A Proposal on a New UV-induced Pigmentation Story: Epidermal Keratinocytes Regulate Dermal Fibroblasts-derived Paracrine Factors Involved in Melanin Production of Melanocytes

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Abstract.

Background: It is well known that keratinocyte-derived paracrine factors, for example Endothelin-1, significantly contribute to UV-induced pigmentation. Recently, there is a great interest in regulation of melanin production in melanocytes thorough fibroblast-derived paracrine factors. However, the involvement of the interaction between the dermal fibroblasts and melanocytes in UV-induced pigmentation via epidermal keratinocytes has been uncleared yet. The purpose of this study was to investigate the involvement of crosstalk between keratinocytes and fibroblasts in melanin production.

Methods: All experiments were conducted with normal human cultured cells. The amount of intracellular melanin was measured using a method of alkaline solubilization and secretory proteins were quantified with ELISA.

Results: The melanin production in melanocytes treated with the conditioned medium of keratinocytes-fibroblasts was markedly reduced compared to that in normal melanocytes. In contrast, this reduction of melanin production was not detected with the conditioned medium of UVB-exposed keratinocyte-fibroblasts. We also found that a reduction in DKK1 of fibroblasts was induced in the inflammatory cytokine of UVB-exposed keratinocytes. In addition, we found that the reduction of DKK1 expression in fibroblasts was resolved by Zinc Glycinate treatment to keratinocytes.

Conclusion: This is a novel finding suggesting that the secretion of paracrine factors of fibroblasts involved in pigmentation is regulated by keratinocytes. The approach about interaction between keratinocytes and fibroblasts may be useful in the prevention of UVB-induced pigmentation.

Keywords: DKK1, Pigmentation, Fibroblasts, Keratinocytes, inflammatory cytokines

1. Introduction.

Keratinocytes can regulate melanocyte proliferation, dendricity and melanogenesis through paracrine factor secretions such as endothelin-1 (ET-1), prostaglandin E_2 (PGE_2), α- melanocyte stimulating hormone (α-MSH) and inflammatory cytokines such as IL-1α [1-3]. Recently, there is a great interest in the regulation of melanin production in melanocytes through fibroblast-derived paracrine factors. For example, several fibroblast-derived paracrine factors such as Dickkopf1 (DKK1), Stromal Derived Factor-1 and others have been identified as inhibitory regulators of melanin production, while Neuregulin-1, Stem Cell Factor and others have been identified as promotive regulators [4, 5]. About the relationship between melanin production and fibroblasts, experiments in the previous study on using Reconstructed Skin Model has revealed that photo-aged fibroblasts have a lower inhibitory effect on melanogenesis than normal fibroblasts [6]. It is also reported that the elimination of senescent fibroblasts from pigmented skin using radiofrequency to correct uneven pigmentation [7]. These findings suggest that the paracrine factors of dermal fibroblasts affect UV-induced pigmentation. Previous studies reported that the keratinocyte-derived IL-1α can stimulate fibroblasts, which in turn secretes melanocyte-stimulating factors such as HGF [8]. These studies suggested that inflammation of keratinocytes induce up-regulation of melanocytes stimulating factors (MSFs) of fibroblasts. However, the involvement of keratinocytes in the process of UV-induced pigmentation through stimulating dermal fibroblasts and the underlying mechanisms was not clearly understood. We recently focused on the DKK1 of fibroblast's secretory protein. DKK1 is a secretory glycoprotein that act on Wnt/β-catenin signaling and is known to be highly expressed in tissues with low pigment formation such as the human palmoplantar [5]. The Wnt/β-Catenin-signaling is not only reported to be activated by inflammatory cytokines but can also inhibit DKK1 expression [9]. In this study, we confirmed that keratinocytes could regulate DKK1 production to contribute to UV-induced pigmentation.

