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Research article

Inhibitory effects of ginsenosides on basic fibroblast growth factor-induced melanocyte proliferation

Ji Eun Lee, Jong Il Park, Cheol Hwan Myung, Jae Sung Hwang*

Department of Genetic Engineering, Graduate School of Biotechnology, Kyung Hee University, Yongin, South Korea

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ABSTRACT

Background: UV-B-exposed keratinocytes secrete various paracrine factors. Among these factors, basic fibroblast growth factor (bFGF) stimulates the proliferation of melanocytes. Ginsenosides, the major active compounds of ginseng, are known to have broad pharmacological effects. In this study, we examined the antiproliferative effects of ginsenosides on bFGF-induced melanocyte proliferation.

Methods: We investigated the inhibitory effects of Korean Red Ginseng and ginsenosides from *Panax ginseng* on bFGF-induced proliferation of melan-a melanocytes.

Results: When melan-a melanocytes were treated with UV-B-irradiated SP-1 keratinocytes media, cell proliferation increased. This increased proliferation of melanocytes decreased with a neutralizing anti-bFGF antibody. To elucidate the effects of ginsenosides on melanocyte proliferation induced by bFGF, we tested 15 types of ginsenoside compounds. Among them, Rh3, Rh1, F1, and CK demonstrated anti-proliferative effects on bFGF-induced melanocyte proliferation after 72 h of treatment. bFGF stimulated cell proliferation via extracellular signal-regulated kinase (ERK) activation in various cell types. Western blot analysis found bFGF-induced ERK phosphorylation in melan-a. Treatment with Rh3 inhibited bFGF-induced maximum ERK phosphorylation and F1-delayed maximum ERK phosphorylation, whereas Rh1 and CK had no detectable effects. In addition, cotreatment with Rh3 and F1 significantly suppressed bFGF-induced ERK phosphorylation. Western blot analysis found that bFGF increased microphthalmia-associated transcription factor (MITF) protein levels in melan-a. Treatment with Rh3 or F1 had no detectable effects, whereas cotreatment with Rh3 and F1 inhibited bFGF-induced MITF expression levels more strongly than a single treatment.

Conclusion: In summary, we found that ginsenosides Rh3 and F1 have a synergistic antiproliferative effect on bFGF-induced melan-a melanocyte proliferation via the inhibition of ERK-mediated upregulation of MITF.

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1. Introduction

Skin pigmentation is primarily the result of the production of the melanin pigment by melanocytes [1]. Melanosomes, melanin containing granules, are transferred from melanocytes into keratinocytes, and thus pigmentation is complete. Therefore, pigmentation is a combination of melanocytes and keratinocytes in the basal layer of the epidermis [2,3]. Hyperpigmentary disorders such as melasma [4], solar lentigo [5], and freckles [6] are characterized by the overproduction of melanin.

Numerous studies have reported that UV light is a well-known extrinsic factor for hyperpigmentation [7,8]. UV-B-exposed keratinocytes also have a potent ability to secrete certain growth factors, cytokines, or chemical mediators, such as nitric oxide [9], interleukin-1 [10], interleukin-6 [11], tumor necrosis factor [12], granulocyte macrophage colony-stimulating factor (GM-CSF) [13], endothelin-1 [14], α-melanocyte-stimulating hormone [15], stem cell factor [16], and basic fibroblast growth factor (bFGF) [17]. Previous studies verified that some paracrine factors produced by human keratinocytes indirectly stimulate the proliferation of human melanocytes and play a key role in controlling melanocyte function [18]. Among these factors, bFGF, which plays an important role in cellular processes such as proliferation and migration, showed the highest rate of increase in proliferation of normal

* Corresponding author. Department of Genetic Engineering, College of Life Sciences, Kyung Hee University, Yongin-si, Gyeonggi-do 17104, South Korea.
E-mail address: jshwang@khu.ac.kr (J.S. Hwang).

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human epidermal melanocytes [19–22]. However, whether bFGF derived from UV-B-induced mouse keratinocytes could stimulate proliferation of mouse melanocytes or how bFGF might act as a regulator of melanocyte proliferation remains unclear.

Panax ginseng Meyer, which is widely used in Chinese medicine and throughout the world, has long been known for its medical properties [23]. In Korea, cultivation of *P. ginseng* began around 11 BC with the transplantation of wild ginseng. Korean Red Ginseng (KRG), a component of Korean herbal medicine, is harvested after 4–6 yr of cultivation, then steamed and dried [24]. The major active components of ginseng are ginsenosides, which contain an aglycone with dammarane sapogenins [25]. More than 30 types of ginsenosides are divided into two major groups based on their chemical structure, panaxadiol and panaxatriol saponin groups [26].

KRG has been shown to have broad pharmacological effects, such as anticancer [27], antitumor [28], and antidiabetic [29], and has demonstrated clinical efficacy for severe climacteric syndromes [30]. Previous studies also verified that ginsenosides had a number of pharmacological actions in cardiovascular disease [31], neurological disorders [32], and ovarian cancer [33]. Previous studies using KRG on skin have been limited to atopic dermatitis [34], wound healing [35], antiallergic, and anti-inflammatory [36] effects. However, the effects of KRG, particularly of ginsenosides on UV-induced skin pigmentation, have not yet been investigated.

In this study, we examined the effect of ginsenosides on melanocyte proliferation induced by bFGF.

2. Materials and methods

2.1. Compound and reagents

Total extract and saponin of KRG were provided by the Korea Ginseng Corporation (Daejeon, Korea). Ginsenoside compounds were purchased from Chengdu Biopurify Phytochemicals, Ltd (Chengdu, Sichuan, China). Newborn calf serum (NBCS) was purchased from Gibco Invitrogen (Carlsbad, CA, USA). Mouse bFGF was purchased from Cell Signaling Technology (Beverly, MA, USA).

