A novel regulatory mechanism underlying asymmetric division of human keratinocytes and the development of skin pigmentation

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Abstract

Background: Recently, the concept of skin beauty has diversified, and the natural beauty of skin has become more desirable. To develop brightening cosmetics, in addition to the conventional brightening function, the ability to protect against daily stress and maintain beautiful skin is important. We focused on the homeostatic function of the epidermis and studied vertical keratinocyte division, asymmetric cell division (ACD), which maintains the epidermal structure and function, to identify new brightening functions by mediating normal homeostasis in the skin.

Methods: The localization of ACD-regulating proteins in human skin was observed by immunostaining. The influence of skin pigmentation stimulants such as UV and skin inflammation on the expression of ACD-regulating proteins in human keratinocytes was confirmed by western blotting. The role of ACD-regulating proteins on melanin accumulation in the epidermis was confirmed in the construction of 3D skin equivalent to *NUMA1* siRNA knockdown keratinocytes. We searched for cosmetic ingredients that suppressed the decrease in *NUMA1* mRNA levels in UVB-irradiated keratinocytes using RT-PCR.

Results: ACD-regulating proteins in pigmented areas were weaker than in non-pigmented areas. The expression of ACD-regulating proteins, especially NuMA, was decreased by UVB irradiation, IL-1β, and MIF treatment as melanogenic stimulants. Downregulation of *NUMA1* in keratinocytes enhanced melanin accumulation in the skin equivalent. Agents that could suppress the UVB-induced decrease in *NUMA1* expression were identified.

Conclusion: Our results indicate that ACD-regulating proteins in keratinocytes suppress skin pigmentation by inhibiting melanin accumulation in the basal layer of the epidermis, suggesting a new mechanism underlying the development of skin pigmentation.

Keywords: asymmetric cell division; keratinocyte; skin pigmentation; NuMA.

Introduction

In recent years, as the concept of beautiful skin has become more diverse, it is expected that new approaches will be developed that can treat skin pigmentation that is suitable for each skin type.

The major factors that regulate skin pigmentation include epidermal melanocytes, which synthesize melanin, and adjacent keratinocytes, which receive melanin and distribute it to the upper layers of the skin [1]. Over 150 genes that directly or indirectly affect skin color have been identified [2]. Some of these genes, such as protease-activated receptor-2 (PAR2), endothelin-1 (ET-1), and stem cell factor (SCF), are increased by UV irradiation and induce melanogenesis in melanocytes [3-5], have been researched and developed as cosmetic products that suppress them. In addition, skin pigmentation develops into a chronic condition with age. The authors determined that it was necessary not only to suppress melanin synthesis, but to also protect the homeostatic function of the epidermis from the stimuli that cause skin pigmentation such as ultraviolet (UV) rays and skin inflammation. Thus, the homeostatic function of the epidermis was studied to develop a more effective treatment for chronic skin pigmentation, while maintaining the natural beauty of the skin. In most cases, approaches that promote keratinocyte proliferation and differentiation are selected. Specifically, because the structure and direction of keratinocyte proliferation in pigmented lesions differ from those in non-pigmented lesions, the authors focused on vertical keratinocyte division , asymmetric cell division (ACD) [6] , which plays an important role in maintaining epidermal functionality [7, 8]. In this study, a novel regulatory mechanism of the ACD protein for suppressing skin pigmentation was identified. Furthermore, we identified agents that can induce the expression of ACD-regulating proteins.

Materials and Methods

Sample preparation

ATP was purchased from Sigma-Aldrich (St Louis, MO, USA); recombinant human macrophage migration inhibitory factor (MIF) (rhMIF) from Shenandoah biotechnology (Warwick, PA, USA); recombinant human interleukin-1β (rhIL-1β) from R&D systems (Minneapolis, MN, USA) and Epigallocatechin gallate from FUJIFILM Wako Pure Chemical (Osaka, Japan).

Human skin samples

Full-thickness normal human abdominal skin (from an 18-year-old female) was obtained from CTI-biotech (Meyzieu, Lyon, France) under ethical considerations.