2. Materials and Methods.

2-1. Cell culture

Normal human epidermal keratinocytes (NHEKs), normal human dermal fibroblasts (NHDFs) were obtained from Kurabo (Osaka, Japan) and normal human epidermal melanocytes (NHEMs) were obtained from Cascade Biologics™ (Oregon, US). NHEKs were cultured using HuMedia-KG2 medium. NHDFs were cultured using Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 5% fetal bovine serum (FBS; GIBCO/Invitrogen, Carlsbad, CA, USA). NHEMs were cultured in Medium 254 (GIBCO) with Human Melanocyte Growth Supplement, (Kurabo). All cells were incubated at 37˚C in 5% CO_2 and 95% air.

2-2. Preparation of conditioned medium from UVB-irradiated keratinocytes

NHEKs were seeded in 12-well microplates at a cellular density of 10×10^4 cells/well using HuMedia-KG2 medium for twenty-four hours. Keratinocytes were then cultured in normal HuMedia-KB2 medium or HuMedia-KB2 medium containing 50 μmol/L Zinc Glycinate for twenty-four hours. Culture medium was then replaced with Hank's balanced salt solution (Ca^{2+}, Mg^{2+} free; HBSS (−)) and the NHEKs were then exposed to UVB at a dose of 20 mJ/cm^2. The UVB-unexposed group was completely shielded from UVB by covering with aluminum foil. After UVB irradiation, Hank's buffer was then replaced with fresh HuMedia-KB2 medium, and the cells were incubated for twenty-four hours. All media were then collected as culture medium from the UVB-irradiated keratinocytes, and culture medium from UVB-unexposed keratinocytes, to be used for fibroblast cultivation.

2-3. Fibroblast cultivation with the conditioned medium from UVB-irradiated keratinocytes

NHDFs were seeded in 12-well microplates at a cellular density of 10×10^4 cells/well using DMEM supplemented with 5% FBS for twenty-four hours. Seeded fibroblasts were subsequently cultured with each conditioned medium of keratinocytes for twenty-four hours. The culture medium was then collected as conditioned medium from fibroblasts to be used for melanocyte cultivation and DKK1 protein detection. The NHDFs treated with each conditioned medium were also harvested for DKK1 mRNA detection.

2-4. Melanocyte cultivation with the conditioned medium from fibroblasts

NHEMs were seeded in 12-well microplates at a cellular density of 20×10^4 cells/well in M254 supplemented with HMGS for twenty-four hours. Melanocytes were then cultured with the conditioned medium from fibroblasts for Seventy-two hours and then analyzed for melanin content.

2-5. Detection of IL-1α protein

Culture supernatants from UVB-irradiated keratinocytes and from UVB-unexposed keratinocytes were collected and subjected to ELISA, and interleukin-1α (IL-1α, R&D Systems) was quantified according to manufacturer's protocol. NHEKs was dissolved in 0.5% Triton X-100 and then BCA™ Protein Assay (Thermo Scientific) were used to quantify cellular proteins. The amount of IL-1α was calculated as the amount per unit cellular protein.

2-6. Fibroblast cultivation with the rhIL-1α

NHDFs were seeded in 96-well microplates at a cellular density of 1.5×10^4 cells/well using DMEM supplemented 5% FBS for twenty-four hours. Fibroblasts were subsequently cultured with rhIL-1α (R&D Systems) for twenty-four hours. The culture supernatant from rhIL-1α treatment were collected for DKK1 protein detection, and NHDFs were harvested for DKK1 mRNA detection.

2-7. Detection of DKK1 mRNA expression

NHDFs treated with rhIL-1α or each conditioned medium of keratinocytes were washed with cold PBS. cDNA from treated NHDFs was prepared using TaqMan™ Gene Expression Cells-to-C_T™ Kit (Ambion) according to the manufacturer's protocol. Using this cDNA as a template, TaqMan™ Fast Universal PCR Master Mix (Applied Biosystems) Real time RT-PCR was performed using TaqMan™ Gene Expression Assays (DKK1, Applied Biosystems). TaqMan™ GAPDH CONTROL MIX (Applied Biosystems) was used as a control-house-keeping. Real time RT-PCR used StepOnePlus with™ Real Time PCR System (Applied Biosystems). Analyses were performed using the $^{\Delta\Delta}$CT method and expressed as a relative value with the corrected value of 1.00 for the test sample untreated cells.