2.2. Cell culture

Murine melan-a melanocytes were kindly donated by Professor Dorothy C. Bennett (St. George's Hospital, London, UK). Melan-a cells were cultured in Ham's F-10 medium supplemented with 10 mg/mL insulin, 0.5 mg/mL bovine serum albumin, 1mM ethanolamine, 1mM phosphoethanolamine, 10nM sodium selenite, 100 mg/mL transferrin, 0.5mM dibutyryl-cyclic adenosine monophosphate, and 1% penicillin–streptomycin. Murine SP-1 keratinocytes were derived from SENCAR mice and were generously provided by Dr Stuart H. Yuspa (Laboratory of Cellular Carcinogenesis and Tumor Promotion, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA). SP-1 keratinocytes were grown in Eagle's minimum essential medium (EMEM) containing 0.05mM Ca^{2+}, 8% Chelex-treated heat-inactivated NBCS, and 1% penicillin–streptomycin.

2.3. Cell viability assay

Melan-a melanocytes were seeded onto 96-well plates (2×10^4 cells/well). After 24 h at 37°C, the media were replaced with Ham's F-10 media containing ginsenosides and bFGF diluted to the appropriate concentrations. Control cells were treated with dimethyl sulfoxide (DMSO) or phosphate-buffered saline (PBS) at a final concentration of 0.1%. After 24 h, the media containing the compounds or DMSO were replaced with media containing 10% EZ-CyTox (Daeil Lab Service, Seoul, Korea). The cells were then incubated at 37°C for 30 min and the absorbance was measured using a microplate reader (Tecan, Mannedorf, Switzerland) at a wavelength of 450 nm. All assays were performed in triplicate. The cytotoxic

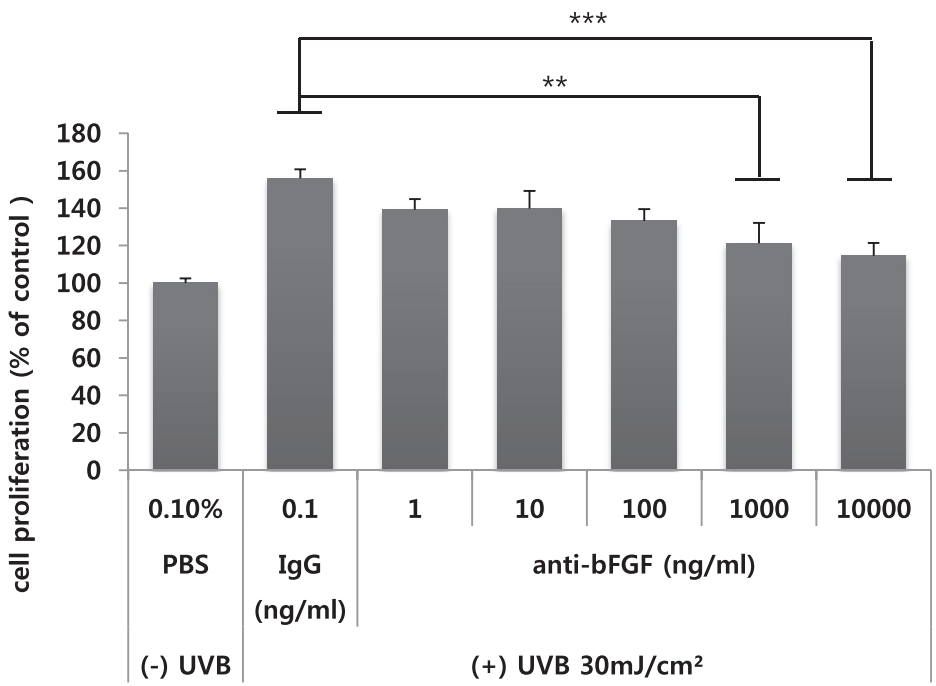

Fig. 1. Effects of anti-bFGF on melanocyte proliferation induced by UVB-irradiated SP-1 keratinocyte media. SP-1 keratinocytes were irradiated with UVB at a dose of 30 mJ/cm^2. Conditioned media were collected after 24 h of incubation from UVB-irradiated SP-1 keratinocytes. IgG or Anti-bFGF was added to the conditioned media and the media were stored for 24 h at 4°C. Melan-a melanocytes were treated with the conditioned media for 3 days. The experiment was repeated three times. Results are presented as percentages of the control and the data were analyzed using Student's unpaired t-tests. $^{**}p < 0.01$, $^{***}p < 0.001$.

