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Full paper

Protective and therapeutic effects of fucoxanthin against sunburn caused by UV irradiation

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A B S T R A C T

Mild exposure to ultraviolet (UV) radiation is also harmful and hazardous to the skin and often causes a photosensitivity disorder accompanied by sunburn. To understand the action of UV on the skin we performed a microarray analysis to isolate UV-sensitive genes. We show here that UV irradiation promoted sunburn and downregulated filaggrin (Flg); fucoxanthin (FX) exerted a protective effect. *In vitro* analysis showed that UV irradiation of human dermal fibroblasts caused production of intracellular reactive oxygen species (ROS) without cellular toxicity. ROS production was diminished by *N*-acetylcysteine (NAC) or FX, but not by retinoic acid (RA). *In vivo* analysis showed that UV irradiation caused sunburn and Flg downregulation, and that FX, but not NAC, RA or clobetasol, exerted a protective effect. FX stimulated Flg promoter activity in a concentration-dependent manner. Flg promoter deletion and chromatin immunoprecipitation analysis showed that caudal type homeo box transcription factor 1 (Cdx1) was a key factor for Flg induction. Cdx1 was also downregulated in UV-exposed skin. Therefore, our data suggested that the protective effects of FX against UV-induced sunburn might be exerted by promotion of skin barrier formation through induction of Flg, unrelated to quenching of ROS or an RA-like action.

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1. Introduction

Human skin is a potential anatomical barrier against pathogens and damage, and acts as an important boundary between the internal and external environment in defense of the body. UV irradiation from sunlight induces photo-damage of the skin, resulting in wrinkles, laxity, coarseness, mottled pigmentation (1), and histological changes that include increased epidermal thickness and alterations to connective tissue (2). Continuous exposure to UV irradiation leads to skin cancer and other photoaging complications. Typically, these have been thought to be mediated by reactive oxygen species (ROS) generated in the oxidative pathways. Several anti-oxidative and anti-photoaging compounds have been identified previously (3), with just a few such as vitamin E, vitamin C and its derivatives achieving market penetration. In many cases, the main symptom of the initial stage manifests as sunburn.

Filaggrin (Flg) has a pivotal role in the terminal differentiation of skin and formation of the cornified envelope (4). Closer to the skin surface, the cells of the granular layer contain dense cytoplasmic granules primarily composed of profilaggrin, with other protein

Abbreviations: UV, ultraviolet; FX, fucoxanthin; Flg, filaggrin; Lor, loricrin; ROS, reactive oxygen species; NAC, *N*-acetylcysteine; RA, retinoic acid; Cdx1, caudal type homeo box transcription factor 1.

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components required for the formation of squames, the flattened, dead cells of the outermost stratum corneum that are responsible for the barrier function of the skin (4). Profilaggrin is expressed late in the epidermal differentiation program in the granular cell layers of stratified, cornified epithelia (4). Keratohyalin granules act as a reservoir of inactive profilaggrin, along with other proteins that are important for squame formation and maturation, such as loricrin (Lor) (5). Human LOR comprises 80% of the total protein of the cornified envelope. The end products of Flg processing are found within the residual cytoplasm of these squames (6). The multifunctionality of Flg is clearly illustrated by examining flaky tail mice (which have a loss-of-expression mutation in Flg). In flaky tail mice, loss of profilaggrin and all downstream events leads to aberrant biogenesis of the stratum corneum (ichthyosis) as well as abnormally dry skin (xerosis) (7). Flg deficiency has been experimentally shown to lead to failure of the barrier function of the skin. The epidermis of Flg-deficient mice allows passive transfer of protein allergens (8), while Lor-deficient mice showed a phenotype of mild skin damage (5).

Fucoxanthin (FX) is one of the carotenoids accumulated by marine plants and was thought to possess bioactive characteristics such as anti-oxidant and provitamin A effects (3, 9). Recent work showed the protective effect of FX against UV-B-induced skin photoaging (10). The antioxidant and angiogenic activity of FX might be involved. In addition, previous reports showed that FX exerted multiple actions such as collagenase inhibition (10), radical scavenging (11), an anti-obesity effect (12), and protective activities in hypertension, hyperglycemia (13) and the metabolic syndrome (14). However, the mechanism of action of FX on skin is not completely understood.

In this report, we show that the sunburn and Flg down-regulation by UV were restored by FX, but not by *N*-acetylcysteine or retinoic acid. Clobetasol and β-carotene failed similarly. Flg promoter deletion and chromatin immunoprecipitation analysis revealed that Flg gene promoter activation via recruitment of transcription factor Cdx1 by FX promoted Flg gene expression. Therefore, we hypothesize here that 1) Flg is a sensitive molecular indicator of UV damage in skin, and 2) FX may have therapeutic potential in skin disorders through activation of the Cdx1-Flg axis.

2. Materials and methods

2.1. Materials, cell culture and animals

A non-edible portion (mikine part) that becomes the industrial waste discharged in the farming of seaweed (*Undaria pinnatifida*), was supplied from Naruto City (Tokushima, Japan), fucoxanthin was purified from them in our laboratory (Refer to supplementary information). Human dermal fibroblasts (HDF) were purchased from Takara Bio Co. Ltd. (Shiga, Japan), A431 cells were purchased from RIKEN Cell Bank (Tsukuba Science City, Japan), and cultured in Dulbecco's Modified Eagle Medium (DMEM) (WAKO Pure Chemicals, Osaka, Japan) containing 10% fetal bovine serum (FBS). Unless otherwise indicated, all other reagents were obtained from Wako Pure Chemicals or Nacalai Tesque (Kyoto, Japan).

Primary skin fibroblastic cells were prepared from E15.5 embryo skin. Briefly, skin samples were digested in 0.1% collagenase/0.1% trypsin solution, and cells were then purified using a cell strainer (70 μm mesh). Cells were plated at cell densities adequate for the respective assays. All animals were obtained from a commercial vendor (SLC Japan, Shizuoka, Japan) and housed in a semibarrier animal room (Light/dark cycle 12:12, free-feeding). The protocol used meets the guideline of the Japanese Society for Pharmacology and was approved by the Committee for Ethical Use of Experimental Animals at Ritsumeikan University (BKC2013-017).

2.2. Intracellular ROS monitoring and cytotoxicity assay

HDF were seeded at a density of 3×10^4 cells/well and were grown to confluence in a 96-well plate (Thermo Fischer Scientific, Inc., Yokohama, Japan). The medium was replaced by serum free-DMEM (SF-DMEM), and cells were then incubated with 100 μM of 2′,7′-Dichlorodihydro fluorescein diacetate (DCFH-DA) (Wako Pure Chemicals) before stimulation for 1 h. Cells were washed five times with SF-DMEM and stimulated by 1 mM hydrogen peroxide exposure or ultraviolet radiation (365 nm: UV-A, 2.7 J/cm^2). Cells were then lysed using 50 μl of passive lysis buffer (Promega, Madison, WI). Fluorescence intensity was measured using a fluorescence microplate reader (Corona Electric model:SH-8100lab, Ibaraki, Japan) at wavelengths of Ex:480 nm/Em:530 nm. Condition settings were determined using a JuLi cell analyzer (AR Brown, Tokyo, Japan) (Refer to Supplementary information).