2-8. Detection of DKK1 protein

Culture supernatants from NHDFs (treated with rhIL-1α or each conditioned medium of keratinocytes) were collected and subjected to ELISA; and Dikkopf1 protein (DKK1, R&D Systems) was quantified according to the protocol of the kit. NHDFs was dissolved in 0.5% Triton X-100 and BCA™ Protein Assay were then used to quantify the cellular proteins. The amount of DKK1 was calculated as the amount per unit cellular protein.

2-9. Detection of Melanin contents

NHEMs were harvested with PBS and centrifuged for five minutes at 1,000 rpm. The supernatants were poured off and 200 μL ethanol/ether 1:1 (v/v) were added to remove opaque substances other than melanin. Finally, the cells were dissolved in 100 μL of 1 mol/L NaOH at 95˚C for ten minutes. The melanin contents were then measured with microplate reader at Abs 405 nm. BCA™ Protein Assay was used to quantify the cellular proteins. The amount of melanin was calculated as the amount per unit cellular protein.

2-10. Statistical Analysis

Data are expressed as Means ± S.D. Statistical analysis was performed by Student's t-test with p-value < 0.050 (*p<0.050) and p-value < 0.010 (**p<0.010) considered statistically significant differences.

3. Results.

3-1. The regulatory function of fibroblast melanogenesis is affected by UV radiation through keratinocyte paracrine factors

To investigate the crosstalk among keratinocytes, fibroblasts, and melanocytes, we observed the melanin production in melanocytes as indicated in the experimental design (Fig. 1a). Melanocytes treated for seventy-two hours with the conditioned medium (CM) of fibroblasts supplemented with UVB-unexposed keratinocyte secretory factor (Control) significantly suppressed melanin production compared to non-treatment melanocytes as previously reported in vitro [10]. In contrast, the amount of melanin production in melanocytes treated with the CM of fibroblasts supplemented with UVB exposed-keratinocyte secretory factor was comparable with the non-treated condition (Fig. 1b). This indicates that there are factors secreted by the fibroblast that can contribute to the

decrease of the amount of melanin. DKK1 is an anti-melanogenic protein secreted by the fibroblast. Here, we investigate if the amount of DKK1 protein was altered by the factors secreted by keratinocytes upon UV irradiation. Under UVB-exposed conditions, DKK1 in CM of NHDFs were found to decrease significantly in comparison to the Control (Fig. 1c).

3-2. Fibroblast's paracrine factor related to the melanogenesis is affected by keratinocyte's inflammatory paracrine factor

It is well known that DKK1 are regulated through the Wnt/β-catenin signaling. In addition, the Wnt/β-catenin signal can be activated by inflammatory cytokine such as IL-1β and IL-6 [9]. These inflammatory cytokines can be related to the suppression of DKK1 expression since under UVB exposed conditions, IL-1α is significantly induced (Fig 1d). We investigated whether IL-1α is involved in the reduction of DKK1. The recombinant IL-1α significantly inhibited the DKK1 mRNA expression and protein secretion (Fig. 2) These results suggest that UV-induced keratinocyte-derived paracrine factor, such as IL-1α, controls the ability of fibroblasts to regulate melanin production. This finding may be a novel mechanism supporting the promotion of epidermal inflammation-induced pigmentation.

3-3. Zinc Glycinate restored DKK1 expression in fibroblasts through suppressing IL1α secretion in UVB-exposed keratinocytes

Finally, we found that the intervention in inflammatory cytokine of keratinocytes was effective to regulation of the DKK1 secretion in fibroblasts. In this study, we used Zinc Glycinate, a skin-brightening active, to suppress UVB-derived inflammatory cytokines. In this study, we found that Zinc Glycinate suppressed the increase in IL-1α secretion in UVB-exposed conditioned (Fig. 3b) and recovered DKK1 expression in UVB-exposed condition (Fig. 3c).