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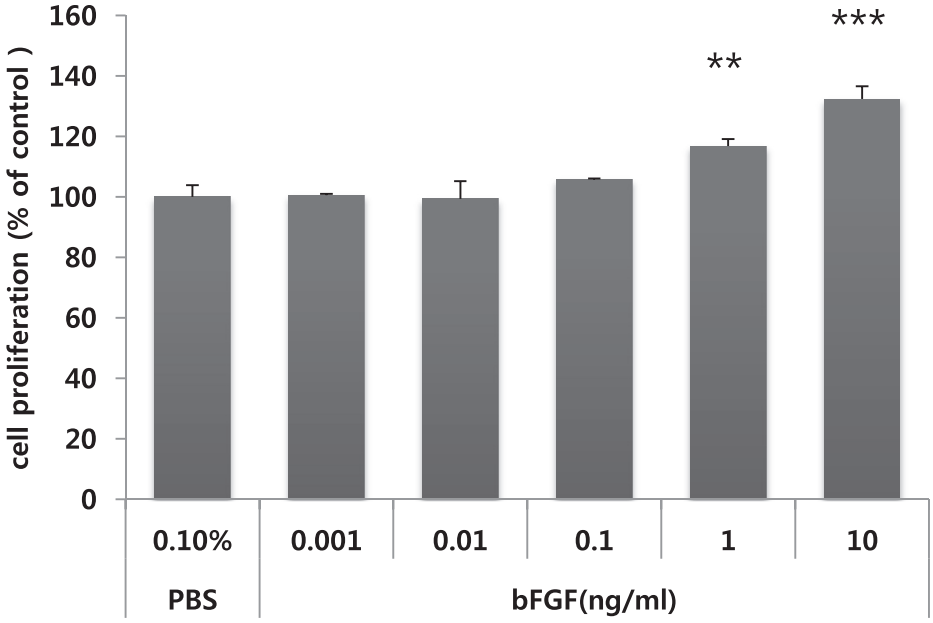

Fig. 2. Effects of bFGF on melan-a melanocyte proliferation. Melan-a melanocytes were cultured in 48-well plates (1x10^4 cells/well). After incubation with indicated concentrations of bFGF for 5 days, cell proliferation was measured using the cell proliferation assay described in the Materials and Methods section. Results are presented as percentages of the control and the data were analyzed using Student's unpaired t-tests. $^{**}p < 0.01$, $^{***}p < 0.001$.

effect of each treatment was expressed as a percentage of cell viability relative to the untreated control cells.

2.4. Neutralization assay

SP-1 keratinocytes were seeded in 60-mm culture dishes (1.5×10^6 cells/dish). After 24 h, the cells were washed with PBS and replenished with serum-free EMEM. After starvation for 24 h, SP-1 keratinocytes were washed with PBS and exposed to a radiation dose of 30 mJ/cm^2 of UV-B light (290–320 nm) by a UV irradiation system. After irradiation, the cells were replaced with 2% NBCS EMEM containing immunoglobulin-G (IgG) or anti-bFGF (Millipore, Temecula, CA, USA). After 24 h, melan-a melanocytes were seeded onto 24-well plates (1×10^4 cells/well) and treated with the conditioned media for 72 h.

2.5. Cell proliferation assay

Melan-a melanocytes were seeded in 48-well plates (1×10^4 cell/well). After 24 h, the cells were washed with PBS and

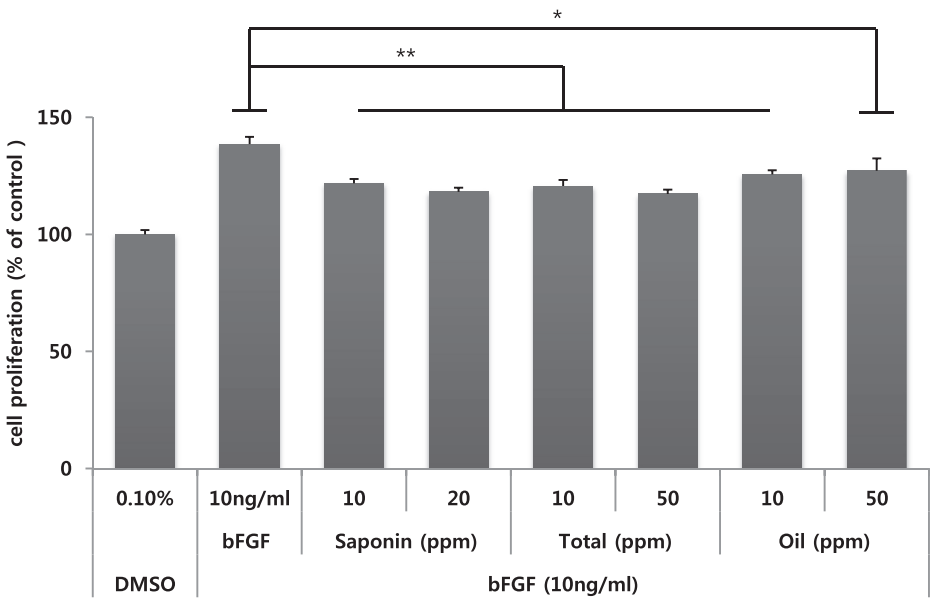

Fig. 3. Effects of KRGE on bFGF-induced melanocyte proliferation. Melan-a melanocytes were cultured in 48-well plates (1x10^4 cells/well). After incubation with bFGF (10 ng/mL), total extract, saponin and oil of Korean Red ginseng (KRG) (10, 20 and 50 ppm) for 5 days, cell proliferation was measured using the cell proliferation assay described in the Materials and Methods section. Results are presented as percentages of the control and the data were analyzed using Student's unpaired t-tests. $^{*}p < 0.05$, $^{**}p < 0.01$.

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replenished with Ham's F10 media containing bFGF (10 ng/mL). After 72 h, the media containing the compounds or DMSO were replaced with media containing 10% EZ-CyTox (Daeil Lab Service). The cells were then incubated at 37°C for 2 h, and the absorbance was measured using a microplate reader at a wavelength of 450 nm. All assays were performed in triplicate. The proliferation effect of each treatment was expressed as a percentage of cell viability relative to the untreated control cells.