Cell viabilities were measured using a colorimetric Cell Count Reagent SF kit (Nacalai Tesque) according to the manufacturer's instructions. After cells were incubated with WST-8 for two hours, cell viabilities were measured on a microplate reader at 450 nm (model 680, Bio-Rad, Tokyo, Japan).

2.3. UV exposure and histological analysis

First, hair was removed from the backs of female ddY strain mice (11–15 weeks old, female, n = 6) using hair remover containing thioglycolic acid. In protective experiments, for four days prior to UV exposure (365 nm: UV-A), Vaseline control was applied to the left of the spinal column; Vaseline containing experimental compounds was applied on the right. To prevent uneven coating of the compound to be applied, we used vaseline as control study. In addition, vaseline did not influence skin condition including mRNA expression levels in our experimental condition. UV exposure was performed at an intensity of 2.7 J/cm^2 for 1 h. In Japan, sunlight intensity during one hour of daylight is approximately 7.2 J/cm^2 (15). For therapeutic experiments, FX treatment was started on day 5, after UV exposure for four days. Two hours after UV exposure, UV-induced sunburn was monitored using a Dolphin skin scope (COREFRONT, Tokyo, Japan).

Murine dorsal skin was harvested after marking the dorsal midline. Skin was first placed flat in a cold Tissue-Tek® OCT compound, followed by placement in a small mold superficial side up at −20 °C, with subsequent storage at −80 °C until sectioned. Frozen sections were allowed to react with respective antibodies and analyzed as described previously (16). Sections (20 μm thick) were subjected to immunohistochemistry using anti-filaggrin (M-290, 1:500) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-loricrin (AF-62, 1:500) (Covance, Berkeley, CA) antibodies in Blocking One Histo (Nacalai Tesque). Sections were exposed to with a secondary antibody, mouse anti-rabbit IgG-FITC (sc-2359, 1:100) (Santa Cruz Biotechnology). Fluorescence images were analyzed using the EVOS® FL cell imaging system as described previously (17).

2.4. Microarray

Samples were prepared from dorsal skin treated with or without UV for four days. Total RNA was extracted using Sepazol (Nacalai Tesque). We prepared samples from the skin with or without exposure to UV (0, 1, 2, 3, 4 days). After checking the quality of extracted RNAs, cRNA were synthesized. An Agilent microarray plate was scanned and analyzed (Bio Matrix Research Center, Chiba, Japan). Obtained intensities of each spot signal were determined using Excel software and compared. Several candidates were analyzed by qPCR screening (data not shown).

2.5. Quantitative real-time PCR

One μg of total RNA was reverse-transcribed by ReverTra Ace® cDNA synthesis kit (TOYOBO, Osaka, Japan). Quantitative Real-time PCR (qPCR) was performed as described previously (17). Briefly, for quantitative real-time PCR, 5 μl of 2× KAPA® master mix (Nippon Genetics Co. Ltd., Tokyo, Japan) was mixed with cDNA, 0.5 μM primer pair. Quadruplicate of cDNA's were amplified on Piko Real PCR system (Thermo Fisher Scientific Inc., Yokohama, Japan). The experiments were performed at five different cDNA pool dilutions. PCR products were normalized against housekeeping genes (mouse GAPDH or human β-actin), and measurements between samples were compared by cycling threshold (Ct). Primer sequences used are as follows: mGAPDH-F 5′-TGCACCACCAACTGCTTAG-3′, mGAPDH-R 5′-GGATGCAGGGATGATGTTC-3′, hActin-F 5′-AGCCTCGCCTTTGCCGATCC-3′, hActin-R 5′-TTGCACATGCCGGAGCCGTT-3′, mFLG-F 5′-GCGAGCTCTTACAAGCAGCATGAGGTAG-3′, mFLG-R 5′-GCCTCGAGAAGACTGAATCGAATGCAGC-3′, mCdx1-F 5′-CTAGGACAAGTAGCTT GCCCTCTT-3′, mCdx1-R 5′-TCCAACAGGCTCACCACACA-3′, Cdx2-F 5′-CGATACATCACCATCAGGAGG-3′, Cdx2-R 5′-TGGCTCTGCGGTT CTGAAAC-3′, Cdx4-F 5′-GAGGAAGTCAGAGCTGGCAG-3′, Cdx-4-R 5′-GGCTCTGCGATTCTGAAACC-3′, hFLG-F 5′-TGAAGCCTATGACACCACTGA-3′, hFLG-R 5′-TCCCCTACGCTTTCTTGTCCT-3′. The non-regulated housekeeping gene served as an internal control and was used to normalize for differences in input RNA. All measurements were performed in quadruplicate.

2.6. Immunoblot

Immunoblot analyses were performed as previously described (17). Proteins were resolved by SDS-10% polyacrylamide gel electrophoresis. The blots were first incubated with rabbit anti-filaggrin (1:1000), rabbit anti-actin (I-19, 1:1000) (Santa Cruz Biotechnology), anti-loricrin (1:1000), rabbit anti-Cdx1 (ab116111; 1:1000, Abcam, Tokyo, Japan) and then exposed to horseradish peroxidase-conjugated anti-rabbit or anti-goat IgG (Santa Cruz Biotechnology, Inc.). Chemiluminescence signals were obtained from reaction with Chemi Lumi One Plus Reagent (Nacalai Tesque), and monitored using an LAS2000 system (FUJI film, Tokyo, Japan). The relative intensities of the bands were measured using NIH Image software.

2.7. Plasmids and reporter assay

Mouse Flg promoter was cloned by PCR using Flg promoter-F 5′-gcCTCGAGTTACAAGCAGCATGAGGTAG-3′, Flg promoter-R 5′-gcAGATCTAAGACTGAATCGAATGCAGC-3′ pGL4.10 at XhoI-BglII site (Flg2000-luc). Similarly, FLG1200-luc and FLG500-luc were prepared by PCR-based cloning using Flg1200-F 5′-

