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

261

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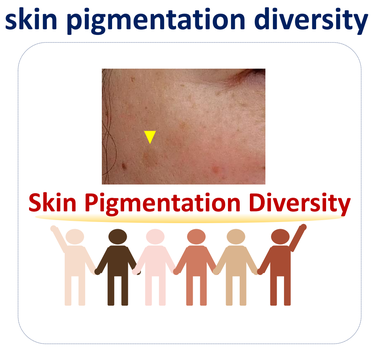
Introduction:

In recent years, 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.

Objective

To find the best approach for skin pigmentation treatments concerning skin pigmentation diversity



Focus on overall treatment

not only Conventional Brightening Function
- Melanogenesis↓
- Inflammation↓
- Melanin transfer↓
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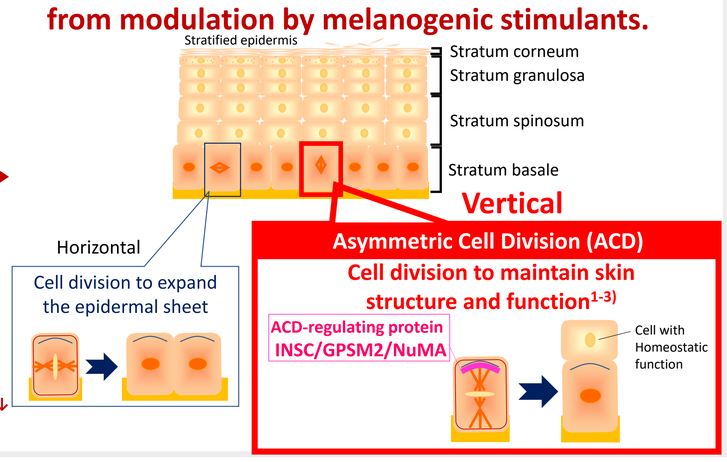
Hypothesis

The best approach is to protect ACD, the cell division that maintains epidermal homeostasis, from modulation by melanogenic stimulants.



Materials & 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).

Observation of skin structure and localization of ACD-regulating proteins in pigmented skin lesions

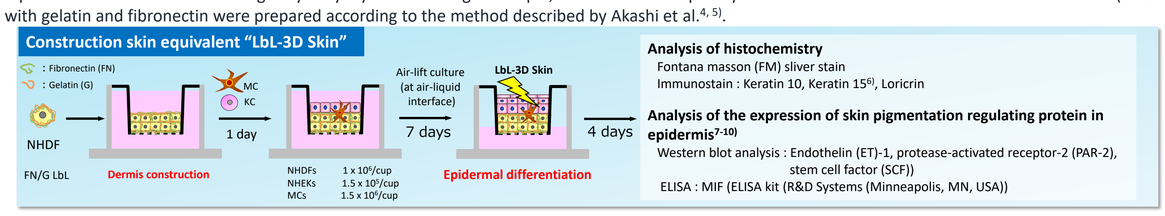
Full-thickness normal human abdominal skin (from an 18-year-old female) was purchased from CTI Biotech Visionary Science (Meyzieu, Lyon, France) under ethical considerations. The 4% paraformaldehyde (PFA) fixed tissue was stained with Fontana Masson stain and immunostained for F-actin and the ACD-regulating proteins, nuclear mitotic apparatus protein (NuMA), G-protein-signalling modulator 2 (GPSM2), and protein inscuteable homologue (INSC).

Analysis of the effects of skin pigmentation Stimulants on the expression of NuMA, GPSM2 and INSC in keratinocytes

Normal human epidermal keratinocytes (NHEK) (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). After two days, the cells were exposed to skin pigmentation stimulants (rhMIF[10)] ,rhIL-1β or UVB (15 mJ/cm^2)). In short, NHEKs were incubated in medium supplemented with rhMIF or rhIL-1β. UVB irradiation was performed using a narrowband UVB lamp (PHILIPS, Amstelplein, Amsterdam, Netherlands) in Hanks (-) buffer, and NHEKs were incubated in fresh medium. After 24 hrs of incubation, the expression of NuMA, GPSM2, and INSC were measured by western blot analysis.

Analysis of the effects of ACD-regulating protein on pigmentation in skin equivalents

NUMA1 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. The skin equivalents were constructed using a layer-by-layer cell coating technique, which was developed by Akashi et al.[4, 5)]. Normal human dermal fibroblasts (NHDFs) coated with gelatin and fibronectin were prepared according to the method described by Akashi et al.[4, 5)].