4. Discussion.

In this study, we considered a hypothesis that keratinocyte-derived inflammatory cytokines, which are increased by UVB exposure, can activate melanin production thorough regulation of secretory protein in fibroblasts. It is well known that melanocytes were regulated by dermal fibroblasts derived factors: stem cell factor, basic fibroblast

growth factor, neuregulin-1, and Wnt signaling pathway inhibitor dickkopf1 (DKK1), among others. In particular, DKK1 was known to modulate melanogenesis in melanocyte. However, there are still any unknown points about the relationship between production of DKK1 in fibroblasts and pigmentation. For example, although it was reported that fibroblasts from photo-aged skin tended to show reduced amounts of DKK1 secretion [6], the differences in DKK1 gene expression between senile lentigo and normal tissue was not observed [7]. It is also reported that there were more differences in NRG-1 gene expression than in DKK1 gene expression between skin phototype [5]. Even though some of the regulatory mechanisms of interactive communications among these cells were still unclear, it is well known that some dermal fibroblast-derived paracrine factors are regulated by epidermal keratinocyte. In particular, wrinkle-related fibroblast-derived paracrine factors such as MMPs have been reported to be upregulated by inflammatory factors in keratinocytes induced by UVB exposure. Although MMPs has undeniable connection to photoaging, it also has a crucial role in wound healing [11]. Granulocyte colony-stimulating factor expression in fibroblasts, which plays a beneficial role in wound healing by stimulating keratinocyte proliferation, were markedly increased by IL-1α from keratinocytes [12]. Keratinocyte growth factor, another factor from fibroblasts that has an effect on melanogenesis, is also stimulated by IL-1 from keratinocytes [13]. In these examples, the crosstalk between keratinocytes and fibroblast are mediated by inflammatory cytokines like IL-1α, among others, usually in a pursuit of a common goal-which is to protect the skin. In the case of UV exposure, in a concerted effort to protect the genes from instability, keratinocytes releases factors to melanocytes to increase melanin production, and at the same time releases factors to fibroblast to suppress it from inhibiting melanin synthesis.

The results of this study showed that inflammatory cytokines in keratinocytes may regulate melanin-modulate paracrine factors such as DKK1 in fibroblasts. These results are likely to be part of the mechanism of increased melanin production in UV-exposed skin. In addition, we found that Zinc Glycinate restored DKK1 expression in fibroblasts through suppressing IL-1α secretion in UVB-exposed keratinocytes. In our previous studies, we showed that Zinc Glycinate has a potential for brightening through stimulating metallothionein expression and inhibiting secretion of melanocyte growth and activating factors in keratinocytes [14]. Thus, our results suggests that, with proper intervention, UV-induced pigmentation can be prevented by containing the inflammatory response of

the skin that could stimulate melanin production in melanocytes and inhibit melanin modulating factors such as DKK1 in dermal fibroblasts.

5. Conclusion.

This is a novel finding suggesting that the secretion of fibroblast's paracrine factors such as DKK1 involved in pigmentation is regulated by keratinocytes. In conclusion, the approach about interaction between keratinocytes and fibroblasts may be useful in the prevention of UV-induced pigmentation.