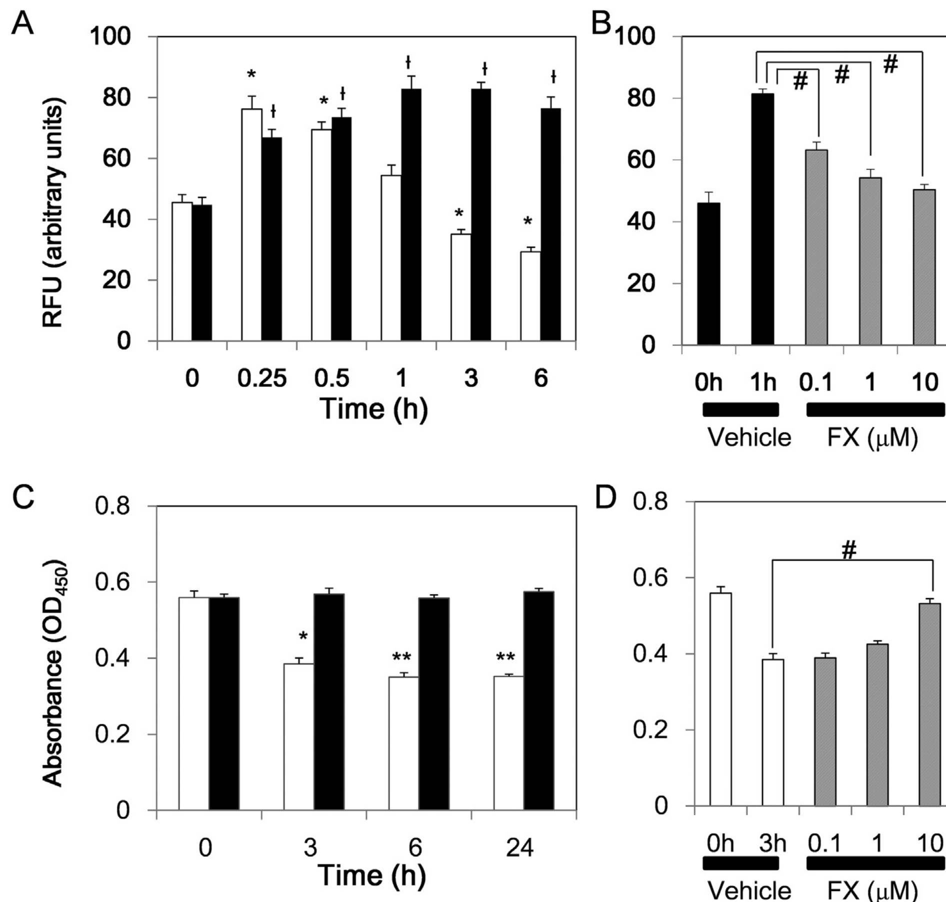

Fig. 1. Intracellular ROS quenching assays in HDF cells. (A), Intracellular ROS levels with H_2O_2 and UV irradiation in HDF cells. Cells were exposed to H_2O_2 (open column) or UV irradiation (closed column) for indicated times after incubation with 100 μM DCFH-DA for 1 h. Relative fluorescence units (RFU) are shown as arbitrary units. *P < 0.05, ƚP<0.05, vs control. n = 16. Similar results were obtained from quadruplicate experiments. (B), Effects of fucoxanthin (FX) on H_2O_2-induced ROS production. Cells were treated with indicated concentration of FX (zebra column). 30 min later, cells were exposed to UV irradiation (closed column). Representative data were shown. (C), Effects of H_2O_2 or UV irradiation on cell viability. Cells cultured in SF-DMEM were exposed to H_2O_2 (open column) or UV irradiation (closed column) for indicated times. Cell viabilities were measured as described in "Materials and Methods". *P < 0.05, **P < 0.001, vs control. n = 8. Similar results were obtained from quadruplicate experiments. (D), Effect of FX on H_2O_2-induced cell toxicity. #P.

gcCTCGAGTATGATGCATGCATAAATGT-3′ and Flg500-F 5′-gcCTCGAGATCAAAATTAATGTGACCAC-3′ with Flg promoter-R primer, respectively. Flg150-luc was prepared by BglII-PstI blunt-in self ligation. Reporter assays were performed by transient transfection of 0.2 μg of luciferase construct, and 0.002 μg of pRL-CMV (Promega) using a Dual Luciferase Reporter Assay System (Promega) as previously described (17). Luciferase activity was measured using a model TD20/20n luminometer (Turner BioSystems, Sunnyvale, CA) and normalized to Renilla luciferase activity under the control of CMV promoter.

2.8. Choromatin immunoprecipitation (ChIP) assay

ChIP assays were performed as described previously (17). Flag-*Cdx1* expression vector was transfected into embryonic skin fibroblasts by electroporation (Bio-Rad, Gene Pulser Xcell™, 250 V, 650 μF). Forty-eight hours later, cells were harvested by fixation. DNA fragments immunoprecipitated with anti-Flag M2 (Sigma Chemicals, Saint Louis, MI), Dynabeads® Sheep anti-Mouse IgG (Invitrogen, Tokyo, Japan) were monitored by qPCR, using Flg-Chip F 5′-TAGAAATAGGAGCCTGGATG-3′, and Flg-Chip R 5′-ATATTTTAATGCCATATATA-3′, as described above.

2.9. Statistical analysis

Data are expressed as mean ± S.E.M. Significance was tested using Student's t-test or, where multiple comparisons were required, One-way analysis of variance (ANOVA). A P-value of less than 0.05 was considered to be significant.

3. Results

3.1. Anti-oxidative activity

Previous reports showed that the skin damage caused by UV radiation may result from injury by ROS (9). To identify ROS-quenching compounds from *seaweed*, we first tested the antioxidant activity of all extracts using ORAC assays (Suppl.1A). As the extract from the hexane fraction was primarily active, further separation procedures were performed by silica gel chromatography. In the nine fractions obtained, fr.7 elicited the most powerful antioxidant activity on ORAC assay (data not shown), and TLC analysis showed that fr.7 contained a band similar to that seen with fucoxanthin (FX) (Suppl.1B). Further purification by silica gel chromatography isolated >95% FX from fr.7 (Suppl.1C, Suppl.2 and Suppl.3). *N*-acetylcysteine (NAC) also quenched radicals in a concentration-dependent manner (Suppl.4A). Such a quenching action was not observed on addition of retinoic acid (RA). In attempts to evaluate by further evaluation using a bioassay, we examined the effect of H_2O_2 and UV exposure (Fig. 1). The addition of 1 mM H_2O_2 to HDF cells caused a transient increase in intracellular ROS maximal at 15 min, and fluorescent activity weakened gradually within 60 min. With these settings, we assessed ROS production using DCFH-DA (Fig. 1A). Intracellular ROS production increased transiently with H_2O_2. To elucidate cytotoxicity of H_2O_2

