Molecules and Cells

Extracellular Vesicles from Korean *Codium fragile* and *Sargassum fusiforme* Negatively Regulate Melanin Synthesis

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Although various marine ingredients have been exploited for the development of cosmetic products, no previous study has examined the potential of seaweed extracellular vesicles (EV) in such applications. Our results revealed that EV from *Codium fragile* and *Sargassum fusiforme* effectively decreased α-MSH-mediated melanin synthesis in MNT-1 human melanoma cells, associated with downregulation of MITF (microphthalmia-associated transcription factor), tyrosinase and TRP1 (tyrosinase-related proteins 1). The most effective inhibitory concentrations of EV were 250 μg/ml for *S. fusiforme* and 25 μg/ml for *C. fragile*, without affecting the viability of MNT-1 cells. Both EV reduced melanin synthesis in the epidermal basal layer of a three-dimensional model of human epidermis. Moreover, the application of the prototype cream containing *C. fragile* EV (final 5 μg/ml) yielded 1.31% improvement in skin brightness in a clinical trial. Together, these results suggest that EV from *C. fragile* and *S. fusiforme* reduce melanin synthesis and may be potential therapeutic and/or supplementary whitening agents.

Keywords: extracellular vesicles, melanin synthesis, seaweed, skin epidermis

INTRODUCTION

Melanin, which is produced in the melanosomes of melanocytes, is a pigment that determines the color of skin, eyes, and hair (Hearing, 2011). Epithelial melanin is transported to neighboring keratinocytes and protects the skin from UV damage (Archambault et al., 1995; Brenner and Hearing, 2008), and abnormal melanogenesis is closely associated with various disorders of hyperpigmentation (e.g., melasma, postinflammatory hyperpigmentation, freckles, and lentigines) and hypopigmentation (e.g., vitiligo and albinism) (Bastonini et al., 2016; Nicolaidou and Katsambas, 2014).

Various systemic agents, such as arbutin, kojic acid, hydroquinone, etc., are commonly used to cure these disorders (Parvez et al., 2006). Unfortunately, topical treatments show limited efficacy. The dermal epidermis has five major keratinocyte layers: the stratum basale, stratum spinosum, stratum granulosum, stratum lucidum, and stratum corneum (Baroni et al., 2012). The thick structure of the skin epidermis limits the ability of topical agents to penetrate/absorb into the skin, accounting for their generally low efficacy. In addition, these drugs have been associated with negative side effects, such as dryness, redness, inflammation, and even a risk of cancer

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(Pillaiyar et al., 2017; Solano et al., 2006). Therefore, it would be beneficial to develop efficient and safe skin-permeating agents. In this context, many researchers have become interested in using extracellular vesicles (EVs) as an alternative agent (Azmi et al., 2013; van den Boorn et al., 2011).

EVs are nano-size membrane vesicles that carry various biomolecules, such as lipids, proteins, and RNAs (Naval and Chandra, 2019). Most forms of life, from microorganisms to higher eukaryotes, produce these vesicles and use them as a means of communicating between cells (Valadi et al., 2007) like intracellular-cellular vesicle in trafficking (Kwon et al., 2020). The physical properties of EVs make them useful carriers of biomolecules. In fact, recent studies have shown that EVs and/or EV-inspired vesicles can be therapeutically effective as natural drug delivery vehicles (Antimisiaris et al., 2018; Gomari et al., 2018). Therefore, it is very likely that EVs could improve the skin penetration of active ingredients.

Marine resources and compounds have been found to have various biological activities, including anti-cancer and anti-inflammatory activities. For example, *Sargassum* seaweed contains anti-inflammatory substances, such as terpenoids, fucoxanthin, and lipid-soluble compounds (Saraswati et al., 2019) Kahalalide F from a species of *Bryopsis* has anti-tumor and anti-fungal effects (Shilabin and Hamann, 2011) and seaweed-derived fucoxanthin and phloroglucinol have shown anti-cancer activity against glioma (Ferreira et al., 2018). In addition, recent studies have shown that marine substances can regulate the pigmentation of the skin. For example, 1,9-dihydroxycrenulide and epiloliolide from *Dictyota coriacea* (Holmes) were found to have anti-melanogenic effects in B16F10 mouse melanoma cells (Ko et al., 2013). Extracts from *Ecklonia cava,* a type of brown seaweed, inhibited melanin synthesis by downregulating key melanogenic proteins, such as microphthalmia-associated transcription factor (MITF), tyrosinase, and tyrosinase related protein-1 and -2 (TRP-1 and -2) (Yoon et al., 2009). In addition, ethanolic extracts of *Sargassum polycystum* inhibited tyrosinase activity in B16F10 cells (Chan et al., 2011). To date, most of the marine ingredients that have been exploited for the development of cosmetic products have been in the form of purified chemicals or cell extracts. Indeed, no previous study has examined the potential use of seaweed EVs for cosmetic applications. Since EVs are known to have useful skin penetration capacity, we set out to test whether EVs purified from seaweed could potentially be used for skin whitening. Here, we developed a method for isolating EVs from seaweeds and examined their potential use as new melanin-reducing agents.

MATERIALS AND METHODS

Reagents and antibodies

The polyclonal antibody against tyrosinase and TRP-1, and the monoclonal antibody against β-actin were purchased from Santa Cruz Biotechnology (USA). The polyclonal antibody against MITF was purchased from Proteintech (USA). The α-MSH, L-DOPA, kojic acid, and arbutin were purchased from Sigma (USA).