2.6. Western blot analysis

Melan-a melanocytes were seeded in 60-mm dishes (1×10^6 cells/dish). Cells were pretreated with saponin of KRG (20 ppm) or ginsenoside (10nM) and then treated with bFGF (10 ng/mL) for the indicated times. Following washing with PBS, cells were lysed in an extraction buffer (0.1M Tris-HCl, pH 7.2, 1% TritonX-100, 200mM NaCl, and protease inhibitor cocktail) at 4°C for 30 min. Each cell lysate was loaded onto NuPAGE 10% Bis-Tris sodium dodecyl sulfate/polyacrylamide gels and run with a 3-(N-morpholino)propanesulfonic acid sodium dodecyl sulfate running buffer (Invitrogen, Carlsbad, CA, USA) for electrophoresis and then transferred to a polyvinylidene fluoride transfer membrane (PALL Corporation, Port Washington, NY, USA). Membranes were blocked with 5% skim milk or 5% bovine serum albumin in Tris-buffered saline containing 0.1% Tween-20, 100mM NACl, and 10mM Tris-HCl (pH 7.5) for 1 h at room temperature, before overnight incubation with a primary antibody using mouse monoclonal anti-β-actin antibody (Sigma Aldrich, St. Louis, MO, USA), rabbit monoclonal anti-p42/44 mitogen-activated protein kinase [MAPK; extracellular signal-regulated kinase (ERK)1/2] antibody (Cell Signaling, Beverly, MA, USA), rabbit monoclonal anti-phospho-42/44 MAPK (ERK1/2) (Cell Signaling), and antimicrophthalmia-associated transcription factor (MITF; C5; NeoMarkers, Fremont, CA, USA) at 4°C. After incubation, membranes were rinsed three

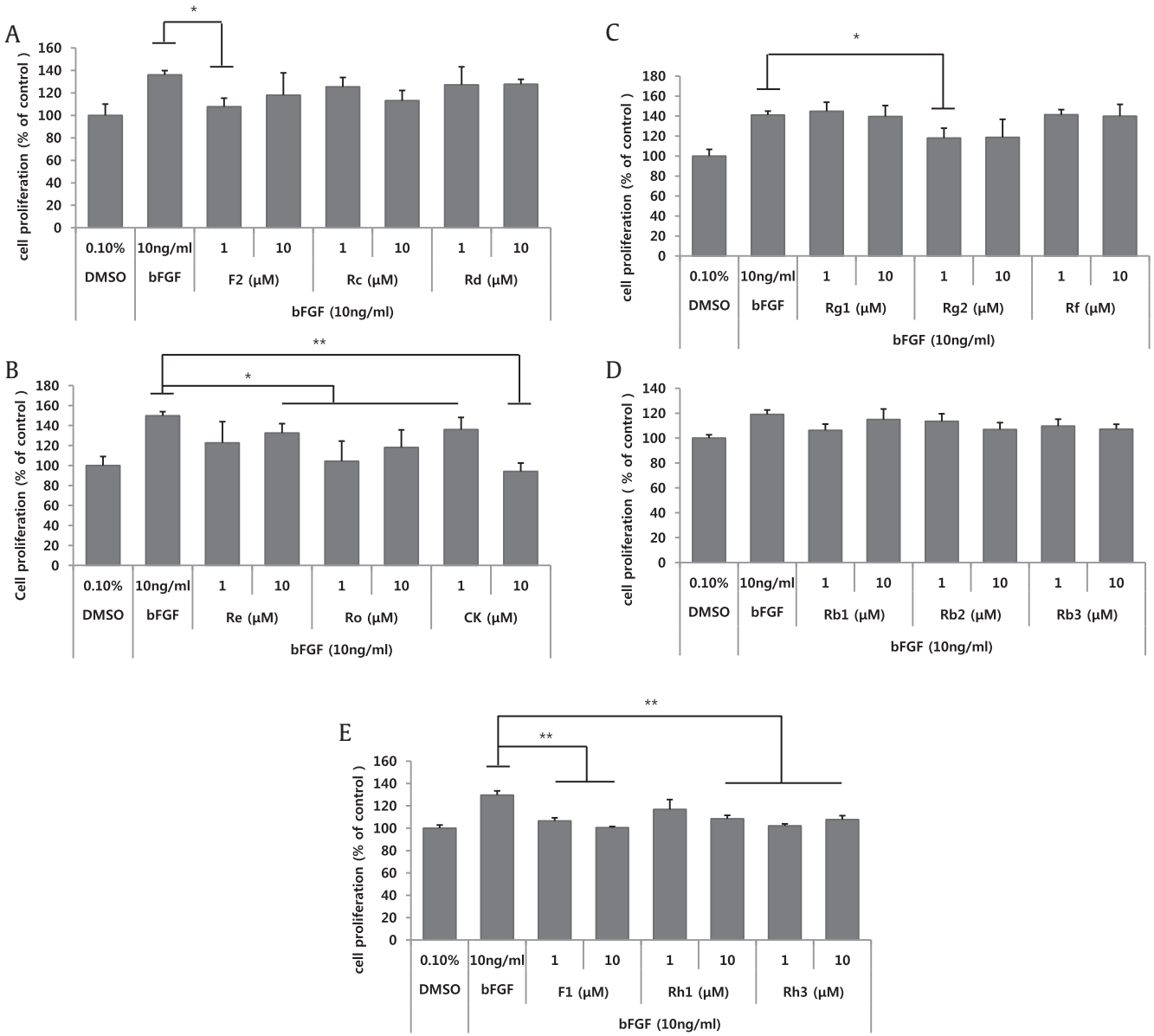

Fig. 4. Effects of ginsenosides on bFGF-induced melanocyte proliferation. Melan-a melanocytes were cultured in 48-well plates ($1x10^4$ cells/well). After incubation with bFGF (10 ng/mL) and ginsenosides (A) F2, Rc, Rd, (B) Re, Ro, CK, (C) Rg1, Rg2, Rf (D) Rb1, Rb2, Rb3, (E) F1, Rh1, Rh3 (1, 10 μM) for 5 days, cell proliferation was measured using the cell proliferation assay described in the Materials and Methods section. Results are presented as percentages of the control and the data were analyzed using Student's unpaired t-tests. $^{*}p < 0.1$, $^{**}p < 0.01$.