Evaluation of ACD inducers in keratinocytes

Human keratinocytes were pre-treated with cosmetic ingredient-related substances and irradiated with ultraviolet B. Subsequently, the irradiated cells were incubated with fresh samples. After 24 hrs of incubation, ACD-regulating mRNA and protein expression was assessed using reverse transcription-polymerase chain reaction and western blotting, respectively.

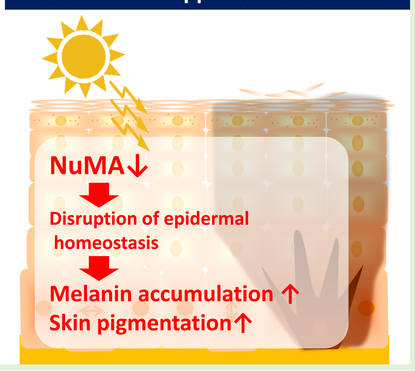
Statistics

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.

Conclusions:

One of the ACD-regulating proteins, "NuMA" in keratinocytes suppresses skin pigmentation by inhibiting melanin accumulation in the basal layer of the epidermis suggesting a new mechanism underlying the development of skin pigmentation.
ATP and Epigallocatechin gallate inhibits on UVB-induced NUMA reduction in keratinocytes.
Our findings provide new insights into how to develop new brightening products consideration of skin diversity, to suppress melanogenesis and normalize epidermal function.

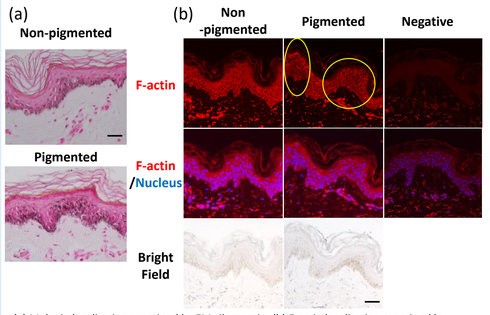
Mechanism of skin pigmentation via UV-induced suppression of NuMA



Results & Discussion:

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

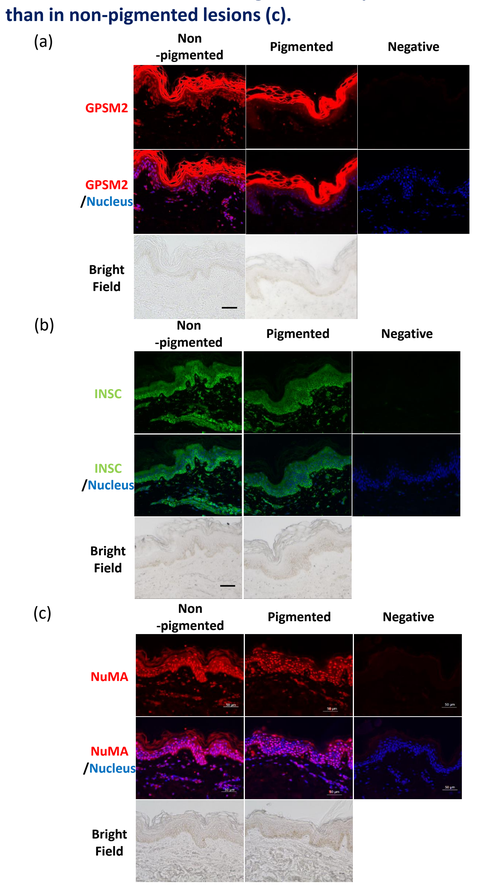
FM staining showed more melanin in the pigmented lesions. F-actin immunostaining confirmed a more disturbed actin staining structure in pigmented than in non-pigmented lesions .



(a) Melanin localization examined by FM silver stain, (b) F-actin localization examined by immunohistochemistry (IHC) in human skin. Bar=50 μm. Yellow circled area : melanin-deposit area.

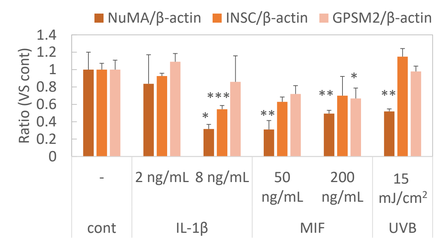
2. Less localization of ACD-regulating proteins in skin lesions with melanin-deposits.