Cell culture

MNT-1 cells (a human melanoma cell line) were cultured in minimal essential medium (MEM; WelGene, Korea) supplemented with 20% fetal bovine serum (Gibco, USA), 10% Dulbecco's modified Eagle's medium (DMEM; Hyclone, USA), 20 mM HEPES, and 50 μg/ml gentamicin (Sigma). The cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Isolation of extracellular vesicles

EVs were purified using ultracentrifugation following a standard protocol (Lane et al., 2015). Briefly, 1× phosphate-buffered saline (PBS) was added to semi-dried and sliced seaweeds (1:10, v:w) and hand-held food blenders were used to thoroughly disrupt tissue connections. The samples were centrifuged at 5,000 × *g* for 1 h to remove tissue, cell debris and insoluble materials, followed by successive centrifugations at 20,000 × *g* for 2 h to remove any remaining insoluble materials. EVs were isolated by centrifugation of supernatants from previous steps at 100,000 × *g* for 4 h. All centrifugations were performed at 4°C. Each EV pellet was resuspended with 1× PBS and centrifuged at 100,000 × *g* (Optima XE-90; Beckman Coulter, USA) for 30 min and the centrifuge tubes were air dried up side-down for 10 min at room temperature. The translucent EV pellets were dissolved in 1× PBS, quantified with Bradford assay (Bio-Rad, USA) and stored at −20°C for up to 1 month. Each purified EVs were filtered through 0.22 um filters and re-quantified with Bradford assay before use. Under our experimental conditions, an average of 1.5 mg of EVs was obtained from 1 kg of *Sargassum fusiforme*, and an average of 1.8 mg of EVs was obtained from 1 kg of *Codium fragile*.

Melanin content assay

Melanin contents were measured as described in a previous study (Jung et al., 2016). Cells were washed twice with PBS, detached by incubation with trypsin/EDTA, and collected by centrifugation at 1,000 x *g* for 3 min. Thereafter, 8×10^5 cells were solubilized in 200 μl of 1N NaOH-10% DMSO at 80°C for 4 h. The dissolved melanin was assessed by absorbance at 405 nm.

Intracellular tyrosinase activity assay

The intracellular tyrosinase activity assay was performed as previously described. Briefly, cells were plated to coverslips in 12-well plates, fixed with 3.5% paraformaldehyde for 10 min, washed with PBS, and incubated in sodium phosphate buffer with 10 mM L-DOPA for 3 h at 37°C. The cells were then washed with PBS, the coverslips were mounted on glass slides, and the slides were observed by microscopy (Jung et al., 2014).

Immunoblotting

Cells were lysed with RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 10 mM NaF, and 2 mM Na_3VO_4, pH 8.0) containing several protease inhibitors (1 μg/ml aprotinin, 1 μg/ml antipain, 1 mM dithiothreitol, 5 μg/ml leupeptin, 1 μg/ml pepstatin A, and 20 μg/ml phenylmethylsulfonyl fluoride). Cell lysates were clarified by centrifugation at 13,000 rpm for

15 min at 4°C, denatured with sample buffer, boiled, and analyzed by SDS-PAGE. Proteins were transferred to nitrocellulose blotting membranes (Amersham Biosciences, USA) and probed with the appropriate antibodies. Signals were detected with an Odyssey CLx imager (LI-COR Biosciences, USA) and analyzed using the Image Studio Lite software (LI-COR Biosciences).

Cell proliferation assay

Cell proliferation was measured using the MTT [3-(4,5-dimethythiazol-2-yl)2,5-diphenyltetrazolium bromide] assay. Briefly, MNT-1 cells were harvested with 0.05% trypsin/EDTA and seeded to 96-well plates at 5×10^3 cells/well. After incubation for 48 h, medium containing 0.5 mg/ml MTT (100 μl; Sigma) was added to each well, and the cells were incubated for 1 h. The medium was then removed and 100 μl of acidic isopropanol (90% isopropanol, 0.5% SDS, 25 mM NaCl) was added to each well. The mean concentration of absorbance at 570 nm in each sample set was measured using a 96-well microtiter plate reader (Dynatech, USA).

MelanoDerm culture and H&E staining

MelanoDerm (MEL-300-B) and the maintenance medium for EPI-100-NMM-113 were purchased from MatTek Corporation (USA). To measure melanin content, MelanoDerm tissue was incubated in 12-well plates containing the pre-warmed maintenance medium according to the manufacturer's recommendations. The medium was changed every other day for 14 days (Park et al., 2018). For H&E staining, a fresh scalpel blade was used to separate the MelanoDerm tissues from the inserts at specified time points. The tissues were immediately fixed in 4% formalin and embedded in paraffin. Each paraffin block was sequentially sectioned at 4 μm, and the sections were mounted on slides and stained using H&E (Abcam, UK). The images were captured at a magnification of ×40, and a minimum of 20 fields per section was assessed using a color image analyzer (Leica DM1000 LED; Leica Microsystems, Germany) (Park et al., 2018).

In vivo clinical study

Healthy Korean (n = 35) between the ages of 29 and 59 years (average 47) were enrolled in the study (Korea Institute of Dermatological Sciences, Korea). For the irritation test, a skin patch containing the test sample was applied to the test site for 24 h. The skin patch was then removed, and the test site was graded for irritation at 30 min, 24 h, and 48 h post-removal using the irritation grading scale of Evaluation followed the SOP of Korea Institute of Dermatological Sciences (Dores and Baron, 2011; Jin et al., 2018; Spínola et al., 2013). Healthy Korean women (n = 21) between the ages of