Keratinocytes culture and UVB irradiation

Normal human epidermal keratinocytes (Thermo Fisher Scientific, Waltham, MA, USA) were cultured in EpiLife™ Medium, with 60 μM calcium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with Humedia-KG supplement (KURABO, Osaka, Japan). Knockdown (KD) experiments were performed using Lipofectamine™ RNAiMAX Transfection Reagent (Thermo Fisher Scientific) and Silencer Select® Validated siRNA (AllStars Negative Control siRNA, FlexiTube siRNA; *NUMA1*_siRNA_9 (Qiagen, Hilden, Germany)), according to the manufacturer's instructions. UVB irradiation was performed using a narrowband UVB lamp (PHILIPS, Amstelplein, Amsterdam, Netherlands) in Hanks (-) buffer, and keratinocytes were incubated in fresh medium. To measure *NUMA1* and *RPS18S* mRNA levels, keratinocytes were cultured for 6 and 24 h, respectively.

Construction of NUMA1 KD skin equivalents and UVB irradiation

The skin equivalents were constructed using a layer-by-layer cell coating technique, which was developed by Akashi et al. [9, 10]. Normal human dermal fibroblasts (HDFs) coated with gelatin and fibronectin were prepared according to the method described by Akashi et al. [9, 10]. Cells were seeded onto 24 well trans-well inserts. After one day, the dermis was coated with type IV collagen. Normal human keratinocytes with or without *NUMA1* KD were seeded onto the surface of the dermis. Normal human melanocytes were seeded at 6 h after keratinocyte seeding. After one day of incubation, the constructs were lifted to the air-liquid interface. After 7 days of incubation, UVB irradiation was performed using a narrowband UVB lamp (PHILIPS, Amstelplein, Amsterdam, Netherlands) in Hanks (-) buffer. After 4 days, the equivalents were divided into the epidermis and dermis.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from melanocytes using TRIzol reagent (Thermo Fisher Scientific) in accordance with the manufacturer's protocol. cDNA was generated from the total RNA using the PrimeScript RT reagent Kit (Perfect Real Time) (Takara Bio, Shiga Japan) on a TaKaRa PCR Thermal Cycler Dice® (Takara Bio) in accordance with the manufacturer's protocol. Real-time PCR was performed using SYBR Premix Ex Taq II (Tli RNaseH Plus) (Takara Bio) on a LightCycler ® 480 System II (Roche Diagnostics,

Rotkreuz ZG, Switzerland). Primers for *NUMA1* and *RPS18S* (ribosomal protein S18) were purchased from Takara Bio. The $2^{-\Delta\Delta CT}$ method was used to analyze the gene expression levels of *NUMA1*, which were normalized to those of *RPS18S* and expressed as a ratio.

Western blot analysis

Whole cell and skin equivalent epidermis were lysed with Laemmli Sample Buffer, and their protein concentrations were determined using the BCA assay (Thermo Fisher Scientific). Equal amounts of homogenates were dissolved in Laemmli sample buffer, 1% 2-mercaptoethanol, and 0.04% bromophenol blue, and the samples were heated to 95°C for 5 min. The samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (DRC, Tokyo, Japan) and electroblotted onto polyvinyl difluoride membranes using a Trans-Blot ® Turbo Transfer System (Bio-Rad, Hercules, CA, USA). The membranes were blocked with StartingBlock™ blocking buffer (Thermo Fisher Scientific), probed with primary antibodies diluted with PBST (1:1,000) , reacted with secondary IgG antibodies diluted with PBST (1:10,000) coupled with horseradish peroxidase and detected using ECL Plus detection reagents (GE Healthcare Bio-Science, Tokyo, Japan). The following primary antibodies were used for immunoblotting: rabbit anti-human NuMA monoclonal antibody, rabbit anti-human PAR2 monoclonal antibody (Cell Signaling Technology, MA, USA), mouse anti-ET-1 monoclonal antibody (Abcam, Cambridge, England), rabbit anti-human INSC polyclonal antibody, rabbit anti-SCF polyclonal antibody (Proteintech, IL, USA), and mouse anti-human β-actin monoclonal antibody (Santa Cruz Biotechnology, CA, USA). The following secondary antibodies were purchased from Thermo Fisher Scientifics (MA, USA) : goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP. Target bands were analyzed using ImageJ software (NIH, Bethesda, MD, USA).