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times with Tris-buffered saline and were incubated with donkey anti-rabbit IgG antibody (Bethyl Laboratories, Montgomery, TX, USA) and goat anti-mouse IgG antibody (Bio-Rad, Hercules, CA, USA) for 1 h at room temperature. After washing, bands were detected with the WEST-ZOL plus Western Blot Detection system (INtRON Biotechnology, Kyungki-Do, Korea) and visualized with ChemiDoc XRS (Bio-Rad).

3. Results

3.1. Effects of anti-bFGF on melanocyte proliferation induced by UV-B-irradiated SP-1 keratinocyte media

We investigated whether bFGF from UV-B-irradiated SP-1 keratinocytes stimulated melan-a melanocyte proliferation. UV-B-irradiated SP-1 keratinocytes conditioned media increased melan-a melanocytes proliferation by ~60% compared with the nonirradiated control. Neutralizing anti-bFGF treatment decreased cell proliferation by approximately 30% at 10 ng/mL. Therefore, we identified bFGF-specific antibodies partially blocked bFGF from UV-B-irradiated SP-1 keratinocytes (Fig. 1).

3.2. Effects of bFGF on melan-a melanocyte proliferation

To investigate the role of bFGF on melan-a melanocytes proliferation, bFGF was treated on melan-a cells (0.001–10 ng/mL) directly. After 24 h, bFGF had no effect on cellular proliferation (data not shown). However, after 72-h culture, we observed that bFGF treatment increased melan-a melanocyte proliferation by approximately 30% at 10 ng/mL compared with the nontreated control (Fig. 2).

3.3. Effects of KRG extract on bFGF-induced melanocyte proliferation

We investigated the effects of KRG on bFGF-induced melanocyte proliferation. Melan-a melanocytes were seeded in 48-well plates, ~1×10^4 cells/well. After 24 h, the cells were replenished by Ham's F-10 media containing bFGF and KRG extract. After 72 h, cell proliferation was detected using an EZ-cytox assay.

Saponin and total extract of KRG inhibited bFGF-induced cell proliferation by ~20% compared with bFGF-treated cells (Fig. 3).

3.4. Effects of ginsenosides on bFGF-induced melanocyte proliferation

To investigate the effects of ginsenosides on bFGF-induced melanocyte proliferation, melan-a melanocytes were seeded onto 48-well plates, ~1×10^4 cells/well, and bFGF and the ginsenosides were treated with melan-a melanocytes. No cytotoxicity was observed after 24 h of ginsenosides treatment (data not shown). After 72-h culture, treatment of ginsenosides Rh3, Rh1, F1, and CK reduced proliferation of bFGF-induced melan-a melanocytes compared with the DMSO-treated control (Fig. 4).

3.5. Effects of saponin of KRG on bFGF-induced ERK phosphorylation in melan-a melanocytes

To investigate the biological mechanisms involved in the proliferation effect of saponin of KRG (SKRG), we investigated the ERK signal. bFGF binds to the FGF receptor, and this leads to the activation of the ERK pathway in melanocytes and melanoma [37]. To determine the effects of SKRG on bFGF-induced ERK-phosphorylation, melan-a melanocytes were pretreated with SKRG at a dose