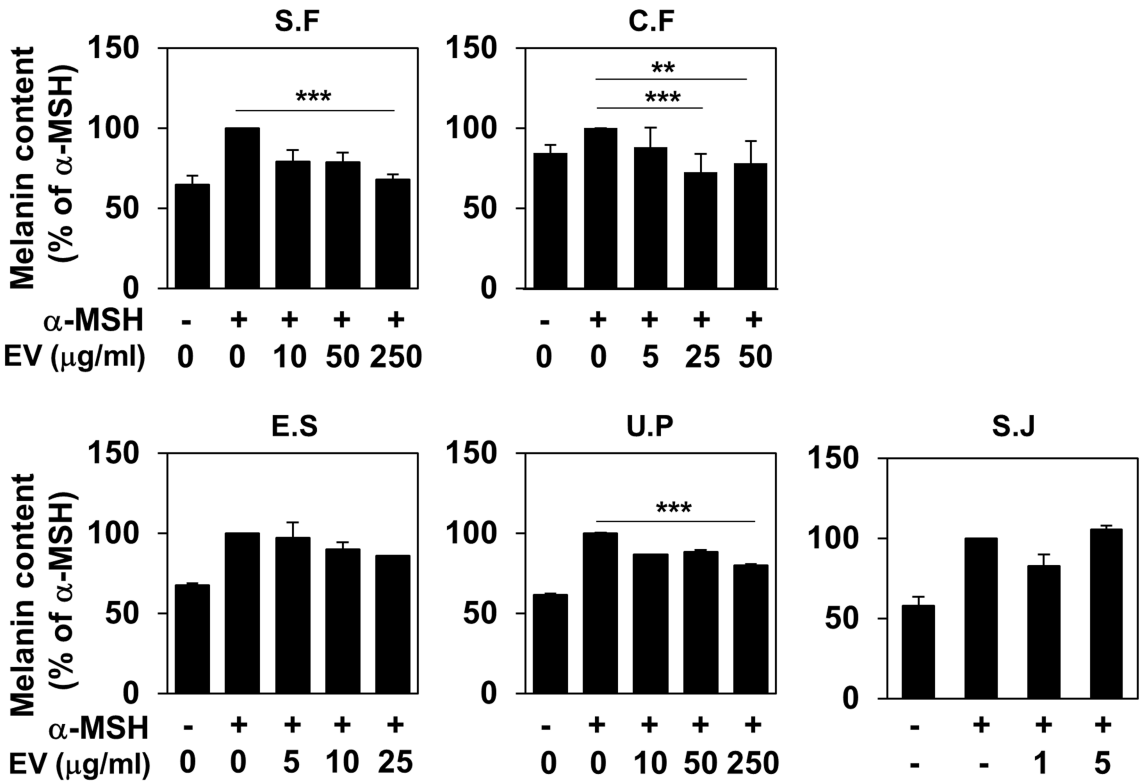

Fig. 1. EVs from *C. fragile* and *S. fusiforme* reduce α-MSH-mediated melanogenesis. EV were isolated from the Korean seaweeds, *S. fusiforme* (S.F), *C. fragile* (C.F), *E. stolonifera* (E.S), *U. pinnatifida* (U.P), and *S. japonica* (S.J). MNT-1 cells were treated with the indicated amounts of seaweed EVs in the presence of 500 nM of α-MSH for 48 h. Cells were harvested and dissolved in 1N NaOH-10% DMSO, and the melanin contents were analyzed by measuring the absorbance at 405 nm. The mean percentages of melanin content are shown. Data are expressed as mean ± SD for three independent experiments; $^{**}P < 0.001$; $^{***}P < 0.0001$ versus DMSO-treated cells.

20 and 50 years (average 46) were enrolled in the study (Korea Institute of Dermatological Sciences). For the skin whitening efficacy test, the test products were each applied to half of the participant's face (on the right for placebo cream, on the left for test cream) once a day for 4 weeks. The skin was imaged by VISIA-CA (VISIA Complexion Analysis; Canfield Scientific, Inc., USA) and the skin whitening was evaluated with a spectrophotometer (CM-2600D; Minolta, Japan).

Statistical analysis

Data are presented as the means from three independent experiments. Statistical analyses were performed using the unpaired Student's *t*-test. A *P* value less than 0.01 or 0.05 was considered statistically significant.

RESULTS

Extracellular vesicles from *Sargassum fusiforme* and *Codium fragile* reduce melanin synthesis in human melanoma cells

We first isolated EVs from five different Korean seaweeds: *S. fusiforme* (S.F), *C. fragile* (C.F), *Ecklonia stolonifera* (E.S), *Undaria pinnatifida* (U.P), and *Saccharina japonica* (S.J). As α-melanocyte stimulating hormone (α-MSH) is known to be a key regulator of melanogenesis in melanocytes (Hedley et al., 1998), we investigated whether the seaweed EVs could suppress α-MSH-induced melanin synthesis. We selected MNT-1 cells (a human pigmented melanoma cell line) as a model system, and treated the cells with 500 nM α-MSH in the absence or presence of various amounts of EVs from the Korean seaweeds (Fig. 1). Interestingly, we found that the EVs isolated from *S. fusiforme* and *C. fragile*, but not those from *U. pinnatifida* and *E. stolonifera*, significantly inhibited the ability of α-MSH to induce melanin synthesis (Fig. 1). Our dose-response experiments revealed that the maximum inhibitory effect on melanin synthesis was seen with 250 μg/ml *S. fusiforme* EVs, but only 25 μg/ml *C. fragile*. Thus, *C. fragile* EVs appeared to have a better anti-melanogenic effect on MNT-1 cells.