ELISA

After treatment, the concentration of MIF in the cell lysate was measured using commercial ELISA kits (MIF; R&D Systems (Minneapolis, MN, USA) according to the manufacturer's instructions.

Immunohistochemistry

Skin tissue specimens and skin equivalents were fixed in 4% paraformaldehyde (PFA) and embedded in paraffin. The tissues were cut into 5 μm sections, deparaffinized, and hydrated. Antigen retrieval was performed using a Decloaking Chamber NxGen (BIOCARE Medical, Pacheco, CA, USA) at 95°C for 20 min in pH 9.0 Tris/EDTA buffer.

After washing with tris buffered saline (TBS) (-), slides were blocked with Protein Block (Abcam), anti-cytokeratin 10 and anti-F-actin (Abcam), anti-cytokeratin 15, anti-INSC, anti-NuMA (Proteintech), Anti-GPSM2 (Aviva Systems Biology, CA, USA), anti-Loricrin (Biolegend, CA, USA), and corresponding Alexa antibodies.

Fontana-Masson silver stain

Paraffin-embedded tissues were cut into 5 µm sections, deparaffinized, hydrated, and prepared for routine Fontana-Masson silver staining. Briefly, the sections were transferred to Fontana silver solution and soaked for 18 h. After washing with distilled water, the sections were fixed with 5% sodium thiosulfate. After washing with tap water, the sections were counterstained with 0.1% nuclear fast red. After rinsing quickly with distilled water, the sections were dehydrated and mounted in medium (Permount).

Statistics

Variables are expressed as mean ± standard deviation (SD) values of the respective test or control group. All quantitative data were analyzed using the Ekuseru-Toukei 2012 software (Social Survey Research Information). Differences between the control and test groups were evaluated using one-way analysis of variance (ANOVA ; post hoc Dunnett's multiple comparison test). Statistical P values < 0.05 was considered significant.

Results

Structure of epidermis and keratinocyte proliferation is not uniform in pigmented skin lesions

To determine whether the direction of keratinocyte division is disturbed in pigmented skin lesions, we first compared the structure of the epidermis of pigmented and non-pigmented lesions of human skin. Fontana-Masson staining showed more melanin in the pigmented lesions. F-actin immunostaining confirmed that the actin staining structure was more disturbed in pigmented lesions than in non-pigmented lesions (Fig. 1).