Conflict of Interest Statement. NONE

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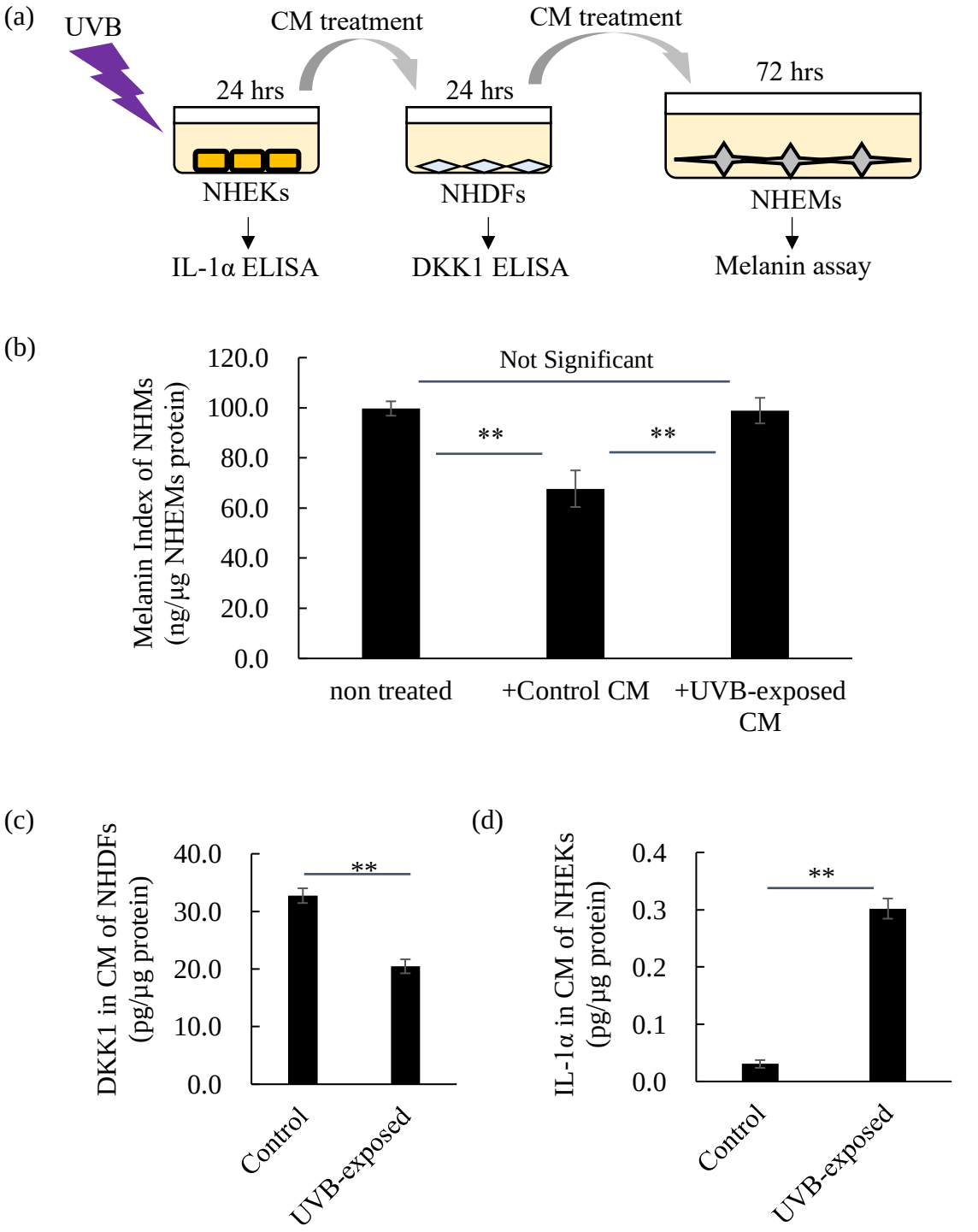

Figure 1. Comparison of Melanin Index in NHEMs treated by each conditioned medium The experiment design (a). NHEMs were seeded in culture-plate for overnight. NHEMs were then cultured with each conditioned medium from keratinocyte-fibroblasts, (Control) and (UVB-exposed) for 72 hrs and then analyzed for Melanin Index in NHEMs (b). Comparison of DKK1 protein secretion in fibroblasts treated by each conditioned medium of keratinocytes (c). Comparison of IL-1α protein secretion in keratinocytes stimulated by UVB (d). Significance; $p^{**} < 0.01$