Characterization of extracellular vesicles from *Sargassum fusiforme* and *Codium fragile*

To further characterize the isolated EVs from *S. fusiforme* and *C. fragile*, the size and morphology of the isolated EVs were

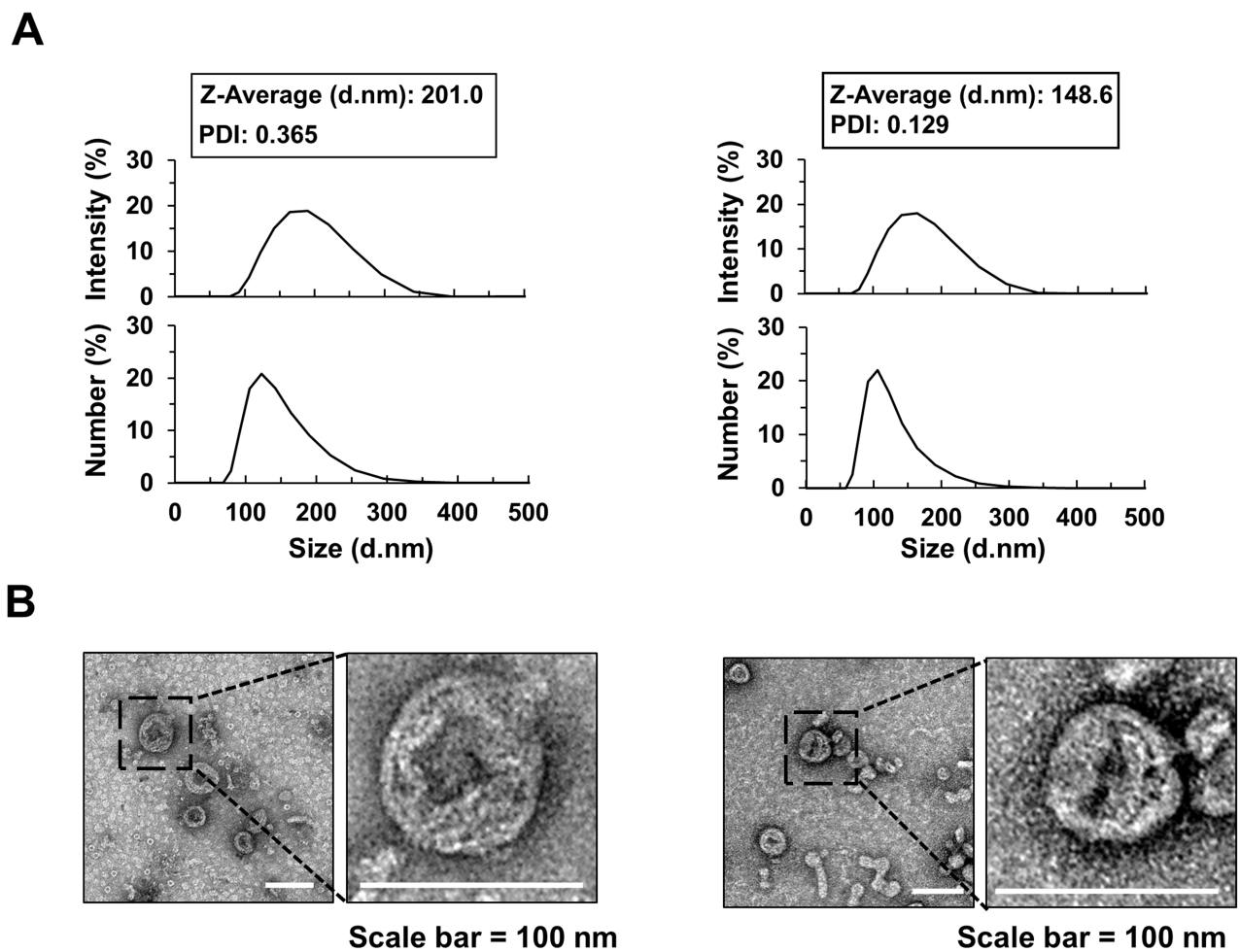

Fig. 2. Characterization of EVs from *C. fragile* and *S. fusiforme*. EV were isolated from *S. fusiforme* (left panel) and *C. fragile* (right panel). The mean radius distributions of purified EVs were determined by DLS (A) and TEM (B). d, diameter (nm).

determined by dynamic light scattering (DLS) and transmission electron microscopy (TEM) (Fig. 2). The PDI (polydispersity index) of Sargassum EV was 0.365 ± 0.058 and the average particle size was 201.1 ± 15.04 nm, and the PDI of Codium EV was 0.129 ± 0.042 and the average particle size was 148.6 ± 3.119 nm (Fig. 2A). Transmission electron microscopic analysis confirmed the overall spherical shape of the EVs and the presence of membranous vesicles smaller than 200 nm in diameter (Fig. 2B), validating that the isolated EVs showed good integrity.

Seaweed extracellular vesicles downregulate melanogenesis-related proteins

Next, we investigated whether EVs from *S. fusiforme* and *C. fragile* could affect the expression of various melanogenic proteins, including tyrosinase and, TRP1 (tyrosinase-related proteins 1) and MITF, which plays a critical role in melanogenesis (Fig. 3). As expected, 25 μg/ml of *C. fragile* significantly downregulated the expression levels of tyrosinase, TRP-1 and MITF in MNT-1 cells treated with 500 nM of α-MSH, whereas 250 μg/ml of *S. fusiforme* mildly slightly downregulated the expression of TRP-1 and MITF, but not tyrosinase (Fig. 3A). When we further examined the effect of EVs on basal melanogenesis, 25 μg/ml of *C. fragile* reduced the expression levels of tyrosinase, TRP-1 and MITF in MNT-1 cells more efficiently (Fig. 3A), supporting that *C. fragile* EVs have a better anti-melanogenic effect on MNT-1 cells.