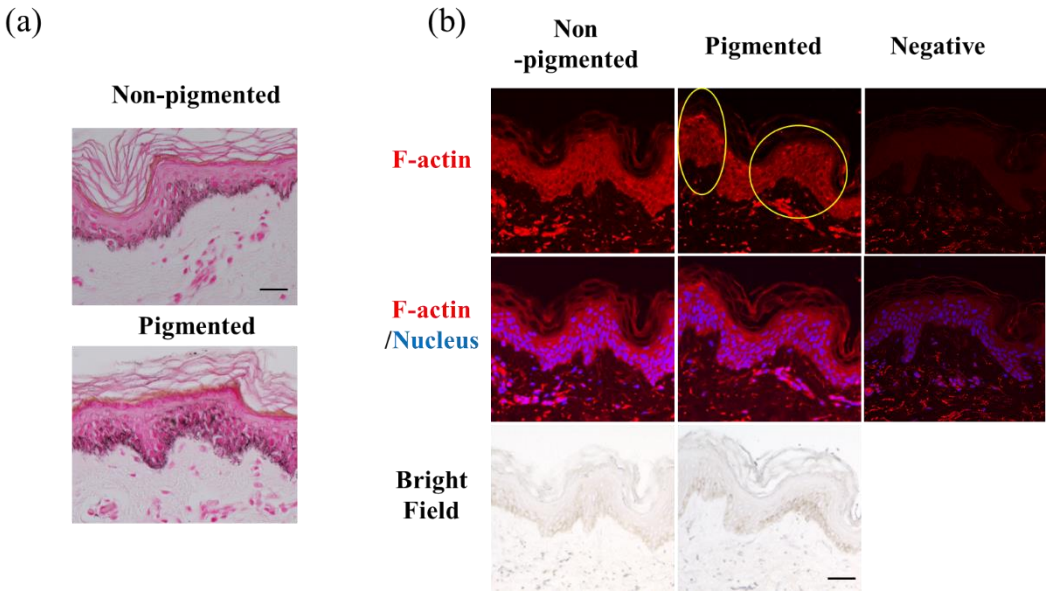

Fig. 1 Skin structure and the direction of keratinocyte division is disturbed in pigmented skin lesions.

(a) Melanin localization examined by Fontana-Masson silver stain, (b) F-actin localization examined by immunohistochemistry (IHC) in human skin. Bar = 50 μm.

Less localization of ACD-regulating proteins in skin lesions with melanin-deposits

The localization of ACD-regulating proteins in pigmented lesions of the human epidermis was confirmed by immunohistochemical staining. Skin tissues were immunostained for ACD-regulating proteins such as G-protein-signaling modulator 2 (GPSM2), protein inscuteable homologue (INSC), and nuclear mitotic apparatus protein (NuMA). GPSM2 was localized in the epidermis below the stratum spinosum and fluorescence was low in melanin-deposited lesions (Fig. 2a). INSC was mainly localized in the basal layer of the epidermis, and its expression was low in melanin-

deposited lesions, similar to GPSM2 (Fig. 2b). NuMA was localized throughout the epidermis and was lower in pigmented lesions than in nonpigmented lesions (Fig. 2c).