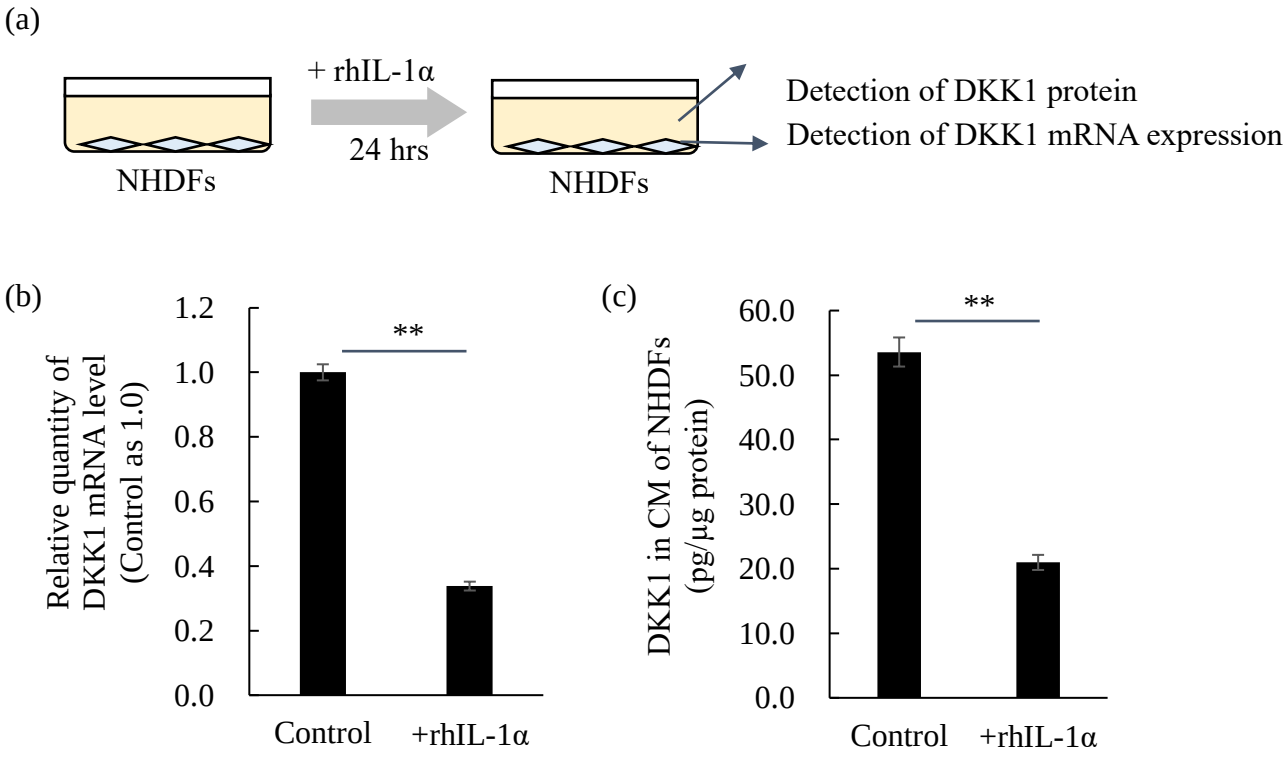

Figure 2. The DKK1 mRNA expression and protein secretion of fibroblasts treated by rhIL-1α The experiment design (a). Comparison of DKK1 mRNA level in NHDFs (b). Comparison of DKK1 protein secretion in culture supernatant of NHDFs (c). Significance; p** < 0.01