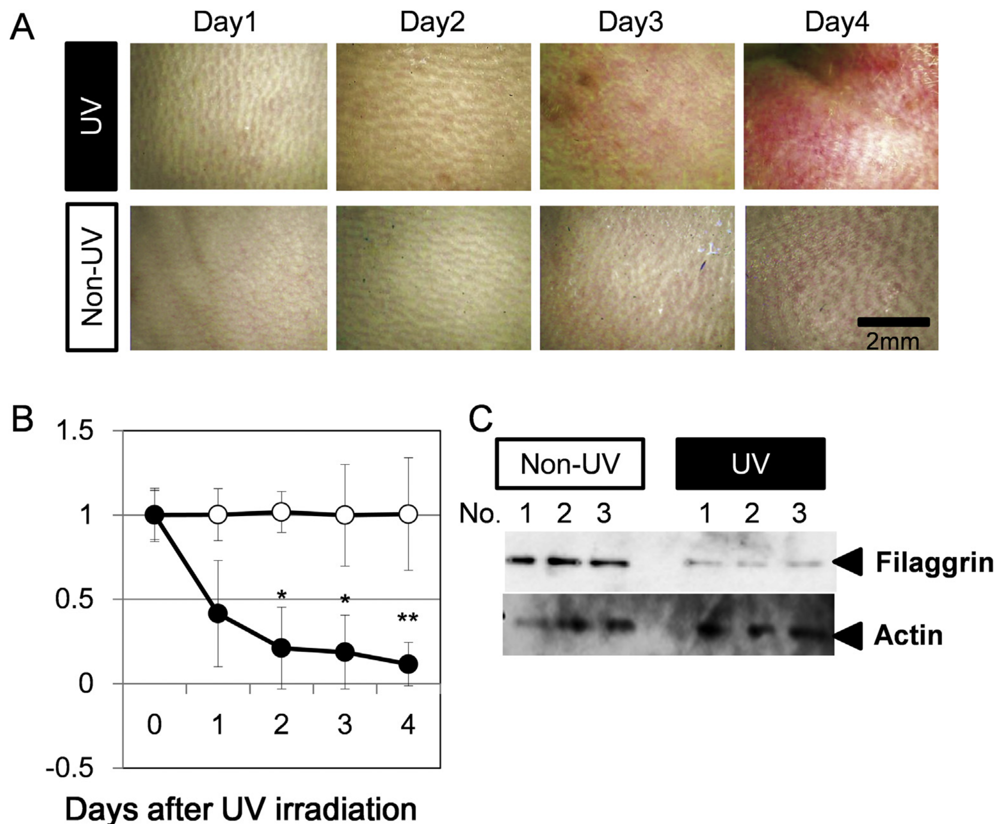

Fig. 2. UV-sensitive gene, Filaggrin (Flg). We examined gene expression profiling in skin with UV exposure (2.7 J/cm^2). (A), UV was exposed for four days (1-h/day) and skin conditions were monitored using a "skin scope". Dorsal skin was shielded on one side from UV irradiation. Non UV control (Non-UV), UV-treated (UV). n = 5. (B, C), UV irradiation caused downregulation of Flg mRNA (B). Flg expression levels were decreased by UV irradiation (closed circles) compared to without UV irradiation (open circles). The value without UV irradiation at day 0 was defined as 1, and relative values are shown. Values are expressed as the mean ± SEM for six mice. Similarly, Flg protein levels were downregulated by UV irradiation (C). *P < 0.05, **P < 0.005 vs UV irradiated samples. For immunoblot analysis, dorsal skin samples were lysed and homogenized with Laemmli buffer (62.5 mM Tris–HCl, 10%glycerol, 2% SDS), and were applied to the gel (50 μg proteins per lane). Reproductive data from three mice are shown. Non UV control (Non-UV), UV-treated (UV).

to HDF cells, WST-8 assays were performed (Fig. 1C). Results demonstrated the delayed cytotoxicity of H_2O_2. On the other hand, UV exposure caused intracellular ROS production persisting from 30 min and cytotoxicity did not appear until 24 h (Fig. 1A and C). FX elicited ROS quenching activities and protective effect on H_2O_2-induced cytotoxicity in HDF cells concentration-dependently (Fig. 1Band D). RA failed to elicit an antioxidant effect in HDF cells exposed to H_2O_2 or UV irradiation (Suppl.4B and data not shown).

3.2. Evaluation of damaged skin and FX action

We attribute the symptoms to persistent inflammation. If we determine the state of the skin before it is damaged, we might be able to ameliorate damage. Therefore, in order to examine the pre-symptomatic state of the skin, we performed microarray analysis. Based on data mining and qRT-PCR screening, we isolated the gene filaggrin (Flg), which was dramatically downregulated by UV exposure with associated sunburn (Fig. 2B and C). Not shown in data, Flg protein levels were not altered during experiments in vaseline-treated control mice. UV exposure apparently caused sunburn on day 4, while UV exposure downregulated Flg gene expressions prior to the appearance of sunburn. Next, to test the availability of FX, NAC, and RA, we examined the *in vivo* effect of compounds on UV-induced skin damage. After removal of dorsal hairs, the left side was treated with Vaseline as a control, and the right side was treated with Vaseline containing 0.5% FX 30 min before UV exposure. Skins were then isolated and analyzed by immunohistochemistry and immunoblot. As shown in Fig. 3A and B, UV exposure caused sunburn and 0.5% FX elicited a dramatic protective effect against UV-induced sunburn. When 10% NAC or 0.5% RA were tested similarly, these compounds failed to protect against sunburn (Fig. 3C, D, I and J). Additionally, 0.05% clobetasol (Dermovate®), a strong steroidal anti-inflammatory drug, also failed to afford protection against sunburn (Fig. 3K and L). We