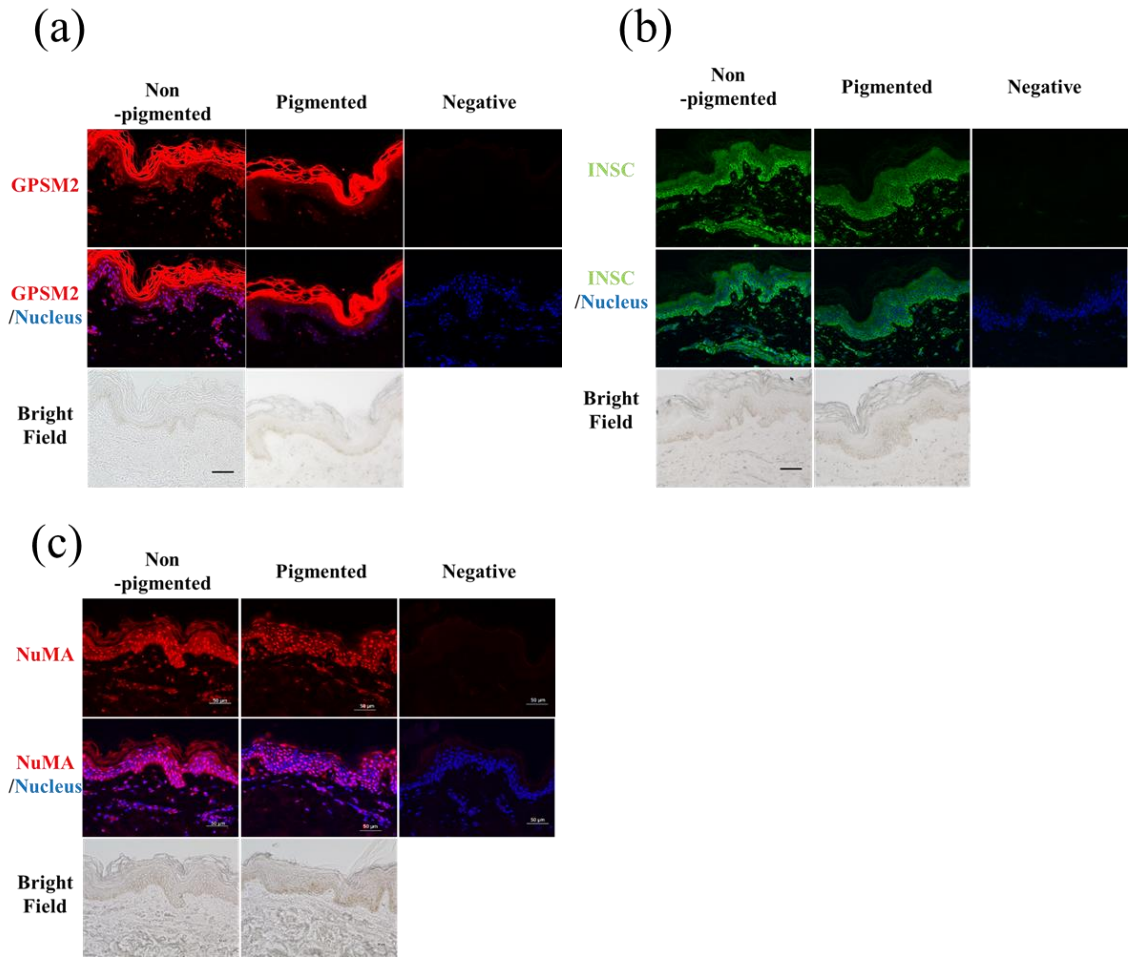

Fig. 2 Less localization of ACD-regulating proteins in pigmented skin lesions.

(a) GPSM2, (b) INSC, and (c) NuMA localization examined by immunohistochemistry (IHC) in human skin. Bar = 50 μm.

Skin pigmentation stimulants decreased ACD related protein expression.

To clarify whether ACD is related to skin pigmentation, we confirmed the effects of interleukin (IL)-1β, MIF [7] as an inflammatory mediator, and UVB rays as stimulants that cause skin pigmentation on the expression of GPSM2, INSC, and NuMA in human keratinocytes. IL-1β and MIF inhibited NuMA expression, while 15 mJ/cm^2 of UVB suppressed only NuMA expression (Fig. 3).

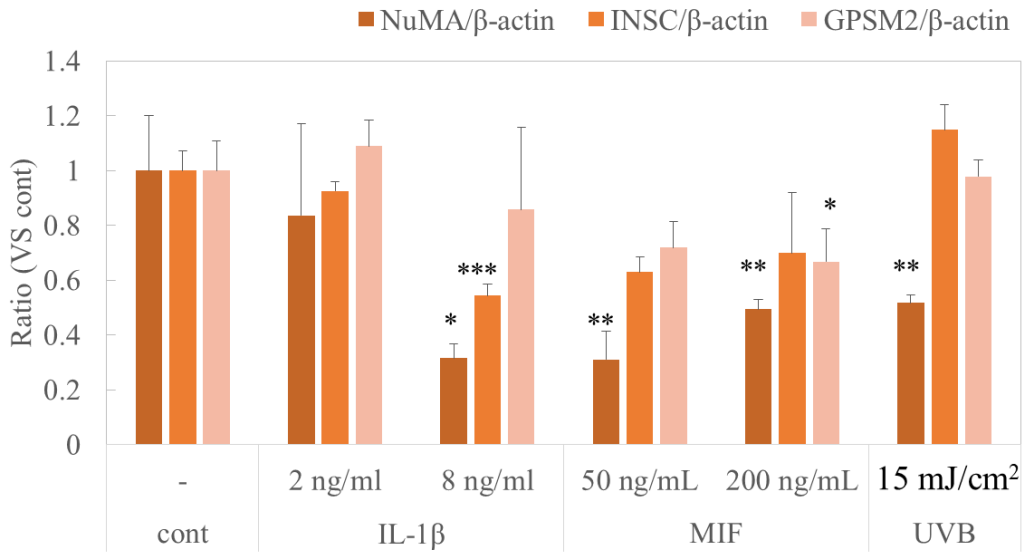

Fig. 3 Skin pigmentation stimulants decreased the expression of NuMA, INSC and GPSM2 in keratinocytes.

Keratinocyte were treated with IL-1β, MIF, and UVB irradiation.