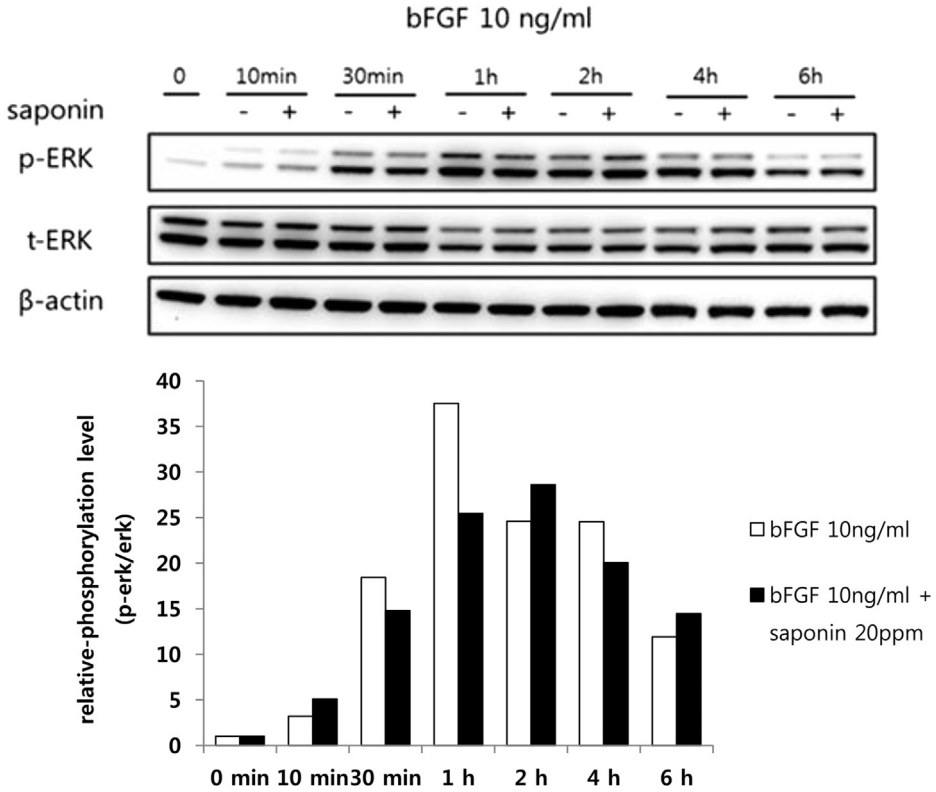

Fig. 5. Effects of SKRG on bFGF-induced ERK activation in melanocytes. Melan-a melanocytes were cultured in 60-mm dishes (1x10^6 cells/dish). After 24 h, cells were pretreated with SKRG (20 ppm) for 24 h. Cells were treated with bFGF (10 ng/mL) at the indicated times for western blot analysis of phospho-ERK (p-ERK) and total-ERK (t-ERK). Band intensity was quantified and normalized relative to ERK using ImageJ (1.47) software (NIH, USA).

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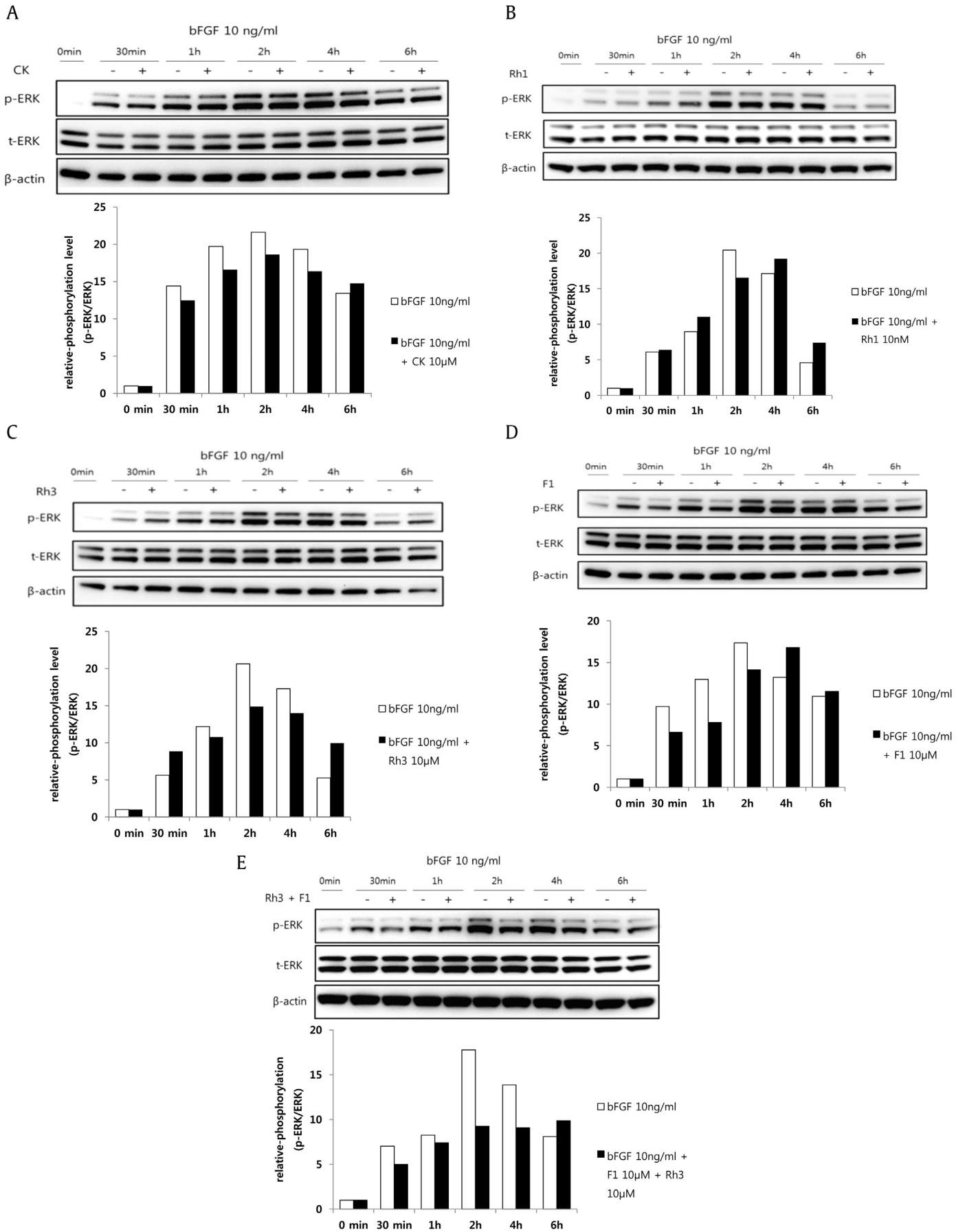

Fig. 6. Effects of ginsenosides Rh1, CK, Rh3 and F1 on bFGF-induced ERK activation in melanocytes. Melan-a melanocytes were cultured in 60-mm dishes ($1x10^6$ cells/dish). After 24 h, cells were pretreated with ginsenoside (10 μM) (A) CK, (B) Rh1, (C) Rh3, (D) F1 and (E) Rh3 and F1 for 24 h. Cells were treated with bFGF (10 ng/mL) at the indicated times for Western blot analysis of phospho-ERK (p-ERK) and total-ERK (t-ERK). Band intensity was quantified and normalized relative to ERK using ImageJ (1.47) software (NIH, USA).