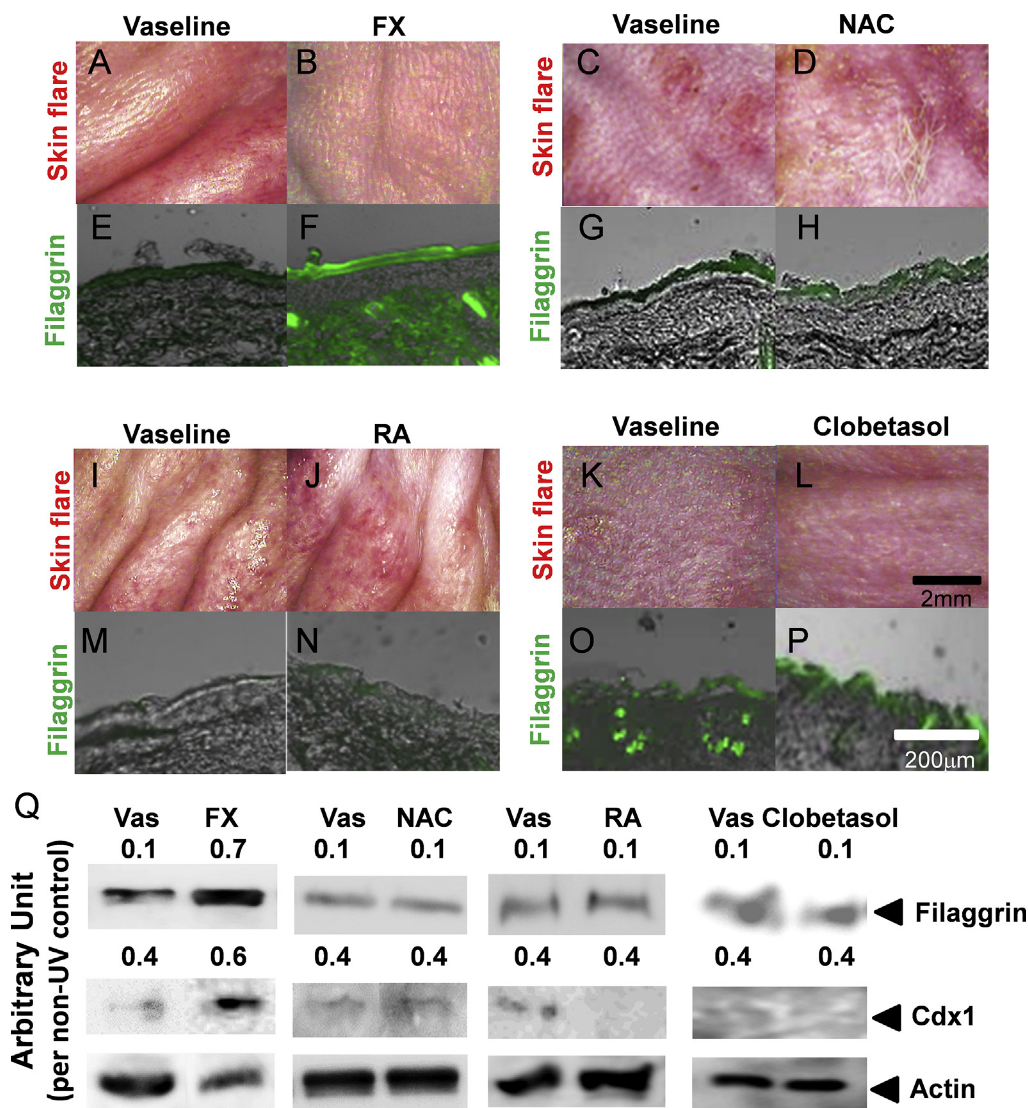

Fig. 3. Effects of compounds on UV-induced skin damage in *in vivo*. FX protected skin from UV-induced sunburn. 0.5% FX (A–B), but not 10% NAC (C–D), 0.5% RA (I–J), or 0.05% clobetasol (K–L) were applied to dorsal skin. Treatment with the respective materials was performed 30 min before UV irradiation. Immunohistochemical and immunoblot analysis. Dorsal skin was fixed with 4% paraformaldehyde in PBS for two days and specimens were prepared as described in "Materials and Methods". For each individual sample, 10–20 specimens were exposed to antibodies — representative images are shown (M–P). Some of the skins were cut into 5 mm squares and dissolved prior to fixation, with storage at −85 °C until needed for immunoblot analysis (Q). Only FX restored the expression of filaggrin, whereas NAC, RA and clobetasol had no effect. Representative data are shown from each of 6 mice samples. The expression level of proteins without UV irradiation was defined as 1, and the relative expression levels of proteins are shown. Vaseline control (Vaseline, Vas), fucoxanthin-treated (FX), *N*-acetylcysteine-treated (NAC), retinoic acid-treated (RA), clobetasol-treated (Clobetasol). n = 5.

examined the damaged state induced by UV exposure and the effect of FX. After UV exposure for four days with or without FX, skin from the back was histochemically analyzed using FITC-dextran treatment for three hours (Suppl.6). On FITC-dextran permeability assay, Vaseline control skins showed fluorescence extending into the epidermis, but in FX-treated skins just the stratum corneum was affected. FITC-dextran did not reach the dermis in either treatment.

3.3. Insufficiency of β-carotene

Next, we examined the effect of FX and β-carotene on Flg promoter activity in skin fibroblastic cells (Fig. 4A). Flg promoter activity was stimulated by FX in a concentration-dependent manner, but not by β-carotene. β-Carotene also failed to protect against sunburn or cause Flg gene restoration with UV (Fig. 3B–E). Loricrin expression was not influenced by any treatment during experiments (Data not shown).

3.4. Regulation of filaggrin gene expression

To further investigate the mechanisms by which Flg expression is regulated, we performed *in silico* analysis of the 2000 bp region proximal to the Flg gene using the TF Search program (http://www.cbrc.jp/research/db/TFSEARCH.html) (Suppl.7). In the proximal 2000 bp promoter region of the mouse Flg gene, we predicted the presence of three clustered RA-responsive elements (Cdx binding site; A, A/T, T, A/T, A, T, A/G). Thus, we examined levels of Cdx1 expression by immunoblot analysis (Fig. 3M, N, and Q). Cdx1 expression was restored by FX alone, and not by RA *in vivo*. Next, we analyzed Flg gene regulation (Fig. 5). To examine the precise mechanism of Flg gene regulation, we designed experimental