L-3,4-dihydroxyphenylalanine (L-DOPA) staining showed that the level of intracellular tyrosinase activity was decreased in seaweed EV-treated MNT-1 cells (Fig. 3B). α-MSH treatment increased the presence of dark spots (pigment, induced by active tyrosinase) in MNT-1 cells, but EVs derived from *S. fusiforme* and *C. fragile* decreased these spots; this was particularly notable for *C. fragile* EVs, which showed this effect at a low concentration of 50 μg/ml (Fig. 3B). Kojic acid and arbutin, which are well-known whitening agents, were used as positive controls (Pillaiyar et al., 2017; Solano et al., 2006). Neither type of EV had any effect on cell viability under our experimental conditions (Fig. 3C). Together, these data suggest that EVs of *S. fusiforme* and *C. fragile* inhibit mela-

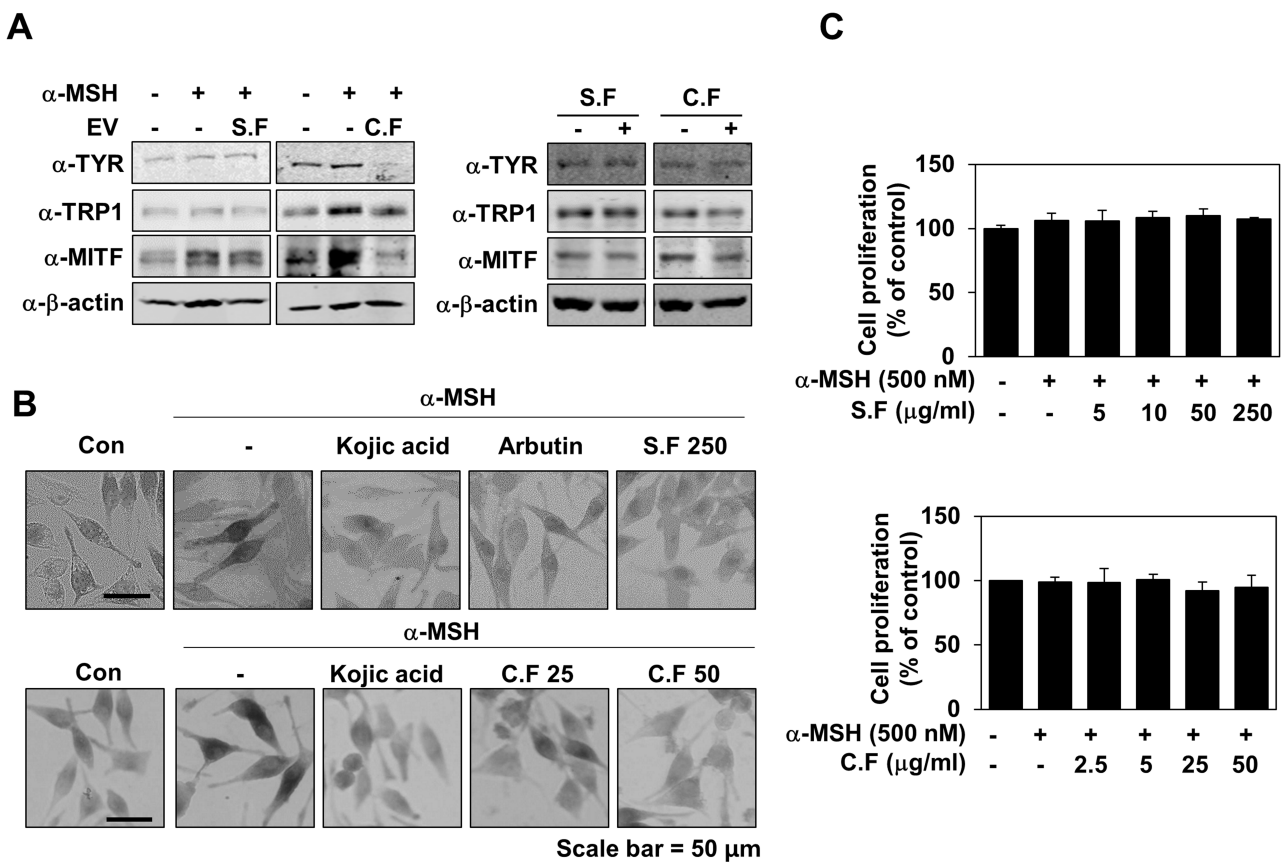

Fig. 3. EVs from *C. fragile* and *S. fusiforme* downregulate melanogenesis-related proteins. (A) MNT-1 cells were treated with the indicated EVs (250 μg/ml of *S. fusiforme*, 50 μg/ml of *C. fragile*) in the presence (left panel) or absence (right panel) of 500 nM of α-MSH for 48 h. Cell lysates were analyzed by immunoblotting using the indicated antibodies. (B) MNT-1 cells (5×10^4 cells/well; 12-well plates) were seeded on cover glasses and treated with the indicated EVs, kojic acid, or arbutin in the presence of α-MSH. After 48 h, the cells were fixed with 3.5% PFA and stained with L-DOPA (10 mM, 3 h). Activated intracellular tyrosinase was observed using a bright-field microscope. Con, control. (C) MNT-1 cells were incubated with various amounts of EVs from either *S. fusiforme* (left) or *C. fragile* (right). After 48 h, cell viability was determined by MTT assay. Percentage values were compared between treated and untreated (control). Data are expressed as mean ± SD for three independent experiments.

nogenesis through inactivating and downregulating melanogenic enzymes.

Extracellular vesicles from *Sargassum fusiforme* and *Codium fragile* reduce pigmented cells in an artificial skin model

To confirm the anti-melanogenic activity of the EVs, we used an artificial skin model that closely mimics human skin (Fig. 4). MelanoDerm tissues models were treated with 50 μl of PBS (control), 50 μg of *S. fusiforme*, 5 μg *of C. fragile* EVs, or 1% of kojic acid (positive control) every other day for 14 days (Fig. 4A). After 2 weeks, MelanoDerm tissues samples were fixed and embedded in paraffin. The skin sections were stained with H&E, and the number of pigmented granules was measured. In macroscopic images, reduced pigmentation was seen in samples treated with 5 μg of *C. fragile* EVs