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of 20 ppm for 24 h. After bFGF exposure for the indicated time, cell lysates were harvested and subjected to western blot analysis using phospho-42/44 MAPK and 42/44 MAPK antibody. We observed that treatment of mouse melanocytes with bFGF over a period of 6 h resulted in rapid phosphorylation of ERK. ERK phosphorylation peaked at 1 h and SKRG inhibited bFGF-induced ERK phosphorylation at 1 h (Fig. 5).

3.6. *Effects of ginsenosides Rh3, Rh1, F1, and CK on bFGF-induced ERK phosphorylation in melan-a melanocytes*

To test the inhibitory effects of ginsenosides Rh3, Rh1, F1, and CK on bFGF-induced ERK-phosphorylation, melan-a melanocytes were exposed to ginsenosides (10μM) for 24 h prior to bFGF (10 ng/mL) treatment. Although ginsenosides CK and Rh1 had no significant effect on ERK activation (Figs. 6A and 6B), Rh3 inhibited bFGF-induced phosphorylation of ERK in melanocytes at 1 h and 2 h (Fig. 6C). We also observed that F1 regulated early phosphorylation and slightly delayed ERK activation (Fig. 6D). These results suggested that ginsenosides CK and Rh1 may affect other signaling pathways of bFGF, such as the phosphatidylinositol 3-kinase-protein kinase B pathway. To investigate whether Rh3 and F1 could have synergistic effects on ERK phosphorylation, cells were treated with Rh3 and F1 simultaneously. We observed that cotreatment with Rh3 and F1 regulated early phosphorylation and strongly inhibited bFGF-induced ERK activation (Fig. 6E). Therefore, we

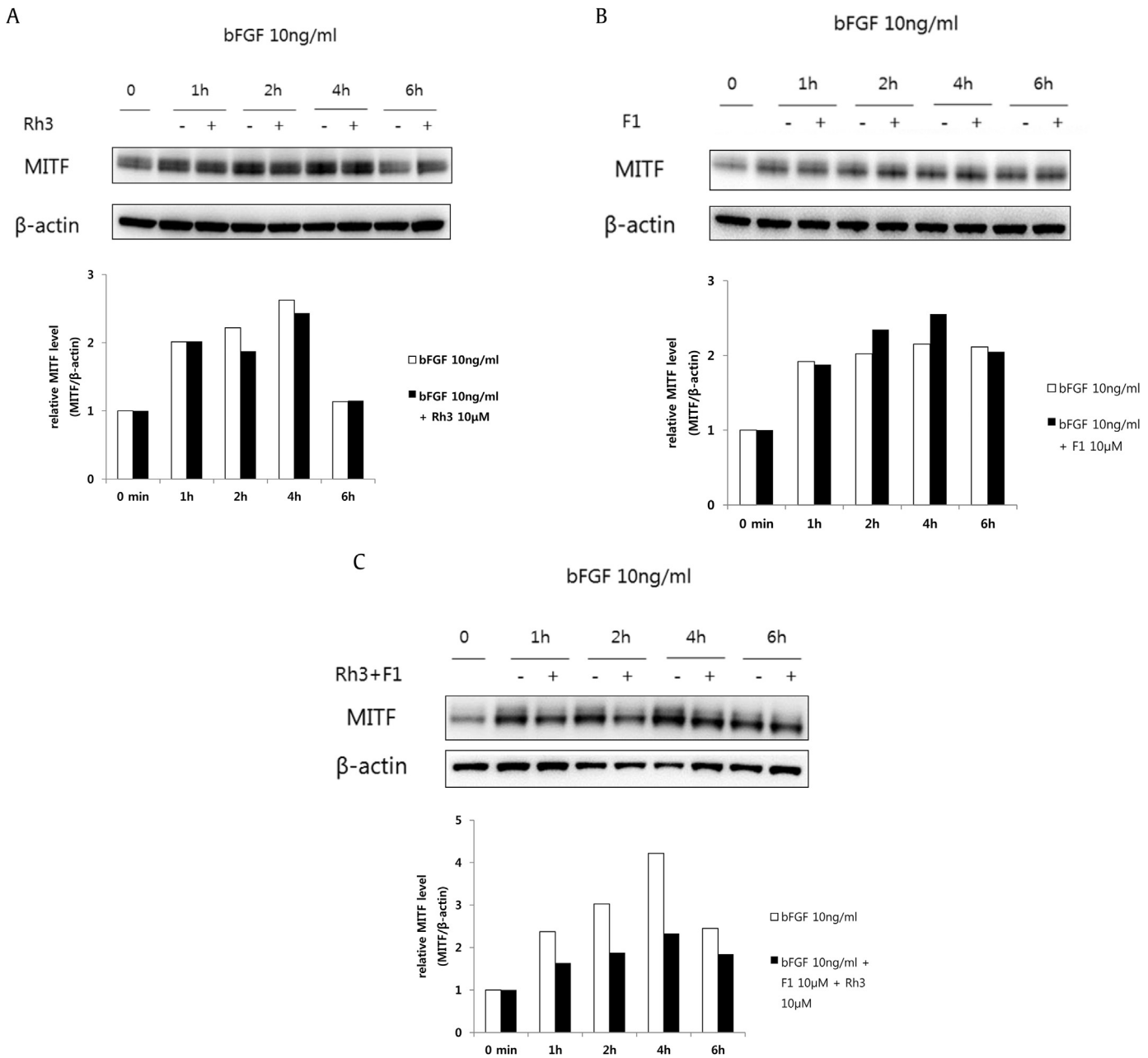

Fig. 7. Effects of ginsenosides Rh3 and F1 on bFGF-induced MITF expression in melanocytes. Melan-a melanocytes were cultured in 60 mm dishes ($1x10^6$ cells/dish). After 24 h, cells were pretreated with ginsenoside (10 μM) (A) Rh3, (B) F1 and (C) Rh3 and F1 for 24 h. Cells were treated with bFGF (10 ng/mL) at the indicated times for Western blot analysis of MITF. Band intensity was quantified and normalized relative to β-actin using ImageJ (1.47) software (NIH, USA).