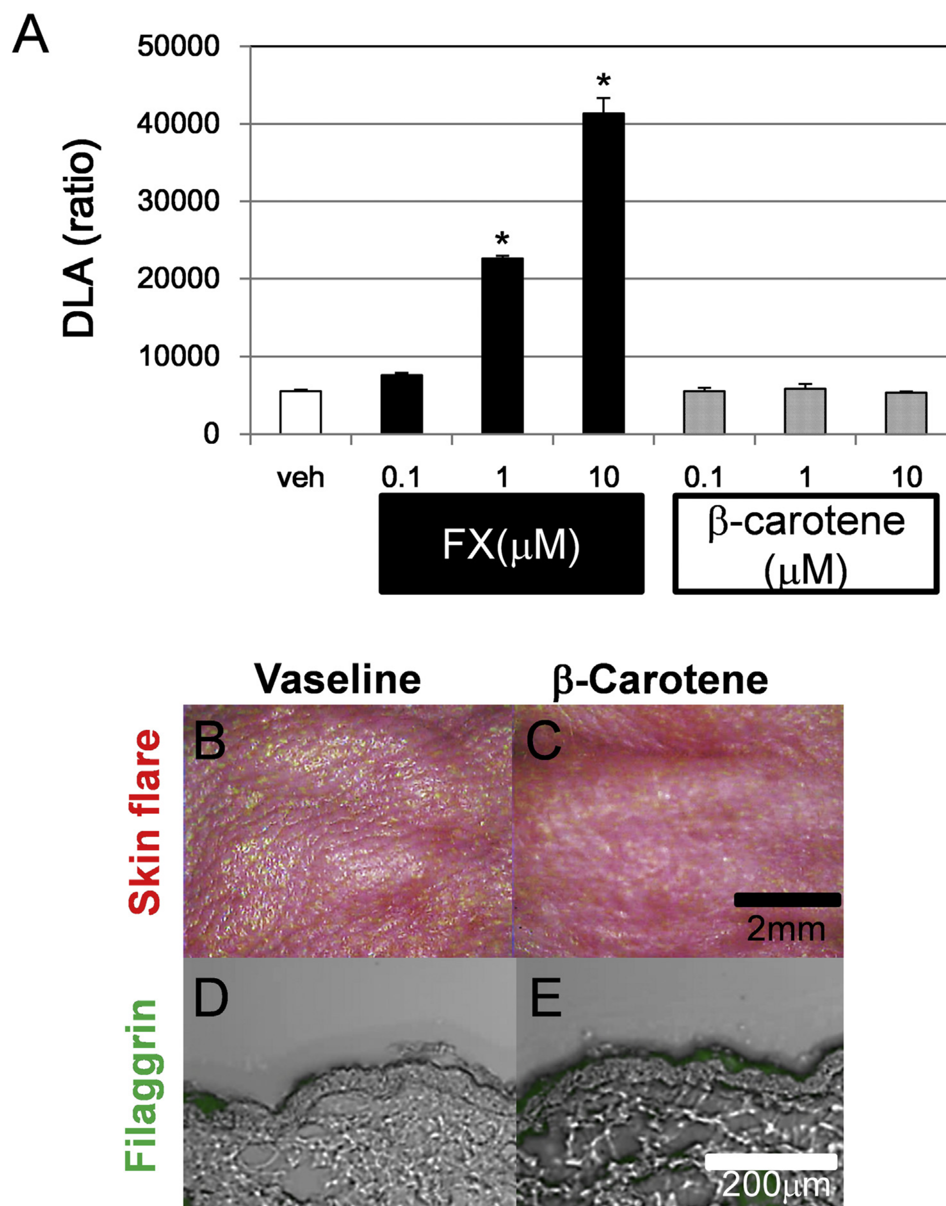

Fig. 4. β-carotene failed to protect against UV-induced sunburn and to induce filaggrin expression. (A), Reporter analysis of FLGp2,000-luc. Luciferase vector transfected into primary skin fibroblast cells. FX or β-carotene (0.1 μM, 1 μM, 10 μM) were treated after three hours of transfection. veh: vehicle control (DMSO). Data are presented as mean ± SEM of four wells. Similar results were obtained from an additional four experiments. *P < 0.001 vs DMSO control. (B–E), Effects of β-carotene on UV-induced skin damage and Flg expressions were analyzed immunohistochemically. Vaseline control (Vaseline), β-carotene-treated (β-carotene). n = 5.

model by UV irradiation using primary cultured skin fibroblastic cells (Fig. 5A). Flg mRNA expression level were dramatically reduced by UV irradiation for 1 h at the start of the study *in vitro* (Fig. 5B). FX attenuated Flg expression under non-UV condition, and FX restored downregulation of Flg expression induced by UV irradiation. On the other hand, FX did not influence Flg expression under non-UV condition, and rescued from UV-induced Flg reduction *in vivo* (Fig. 5C). UV irradiation in primary cultured skin fibroblasts as well as HDF cells had no effect on cell viability (not shown in data). Using this *in vitro* experimental condition impaired by UV irradiation, we examined the effect of FX on expression levels of Cdx family genes (Fig. 5D). All Cdx genes were downregulated by UV irradiation approximately up to 10% compared to non-UV condition (data not shown). Cdx1 were protected by FX under the condition of UV exposure. In the same condition, Cdx2 and Cdx4 were not restored by FX.

To analyze the mechanism of FX-induced Flg gene regulation, we designed deletional constructs of the Flg gene promoter (Fig. 6A). The activity of Flg2000-luc was detected strongly under normal culture condition, whereas reporter activity was reduced in Flg1200-luc. Additionally, the −2000 to −1200 region was responsive to FX in skin fibroblastic cells. Overexpression of *Cdx1* in primary skin fibroblastic cells increased Flg expression (Fig. 6B and C). A chromatin-immunoprecipitation assay supported the hypothesis that *Cdx1* actually interacted with this cluster1 region (Fig. 6D, Suppl.7).