GPSM2 was localized in the epidermis below the stratum spinosum and low fluorescence in melanin-deposited lesions (a). INSC was mainly localized in the basal layer of the epidermis, and showed low expression in melanin-deposited lesions (b). NuMA was localized throughout the epidermis and was lower in pigmented than in non-pigmented lesions (c).



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

IL-1β and MIF inhibited NuMA expression, while 15 mJ/cm^2 of UVB suppressed only NuMA expression. It was speculated that NuMA contributed most to skin pigmentation among the three proteins.

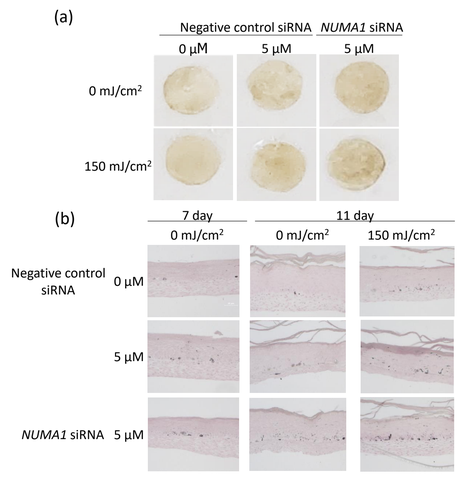


Keratinocyte were treated with IL-1β, MIF and UVB irradiation. Values reported are means ± SD of n=3 replicates, Dunnet's test, *p<0.05, **p<0.01, ***p<0.001

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

4. NuMA KD in keratinocytes induced pigmentation in skin equivalents.

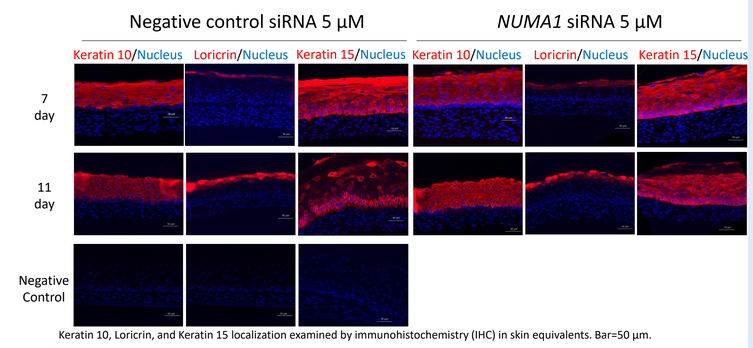
We constructed 3D skin-equivalent models using *NUMA1* KD keratinocytes. The efficiency of *NUMA1* KD was suppressed to about 50% until 11 days after the air lift. *NUMA1* KD skin-equivalents became darker compared to those prepared using control keratinocytes (a). Moreover, melanin accumulation and melanocyte activation in the basal and lower spinous layers of the epidermis were observed in *NUMA1* KD skin equivalents (b).



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

5. NuMA KD in keratinocytes downregulated skin maturation in skin equivalent.

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. The stem cell marker keratin 15[6)] 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.



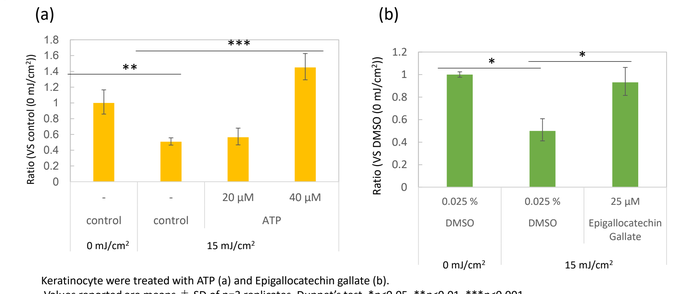
Keratin 10, Loricrin, and Keratin 15 localization examined by immunohistochemistry (IHC) in skin equivalents. Bar=50 μm.

6. NuMA KD in keratinocytes did not alter melanogenesis stimulants expression.

Melanogenesis stimulants derived from keratinocytes, SCF, ET-1, and MIF, and PAR-2 in the skin equivalents did not change in *NUMA1* KD. The amount of these proteins had not changed three days after UVB irradiation (data not shown). These results suggest that NuMA is presumed to be involved in melanin clearance rather than melanin synthesis.

7. Inhibitory effects of ATP and Epigallocatechin gallate on UVB-induced NuMA reduction 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.



Keratinocyte were treated with ATP (a) and Epigallocatechin gallate (b).
Values reported are means ± SD of n=3 replicates, Dunnet's test, *p<0.05, **p<0.01, ***p<0.001

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