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found that ginsenosides Rh3 and F1 were involved in the activation of ERK during the intracellular signaling initiated by bFGF in mouse melanocytes.

3.7. Effects of ginsenosides Rh3 and F1 on bFGF-induced MITF expression in melan-a melanocytes

MITF, a basic-helix-loop-helix/leucine-zipper transcription factor, is essential for melanocyte development and survival and controls proliferation of melanocytic cells [38]. Here, we found bFGF increased MITF protein expression for a period of 4 h. To further elucidate the inhibitory effect of ginsenosides Rh3 and F1, cells were treated with each ginsenoside separately. The results indicated that treatment with either Rh3 or F1 had no effect on bFGF-induced MITF expression in melanocytes (Fig. 7A and 7B). To investigate whether Rh3 and F1 could have a synergistic effect on MITF expression, cells were treated with Rh3 and F1 simultaneously. We observed that cotreatment of Rh3 and F1 inhibited bFGF-induced MITF protein expression in melan-a melanocytes (Fig. 7C).

4. Discussion

Previous studies have reported that UV light is a well-known extrinsic factor for stimulating keratinocytes to produce paracrine factor for melanocytes [8–10]. Paracrine regulation of GM-CSF derived from UVB-irradiated SP-1 keratinocytes was also observed in our previous study [39].

Here, we found that bFGF increased mouse melanocyte proliferation via a paracrine effect. When melan-a melanocytes were treated with UV-B-irradiated SP-1 keratinocyte conditioned media, cell proliferation increased by ~60%. We also found that bFGF derived from UV-B-induced keratinocytes was blocked by anti-bFGF treatment, and melanocyte proliferation decreased by ~40% (Fig. 1), indicating that bFGF secreted from UV-B-irradiated keratinocytes stimulated melanocytes proliferation.

KRG has been shown to have broad pharmacological effects, such as anticancer, antitumor, and antidiabetic effects [27–29]. Ginsenosides, the major active components of ginseng, also have various clinical actions, including antiaging, antidiabetic, and anti-inflammatory effects [40–42]. However, the effects of KRG and ginsenosides on bFGF-induced melanocyte proliferation have not been investigated previously.

In this study, we demonstrated the antiproliferative effects of SKRG and ginsenosides. SKRG at a concentration of 20 ppm did not have cytotoxic effects on melanocytes. However, preincubation with SKRG before bFGF stimulation decreased melanocyte proliferation to 20% after 72 h (Fig. 3). Among those tested ginsenosides Rh3, Rh1, F1, and CK also inhibited bFGF-induced melan-a melanocyte proliferation (Fig. 4).

In melanocytes, bFGF binds to the FGF receptor, and this leads to activation of the MAPK pathway [37]. Western blot analysis showed that bFGF significantly induced ERK phosphorylation in melan-a melanocytes, consistent with previous evidence that bFGF induced melanocyte proliferation through ERK1/2 signaling in human primary melanocyte [22]. Treatment of SKRG inhibited phosphorylation of ERK (Fig. 5). Treatment of Rh3 had the capacity to interrupt maximum ERK phosphorylation and, interestingly, treatment of F1 slightly delayed the maximum ERK phosphorylation, whereas Rh1 and CK had no detectable effects (Fig. 6). Cotreatment of Rh3 and F1 showed greater suppression of ERK phosphorylation than did single treatment of either Rh3 or F1 (Fig. 6). This is likely due to the fact that F1 delayed early activation of ERK and then, synergistically, Rh3 and F1 inhibited phosphorylation of ERK. Rh1 and CK might affect other pathways induced by bFGF in melan-a melanocytes.

In melanocytes, MITF is phosphorylated by MAPK signaling and stimulates its activation while it simultaneously targets its ubiquitin-dependent proteasomal degradation. MITF regulates genes important for melanocyte function, including apoptosis, proliferation, and differentiation [43]. Thus, we next determined whether bFGF increased MITF protein levels. Western blot analysis showed that bFGF stimulated MITF protein expression at 4 h. Separate treatment of Rh3 and F1 had no remarkable effects. However, cotreatment of Rh3 and F1 effectively suppressed bFGF-induced MITF protein expression in melan-a melanocytes (Fig. 7).

In this study, we identified treatment of Rh3 and F1 decreased the phosphorylation of ERK and MITF protein expression in bFGF-treated melan-a melanocytes. Therefore, Rh3 and F1 can act synergistically to inhibit the bFGF-induced proliferation of mouse melanocytes.

Keratinocyte derived paracrine factors stimulate melanocyte proliferation, leading to skin hyperpigmentation [18]. In a previous study, we found that SKRG decreased GM-CSF expression levels in UV-B induced keratinocytes [44] and inhibited the proliferation of GM-CSF induced melanocytes [39]. These findings showed that SKRG had antiproliferative effects on mouse melanocytes and ginsenoside Rh3 and F1 may be effective ingredients that inhibit skin pigmentation stimulated by bFGF following UV-B radiation.

Further studies are needed to identify which transcriptional regulator of MITF is affected by ginsenosides Rh3 and F1 and how Rh1 and CK regulate other signaling pathways induced by bFGF in mouse melanocytes.

Conflicts of interest

The authors have no conflicts of interest to report.

Acknowledgments

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