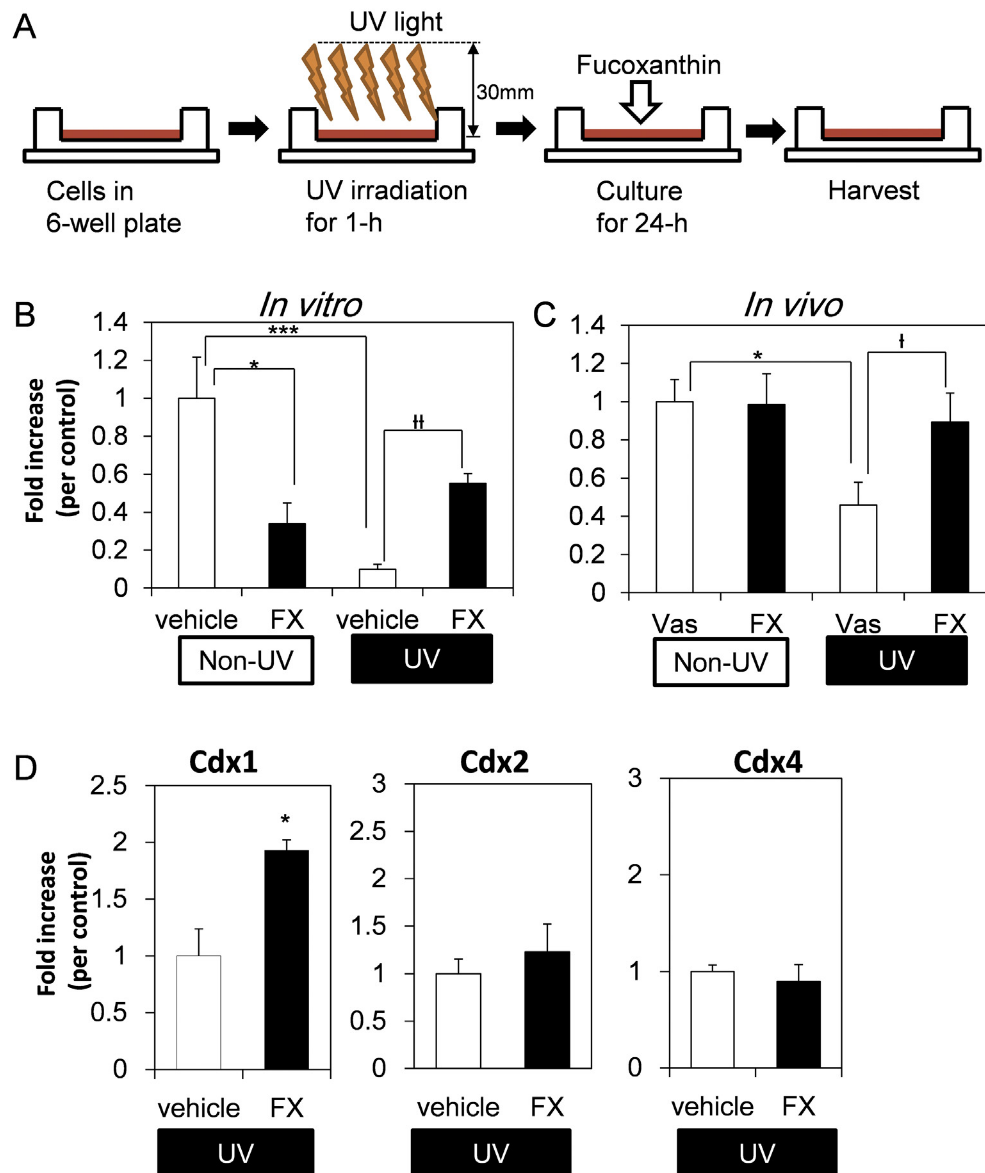

Fig. 5. The effect of FX on Flg gene expression. (A), *In vitro* culture model for evaluation of FX action. Embryonic skin fibroblastic cells were plated in 6-well plate. After confluent, cells were exposed to UV for 1 h from an indicated distance, and then cultured for 24 h with or without FX. Cells were harvested, and gene expression levels were analyzed by qPCR. (B), the effect of FX on Flg gene expression *in vitro*. Values are expressed as the mean ± SEM of 6 wells. *P < 0.05, ***P < 0.001 vs vehicle control (DMSO) in non-UV condition, ǂǂP<0.01, vs vehicle control in UV exposed condition. Similar results were obtained from an additional two experiments. (C), the effect of FX on Flg gene expression *in vivo*. The effect of FX on Flg expression levels in dorsal skins were analyzed under the conditions shown in Fig. 2. n = 5. *P < 0.05, vs vaseline control in non-UV condition, ǂP<0.05, vs vaseline control in UV exposed condition. The expression level of Flg mRNA without UV irradiation was defined as 1, and the relative expression levels are shown (B, C). (D), The effect of FX on Cdx family genes *in vitro*. The expression level of respective Cdx gene mRNAs with UV irradiation was defined as 1, and the relative expression levels are shown. Values are expressed as the mean ± SEM of 6 wells. *P < 0.05, vs vehicle control (DMSO) in UV exposed condition. Similar results were obtained from an additional two experiments.

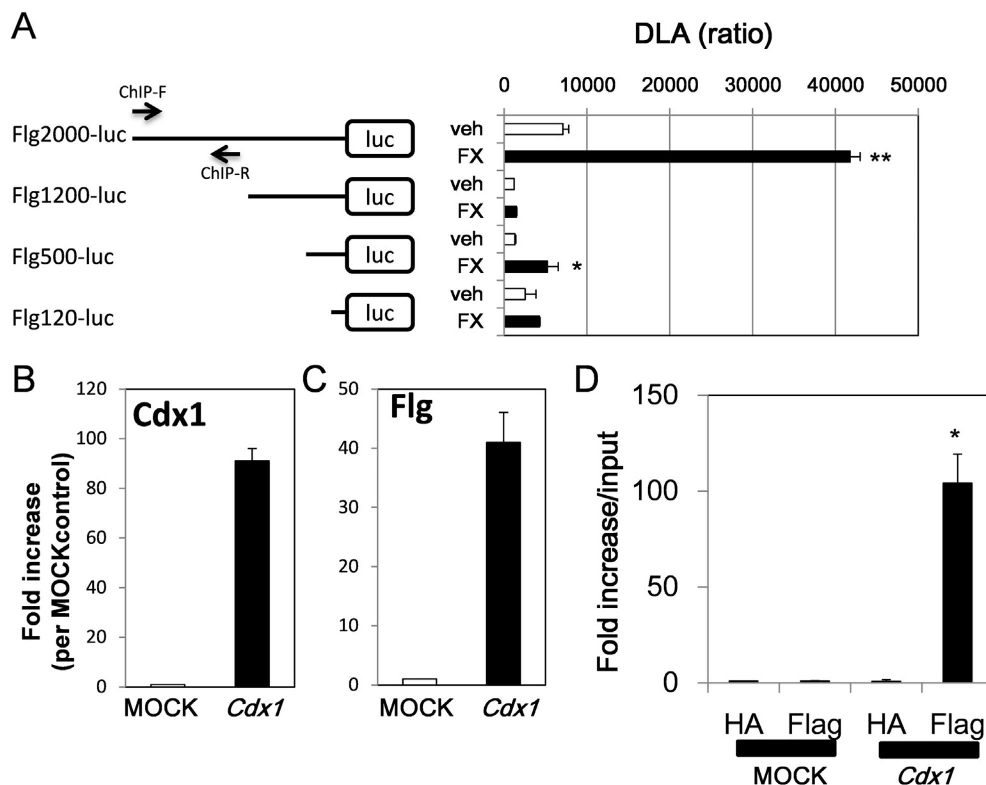

Fig. 6. Direct interaction between Cdx1 and Flg gene promoter. (A), Flg promoter activities regulated by FX. Cells were transfected with indicated Flg-luc constructs (left panel). Three hours after transfection, cells were treated with or without FX. Flg promoter activities were specifically potentiated by FX in Flg2000-luc, while FX had little influence on other deletional constructs (right panel). Values are expressed as the mean ± SEM of 4–8 wells. *P < 0.05 vs vehicle control (DMSO). (B, C), Overexpression of *Cdx1* promoted Flg expression. We tested Flg gene expression levels in both MOCK and *Cdx1* overexpressed cells. qPCR analysis was performed as described in "Materials and Methods". Values are expressed as the mean ± SEM of four wells. (D), The interaction between *Cdx1* and Flg promoter by ChIP analysis. Fragmented chromatins of MOCK or *Cdx1*-transfected cells were immunoprecipitated by FLAG M2 beads. Amplification input DNA was constant. Immunoprecipitated DNA-derived amplification by FLAG-M2 beads was detected only in *Cdx1*-transfected cells. *P < 0.001 vs MOCK control. Similar results were obtained from an additional three experiments.

3.5. Therapeutic effects of FX

Finally, we examined the therapeutic effect of FX. After UV exposure for four days, sunburn was clearly apparent (Fig. 7). Skins were treated with 0.5% FX for four days until day 8. FX treatment cured sunburn (Fig. 7A and B). Immunohistochemical and immunoblot analysis showed that Flg expression, downregulated by UV exposure, was actually restored by FX (Fig. 7C–E). Cdx1 expression was also restored by FX.

3.6. Discussion

Acute skin damage due to tanning manifests as sunburn. In the current study, we designed an experiment that examined the effects of mild, intermittent stress on the skin. Although moderate sunburn does not itself result in drastic adverse events in healthy skin, the cutaneous symptoms may worsen in allergic contact dermatitis (18), atopic dermatitis (19), and seborrheic dermatitis (20). Of interest, all of these conditions benefit from therapeutic UV irradiation protocols (21). The mechanisms of these opposite outcomes in response to cutaneous UV irradiation remain unclear. Benefits that accrue from adequate treatment with UV irradiation should depend on age and severity (22). In healthy skin at least, the major factor that eliminates moisture is thought to be loss of the skin barrier (23, 24). In the current study, we identified the UV sensitive gene; we intended to explore compounds that can be used to restore the function of this gene. We subsequently found that fucoxanthin had a strong effect on recovery of Flg expression, reduced by UV irradiation and sunburn.