Values reported are means ± standard deviation of n=3 replicates, Dunnet's test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$

Knockdown of NuMA in keratinocytes induced pigmentation in skin equivalents

From the results of the experiments that NuMA expression was most reduced by skin pigmentation stimulants, it was speculated that NuMA contributed most to skin pigmentation among the three proteins. We constructed 3D skin-equivalent models using *NUMA1* KD keratinocytes, normal melanocytes, and fibroblasts. The efficiency of *NUMA1* KD was suppressed to about 50% until 11 days after the air lift. *NUMA1* KD skin equivalents became darker compared to those prepared using control keratinocytes (Fig. 4a). Moreover, melanin accumulation and melanocyte activation in the basal and lower spinous layers of the epidermis were observed in *NUMA1* KD skin equivalents (Fig. 4 b). The results of differentiation marker staining showed that the localization of keratin 10 did not change, but the localization of loricrin was weak in the stratum granulosum in *NUMA1* KD skin equivalents (Fig. 5).

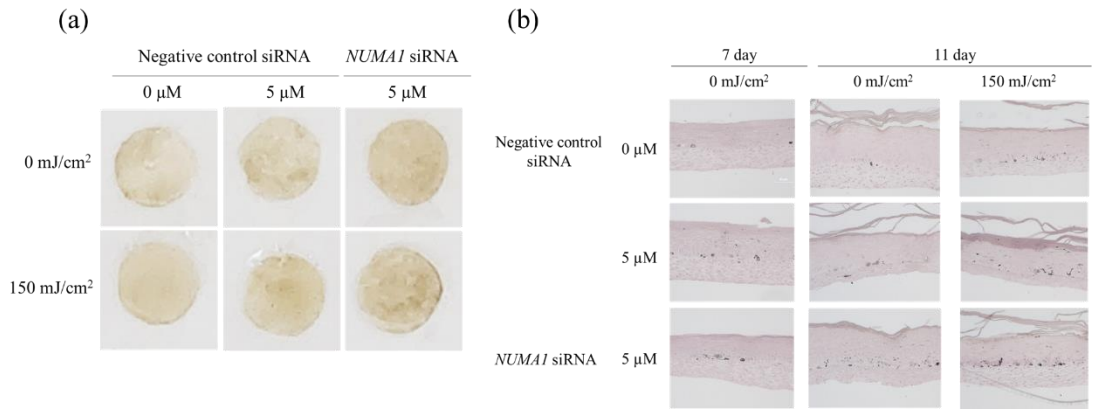

Fig. 4 *NUMA1* knockdown in keratinocytes induced pigmentation in skin equivalents.

(a) Stereomicroscopic images of skin equivalents, (b) melanin localization examined by Fontana-Masson silver stain in human skin. Bar = 50 μm.

The stem cell marker keratin 15 [13] was localized in the basal epidermis as the culture progressed in the negative control exposed skin equivalents. Keratin 15 in the *NUMA1* KD skin equivalent was observed throughout the stratum granulosum until 11 days after the airlift. (Fig. 5). Melanogenesis stimulants derived from keratinocytes, SCF, ET-1, and MIF, and PAR2 in the skin equivalents did not change in *NUMA1* KD. The amount of these proteins was not altered 3 days after UVB irradiation (data not shown).