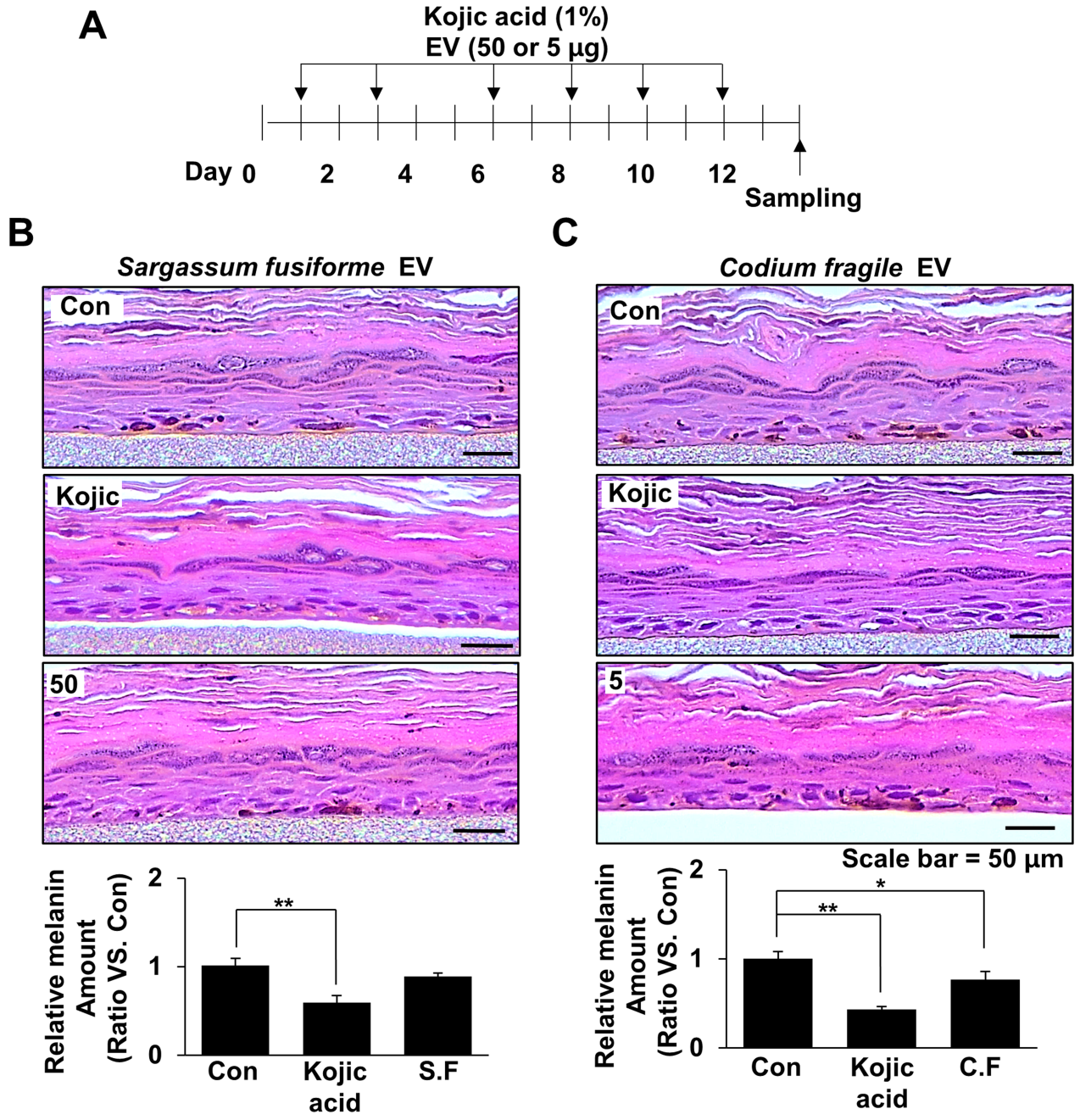

Fig. 4. *C. fragile* EVs inhibit melanin synthesis in an artificial skin model. (A) Seaweed EVs (*S. fusiforme*, 50 μg; *C. fragile*, 5 μg) were treated to MelanoDerm tissues every 2 or 3 days. (B and C) After 14 days, tissues were fixed with formaldehyde and embedded in paraffin. Tissue sections (B, *S. fusiforme*; C, *C. fragile*) were stained with H&E (top). Scale bar = 50 μm. The relative amounts of melanin in H&E-stained sections were analyzed with the ImageJ program (bottom). Con, control. $*P < 0.05$; $**P < 0.001$.

or 50 μg of *S. fusiforme* EVs, compared with PBS-treated samples (Figs. 4B and 4C). Taken together, these results demonstrate that EVs from *S. fusiforme* and *C. fragile* reduce pigmented cells in an artificial skin model.

Codium fragile extracellular vesicles decrease melanin synthesis in human skin

To further confirm the anti-melanogenic activity of *C. fragile* EVs and to clinically validate their anti-melanogenic effect, we produced a prototype cream containing *C. fragile* EVs (final concentration, 5 μg/ml) and performed a clinical study (Fig. 5). We first tested the potential for the prototype cream containing *C. fragile* EVs to irritate human skin. The cream was applied to a test site, which was then graded using the irritation-grading scale of the International Contact Dermatitis Research Group (ICDRG). As shown in Fig. 5A, the mean clinical irritation index did not differ between sites treated with the formulation containing *C. fragile* EVs and those treated with a comparable cream lacking the EVs (control). We then conducted a clinical study to test the effectiveness of skin whitening. The test products were each applied to half of the participant's face (on the right for placebo cream, on the left for test cream) once a day for 4 weeks and then the skin whitening was analyzed (Fig. 5B). Indeed, our results revealed that the prototype cream resulted in improvement of the L*-value (a color brightness value). We observed that sites treated with the prototype EV-containing cream exhibited 0.94% improvement in skin whitening after 2 weeks and 1.31% after 4 weeks (Fig. 5B). These results indicate that *C. fragile* EVs can decrease melanin synthesis in human skin.

