and increase of GSK3β active form, which leads to the decrease of β-catenin in nucleus. These data also suggest that phytosphingosine is able to inhibit MITF transcription by inhibiting the formation of β-catenin and TCF/LEF complex in nucleus. Further study revealed that the regulation of MITF by phytosphingosine is also correlated with the downregulation of MITF upstream transcriptional regulators including CREB, Pax3, and SOX10. We also confirmed that c-Kit-mediated MAPK/PI3K pathways were also inhibited by the treatment of phytosphingosine. In addition, the involvement of ERK and CREB in the regulation of MITF by phytosphingosine was also elucidated with the co-treatment of the ERK and AKT inhibitors. In particular, the analysis of signaling pathways indicate that the downregulation of MITF by phytosphingosine is in part associated with both the suppression of MITF gene expression in a short period time and the degradation of MITF protein *via* ERK-mediated MITF phosphorylation and its subsequent proteasomal degradation in a longer period time (Fig. 9). Furthermore, the *in vivo* human skin mimic model using a reconstructed 3D human skin also exhibited the UVB-induced melanin biosynthesis was effectively inhibited by phytosphingosine.

In conclusion, herein, we for the first time demonstrate the anti-melanogenic activity of phytosphingosine in both cultured melanocytes and a reconstructed 3D human skin models. The plausible mechanisms of actions for the inhibition of melanin biosynthesis in melanocytes by phytosphingosine were also elucidated by analyzing the signaling pathways in melanogenesis.

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