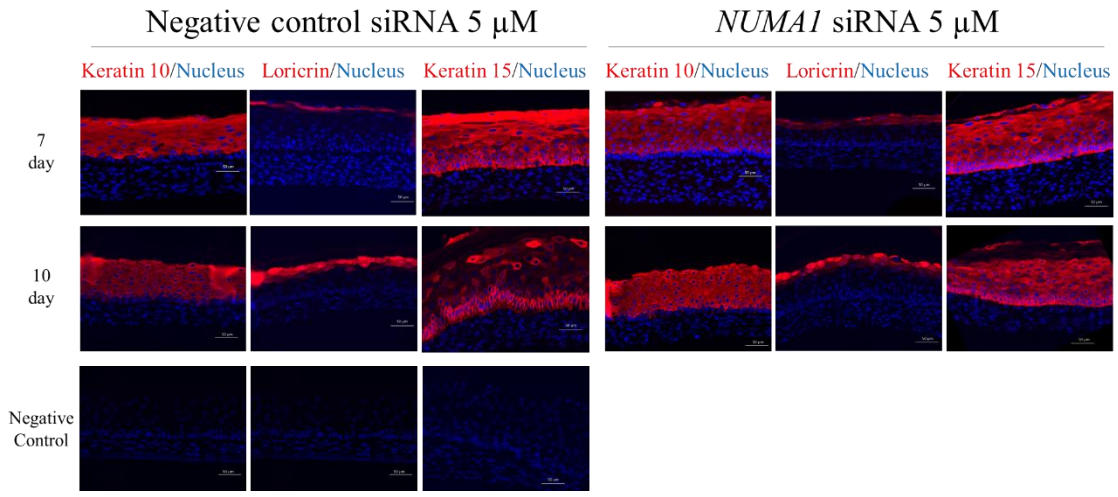

Fig. 5 *NUMA1* knockdown in keratinocytes downregulated skin maturation in skin equivalent.

Keratin 10, Loricrin, and Keratin 15 localization examined by immunohistochemistry (IHC) in human skin. Bar = 50 μm.

Evaluation of ACD inducers in keratinocytes

We searched for cosmetic ingredients that suppress the decrease of *NUMA1* expression in UVB-irradiated keratinocytes from natural compost. Epigallocatechin gallate and ATP suppressed UVB irradiation-induced reduction in *NUMA1* expression (Fig. 6).

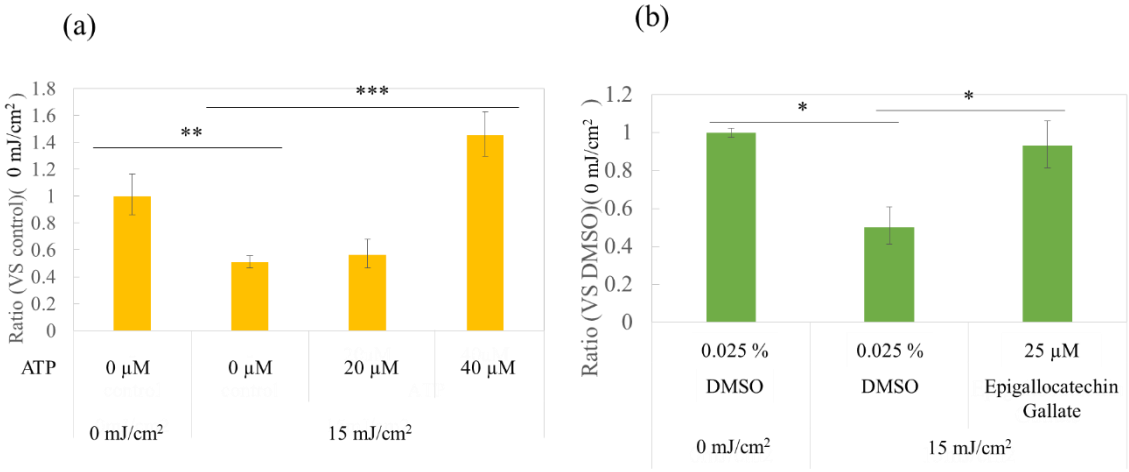

Fig. 6 Inhibitory effects of ATP and Epigallocatechin gallate on UVB-induced NUMA reduction in keratinocytes.

Keratinocyte were treated with (a) ATP and (b) Epigallocatechin gallate.

Values reported are means ± standard deviation of n=3 replicates, Dunnet's test, **P* < 0.05, ** *P* <0.01, ****P* < 0.001