The extracellular vesicles have anti-melanogenic effects of *Codium fragile*

To further investigate the contribution of the EV to the anti-melanogenic effect, a crude extract of *C. fragile* in PBS was ultra-centrifuged into a supernatant (Sup) and pellet that contained the EV, and then the EV pellets were resuspended in PBS with the same volume of crude extract used (EV, Fig. 6A). Isolated EVs showed enriched characteristic protein bands on SDS-PAGE gels (Fig. 6B). When comparing the melanin contents, most of the anti-melanogenic effect of *C. fragile* was contained in the EVs, but not in the supernatant, which lacks the EVs (Fig. 6C). Together, these data suggest that EVs from *C. fragile* exerted anti-melanogenic effects in

A

ICDRG score after removal (h)			Mean score
0.5	24	48	
0	0	0	0

B

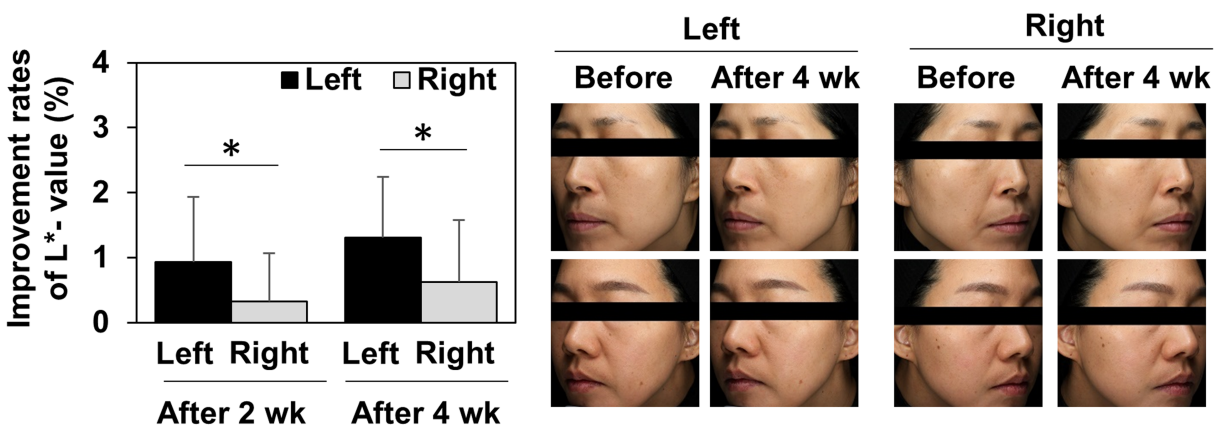

Fig. 5. *C. fragile* EVs decrease melanin synthesis in human skin. (A) Test products (C.F, a prototype cream containing *C. fragile* EVs, final concentration, 5 μg/ml; Control; a comparable cream lacking the EVs) were applied to the test site for 24 h and then each test site was graded for irritation 30 min, 24 h, and 48 h post-removal (age = 47.49 ± 6.91, n = 35, Ethnicity = Korean, Sex = male, female). ICDRG score 0, Negative (-); score 0.5, Doubtful or slight reaction and erythema (±); score 1, Erythema + Induration (+); score 2, Erythema + Induration + Vesicle (++); score 3, Erythema + Induration + Bullae (+++) (B) The skin whitening effect of *C. fragile* EV cream was evaluated using representative clinical images of participants, along with their L*-values (age = 46.29 ± 6.41, n = 21, Ethnicity = Korean, Sex = female; black, *C. fragile* EV cream, left; control cream, right). Improvement rates of L*-value (%), $(L_{2wks\ or\ 4wks} - L_{before})/L_{before} \times 100$ (left). Pictures of a participants prior to and after 4 weeks (right). Data are expressed as mean ± SD; $*P < 0.05$.