We isolated UV sensitive several genes. Among them, Flg gene was affected at an early sensitive gene. In addition, Flg gene was responded to FX and FX restored UV-induced sunburn, suggesting that is an important gene that reflects the state of the skin damage. From *in silico* promoter analysis, we predicted the importance of the Cdx family transcription factor within the Flg promoter (Suppl.7). As shown in Fig. 3Q, the downregulation of Cdx1 by UV exposure was also specifically restored by FX. These FX actions were reproduced using cultured skin fibroblastic cells (Fig. 5A). Although FX alone inhibited Flg mRNA expression in cultured cells, FX itself did not influenced them in *in vivo* (Fig. 5B and C), suggesting that high Flg mRNA inducing activity in cultured cells under normal culture condition might be masked FX action. Further investigation will be required.

Among several species, encoded Flg genes have different genetic loci; the gene promoter region has not been conserved. Flg genes might therefore mediate different responses among species. However, our current study clearly showed that FX promoted Flg gene activation, at least in the rodent.

Members of the caudal gene family (in mice and humans: Cdx1, Cdx2, and Cdx4) have been studied during early development as regulators of axial elongation and anteroposterior patterning (25). Moreover, cdx genes regulate the development of neural and endodermal tissues and play important roles in adult intestinal tissue homeostasis and the pathogenesis of gastrointestinal cancers (26). The role of Cdx genes in the skin is

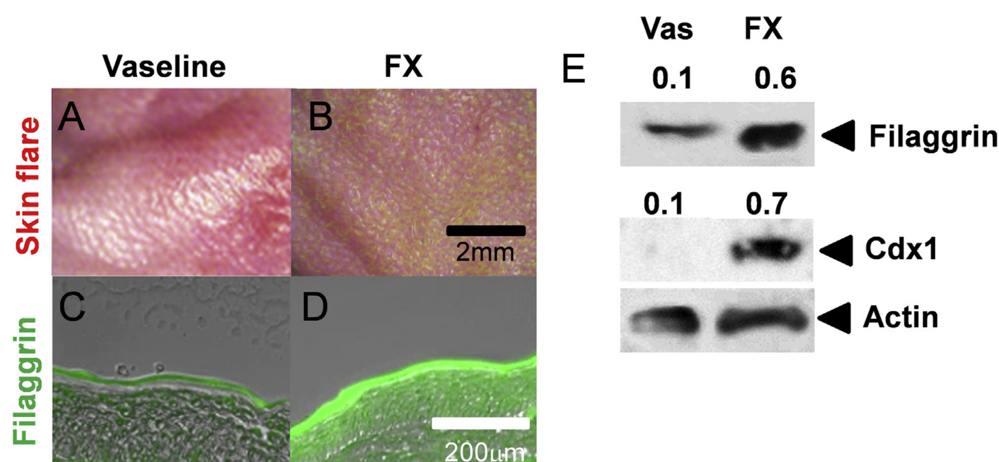

Fig. 7. Restoration of UV-induced damaged skin by FX. (A, B), FX treatment cured sunburn. Skins were treated with 0.5% FX from days 4–8, and UV irradiated from days 1–8. (C–E), Immunohistochemical and immunoblot analysis were performed. FX restored Flg expression that was decreased by UV exposure; Cdx was also restored by FX. The expression level of proteins without UV irradiation were defined as 1, and the relative expression levels of proteins are shown. Vaseline control (Vaseline, Vas), fucoxanthin-treated (FX). n = 5.

less well elucidated. We reports here for the first time that Cdx genes expressed in skin fibroblastic cells and functioned as a regulator for Flg gene. Cdx1 was regulated by FX (Fig. 5D), and *Cdx1* markedly stimulated Flg expression (Fig. 6A–C), suggesting that FX stimulates Cdx1 induction, subsequently inducing the Flg gene. Additionally, *Cdx1* interacted directly with the Flg promoter region (Fig. 5D), suggesting that at least *in vivo*, the Flg gene was tightly regulated by Cdx1.

Intracellular and extracellular oxidative stress initiated by ROS advance skin aging. Because UV enhances ROS generation in cells, skin aging is usually discussed in relation to UV exposure (27). Recent studies indicate that the beneficial effect of lycopene may be due in part to the protection of the cells from ROS through induction of phase II enzymes that detoxify carcinogens (28). FX, a carotenoid, exerted a strong ROS-quenching effect and protective effect on UV irradiation (Suppl.4, Fig. 3). However, the strong ROS quencher NAC failed to protect against sunburn from UV. Antioxidant actions are best known as activities of carotenoids such as β-carotene, astaxanthin, lycopene, and so forth. FX also exhibits a strong antioxidant effect under aerobic conditions (9, 29), suggesting that the protective effect of FX on UV irradiation is not be due to an ROS-quenching effect of FX on UV irradiation is not due to an ROS-quenching effect, at least, in our experimental condition. In addition, since FX showed an absorption peak mainly outside that of UV-A (315–400 nm) (Suppl.3), this strongly suggests that its effects are not due to direct absorption of UV.

We also determined that elastin and col1a1 promoter activities were stimulated by FX in a concentration-dependent manner (Suppl.8), indicating that FX might activate metabolism in the skin through several mechanisms. Although these RA-like effects were observed with FX, RA failed to protect against UV-induced sunburn and Flg downregulation (Fig. 3I, J, M, N and Q), suggesting the protective effect of FX on UV irradiation is not due to RA-like action. FX is similar to β-carotene, as a carotenoid. However, β-carotene failed to protect against sunburn, and did not induce Flg expression and Flg gene promoter activation (Fig. 4). Since β-carotene also has strong antioxidant activity, these results support the finding that the anti-oxidant NAC too was not protective (Suppl.4). In previous reports, β-carotene has been shown to be metabolized to vitamin A after absorption into the body (30). With excessive doses, β-carotene is distributed to and stored in adipose tissue. However, β-carotene applied to the skin is likely to act by different mechanisms. FX has been considered to have a protective effect on the skin without metabolism to RA. Many vegetables or sea foods contains several type of carotenoids including FX that were thought to function as provitamin A (8, 9, 31). An earlier report showed that continuous topical RA promotes growth and differentiation of epidermal keratinocytes (32). Recently, FX has been shown to be present as fucoxanthinol in blood, with subsequent metabolism to amarouciaxanthin A (33). There is thus strong evidence that FX or its metabolites have an effect that is peculiar among the carotenoids. Clobetasol also failed to provide protection (Fig. 3K and L). We therefore believe that skin damage due to UV exposure cannot be ameliorated by steroids, at least in the acute phase.

In this report, we focused on the protective and therapeutic effect of FX accompanied by Flg restoration. An Flg mutation has been identified as a risk factor for atopic dermatitis (34). From the fact that drying of the skin also occurs in atopic dermatitis, FX might be also effective in similar skin conditions, including atopic dermatitis. Further investigation will be required. We here conclude that FX stimulates restoration of the skin barrier protein Flg, likely by enhancement of metabolism in the dermis, leading to it exerting protective and therapeutic effects against sunburn caused by UV.

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Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jphs.2016.08.004.

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