Discussion

In the epidermis, keratinocytes divide horizontally and vertically at the base to maintain a layered structure. Vertical cell division: ACD requires the establishment of cortical cell polarity and mitotic spindle orientation along the cell polarity axis to maintain epidermal organization and barrier function. In this study, we focused on the fact that the skin structure is disturbed in skin pigmentation and hypothesized that correct skin structure through proper cell division could suppress the development of skin pigmentation. First, we compared the skin structures of pigmented and non-pigmented lesions to determine whether the proper direction of cell division affected skin pigmentation development. F-actin immunostaining confirmed that the actin staining structure was more disturbed in the pigmented lesion than in the non-pigmented lesion, and the disorder of skin structure and cell proliferation was speculated. Therefore, we focused on ACD, which regulates

cell division, in this study. The molecular mechanism that orients the mitotic spindle in ACD is regulated by multiple proteins, including NuMA, GPSM2, and INSC. These proteins are physically bound to the NuMA / GPSM2 / αi complex by the adapter protein INSC to form an essential complex for ACD [8]. Next, the localization of NuMA, GPSM2, and INSC was detected in pigmented lesions in the human epidermis by immunohistochemistry. GPSM2 is localized in the epidermis below the stratum spinosum. INSCs are mainly localized in the basal layer of the epidermis. NuMA is localized throughout the epidermis. These results indicate that the base of the epidermis is the location where ACD is active. Moreover, the expression of both genes was lower in pigmented lesions than in non-pigmented lesions. Based on these results, it was speculated that there was a relationship between the decrease in ACD and skin pigmentation. Furthermore, to clarify whether ACD is related to skin pigmentation, the effects of IL-1β, MIF as an inflammatory mediator, and UVB, as stimulants that cause skin pigmentation, were evaluated for the expression of GPSM2, INSC, and NuMA in human keratinocytes. IL-1β and MIF significantly inhibited their expression, whereas UVB only suppressed NuMA expression. Therefore, we hypothesized that a decrease in ACD-related proteins, especially NuMA, would induce skin pigmentation.

Next, we examined whether downregulation of NuMA expression leads to melanin accumulation in the epidermis using reconstituted 3D skin equivalents constructed with *NUMA1* KD keratinocytes, normal melanocytes, and fibroblasts. *NUMA1* KD skin equivalents were darker than those prepared using control keratinocytes. Moreover, melanin accumulation and melanocyte activation in the basal and lower spinous layers of the epidermis were observed in *NUMA1* KD skin equivalents. These results suggest that NuMA is involved in pigmentation regulation. The results of differentiation marker staining showed that the localization of loricrin was weak in the stratum granulosum in *NUMA1* KD skin equivalents. Additionally, the stem cell marker keratin 15 in the *NUMA1* KD skin equivalent was observed throughout the stratum granulosum until 11 days after airlift, unlike in the control group that exhibited a reduced keratin 15 during incubation. However, the levels of SCF, ET-1, MIF, and PAR2, that are involved in skin pigmentation, in the skin equivalents did not change in the *NUMA1* KD. Based on the results that show limited localization of loricrin, a late differentiation marker, and broad localization of keratin 15 in *NUMA1* KD skin equivalents, normal keratinization was inhibited under *NUMA1* KD. Therefore, NuMA is presumed to be involved in melanin clearance, rather than melanin synthesis. Furthermore, our results suggest that ACD-

related proteins in keratinocytes are potential new targets for suppressing the development of skin pigmentation.

Finally, to confirm the potential for developing cosmetics with this new lightening approach that mediates NuMA expression, we evaluated the cosmetic ingredients with an index of the level of suppression of the decrease in NuMA caused by UVB irradiation. We found that epigallocatechin gallate present in green tea (*Camellia sinensis*) extract and ATP present in cosmetic materials act as ACD inducers in keratinocytes. The inducers of ACD-related proteins are expected to suppress the development of skin pigmentation. In future research we will evaluate these skin lightening effects *in vivo* on various types of skin pigmentation, such as sun tanning, inflammatory pigmentation, and aged spots.

Conclusion

Our results indicate that ACD-related proteins in keratinocytes suppress skin pigmentation by inhibiting melanin accumulation in the basal layer of the epidermis, suggesting a new mechanism underlying the development of skin pigmentation. We intend to study this mechanism in greater detail to facilitate the development of new cosmetic products for the treatment of chronic skin pigmentation, by enhancing melanin clearance. It is expected that new brightening products will be developed in consideration of skin diversity, to suppress melanogenesis and normalize epidermal function.

Conflict of Interest Statement. NONE.

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