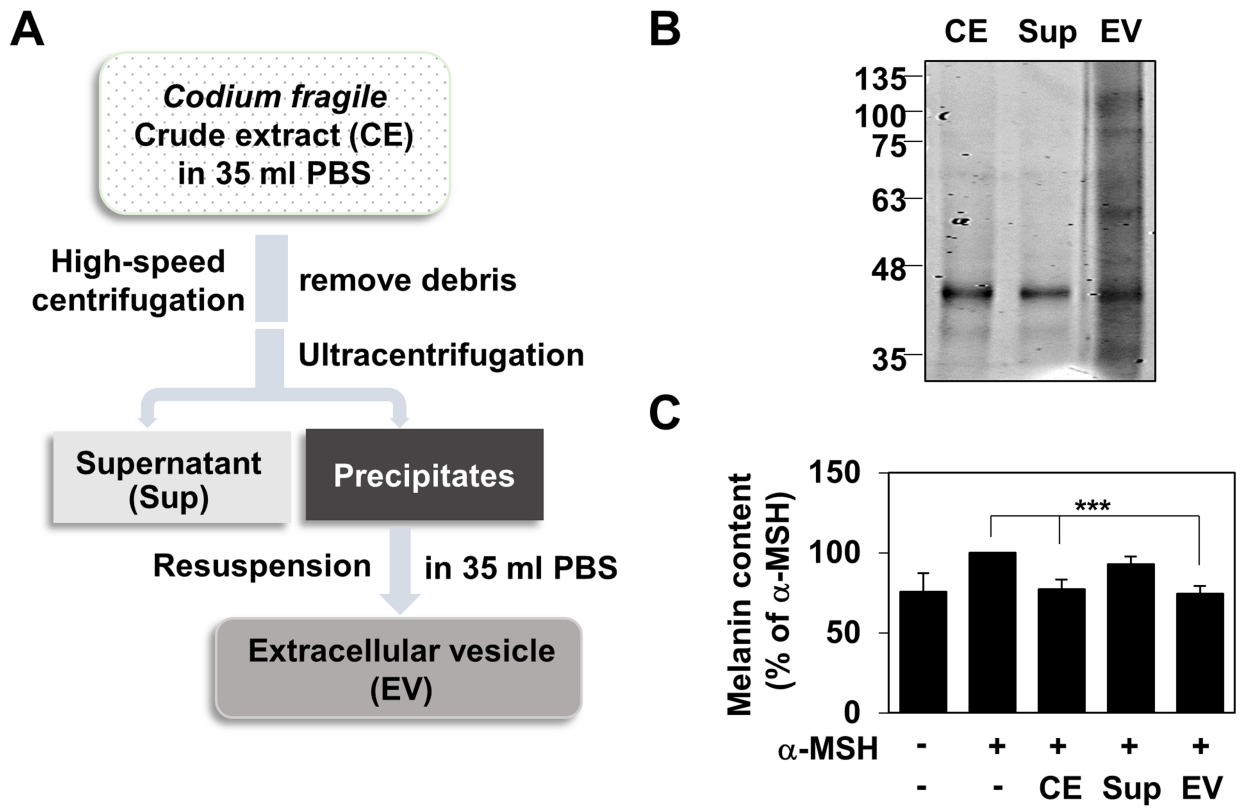

Fig. 6. The EVs have anti-melanogenic effects of *C. fragile*. (A) EVs were isolated from the crude extract of *C. fragile* in PBS as described in 'Materials and Methods' and isolated EVs were resuspended with the same volume of crude extracts used. (B) Crude extracts (CE), supernatant (Sup), and the EV were subjected to 10% SDS-PAGE and stained with Coomassie Brilliant Blue. (C) MNT-1 cells were treated with the 50 μl of CE, Sup, and EV in the presence of 500 nM of α-MSH for 48 h. The melanin contents were analyzed as described in Fig. 3. Data are expressed as mean ± SD for three independent experiments; ***$P < 0.0001$ versus DMSO-treated cells.

this system.

DISCUSSION

Many tyrosinase inhibitors have been developed as whitening agents, but some are limited in their usefulness due to low potency and the potential for side effects. For example, *L*-ascorbic acid and ellagic acid have low stability and solubility (Arulmozhi et al., 2013; Kahan et al., 2009); kojic acid induces contact dermatitis and cancer (Fujimoto et al., 1999); hydroquinone induces dermatitis, inflammation, dryness, and redness (Draelos, 2007); and arbutin is chemically unstable and releases hydroquinone, which may exhibit bone marrow toxicity (Zhou et al., 2009). There is therefore a great need to develop new anti-melanogenic substances with low side effects and high effectiveness. Since EVs contain various biomolecules that can be easily transferred to cells (Antimisiaris et al., 2018; Gomari et al., 2018; Naval and Chandra, 2019), they have been suggested to have potential therapeutic relevance for improving skin conditions. Indeed, EVs from dermal fibroblasts were reported to improve collagen production and repair sun-damaged skin cells (Hu et al., 2019), and stem cell EVs were shown to effectively regenerate skin (Oh et al., 2018). Here, we provide the first evidence indicating that EVs from the Korean seaweeds, *S. fusiforme* and *C. fragile*, have anti-melanogenic effects in skin. Our data revealed that seaweed EVs from *S. fusiforme* and *C. fragile* reduced the melanin synthesis of MNT-1 cells (Figs. 1 and 3B) and expression levels of MITF, tyrosinase and TRP-1, which are key regulators involved in melanin synthesis (Fig. 3A). Moreover, *C. fragile* EVs inhibited melanogenesis in the epidermal basal layer of a three-dimensional human skin model (Fig. 4), and the application of a prototype cream containing 5 μg/ml *C. fragile* EVs yielded an improvement in skin brightness (Fig. 5). These findings strongly suggest that *C. fragile* EVs have anti-melanogenic effects.

We do not know yet how *C. fragile* EVs regulates melanin synthesis. Since an earlier interesting study showed that miRNA in the keratinocyte-secreted exosomes regulated melanogenesis through the MITF signaling pathway (Kim et al., 2014) and our data revealed that *C. fragile* EVs inhibited the expression of MITF in MNT-1 cells, it is very likely that miRNA in the *C. fragile* EVs could be involved in this regulation, which has to be identified in the near future. However, this finding may have significant commercial implications for the cosmetic use of seaweed-containing products. Seaweed is known to have whitening components. For example, seaweed EVs from *E. cava*, *Sargassum siliquastrum*, and *E.*

stolonifera reportedly reduce melanin synthesis and tyrosinase activity (Cha et al., 2011; Kang et al., 2004; Yoon et al., 2009), and diverse seaweed polysaccharides (e.g., alginate, carrageenan, ulvan, and laminarin) have shown potential for various applications, including the design of drug delivery systems (Cunha and Grenha, 2016; Venkatesan et al., 2016). Most of the effective skin whitening compounds identified to date are limited by their low degree of skin penetration and must be mixed with other components to efficiently overcome this barrier. Our present results suggest that seaweed EVs could offer better whitening effects and *C. fragile* EVs could prove very valuable in the cosmeceutical industry.

In summary, we herein show for the first time that *C. fragile* EVs exert anti-melanogenic effects and exhibit commercially acceptable skin permeability. Although further studies will be required to fully elucidate the mechanisms underlying the *C. fragile* EV-mediated inhibition of melanin synthesis, our present findings could guide cosmeceutical companies in developing new skin-whitening agents for therapeutic use.

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Written informed consent was obtained from the participants for publication of this article and accompanying images.

AUTHOR CONTRIBUTIONS

H.C. (Heesung Chung), B.J., S.Y., and E.S.O. wrote the manuscript. H.C. (Heesung Chung), B.J., H.J, H.K.S., E.P., and K.J. performed the experiments. H.C. (Han Choe) and H.S.C. helped analyzed EV. S.Y. provided reagents. H.C. (Heesung Chung), B.J., S.Y., and E.S.O. designed the experiments and expertise and feedback.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

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