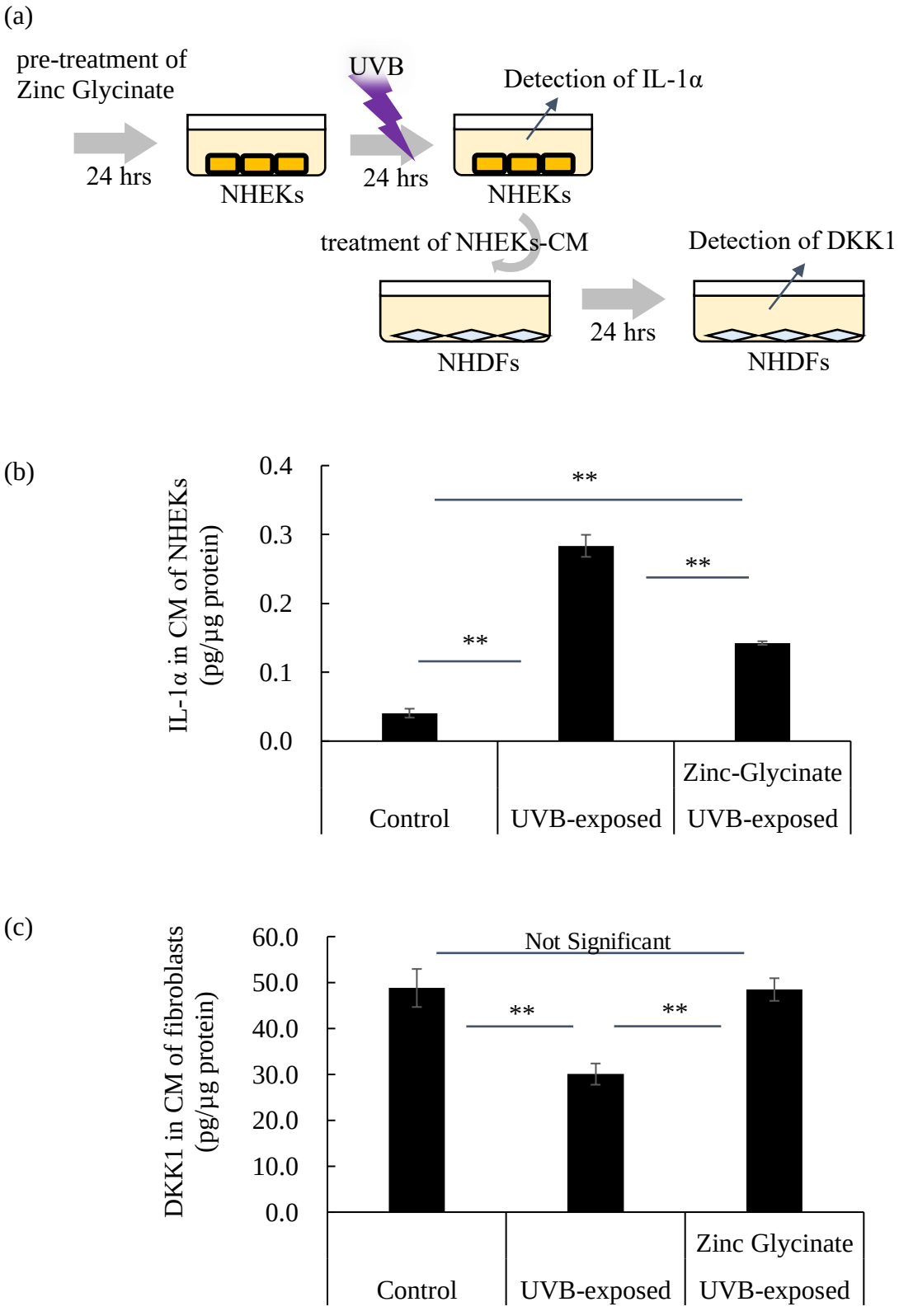

Figure 3. The amount of IL-1α in keratinocytes and amount of DKK1 in fibroblasts treated by Zinc Glycinate The experiment design (a). Comparison of IL-1α protein level in culture supernatant in NHEKs (b). Comparison of DKK1 protein secretion in culture supernatant of NHDFs (c). Significance; p** < 0.01

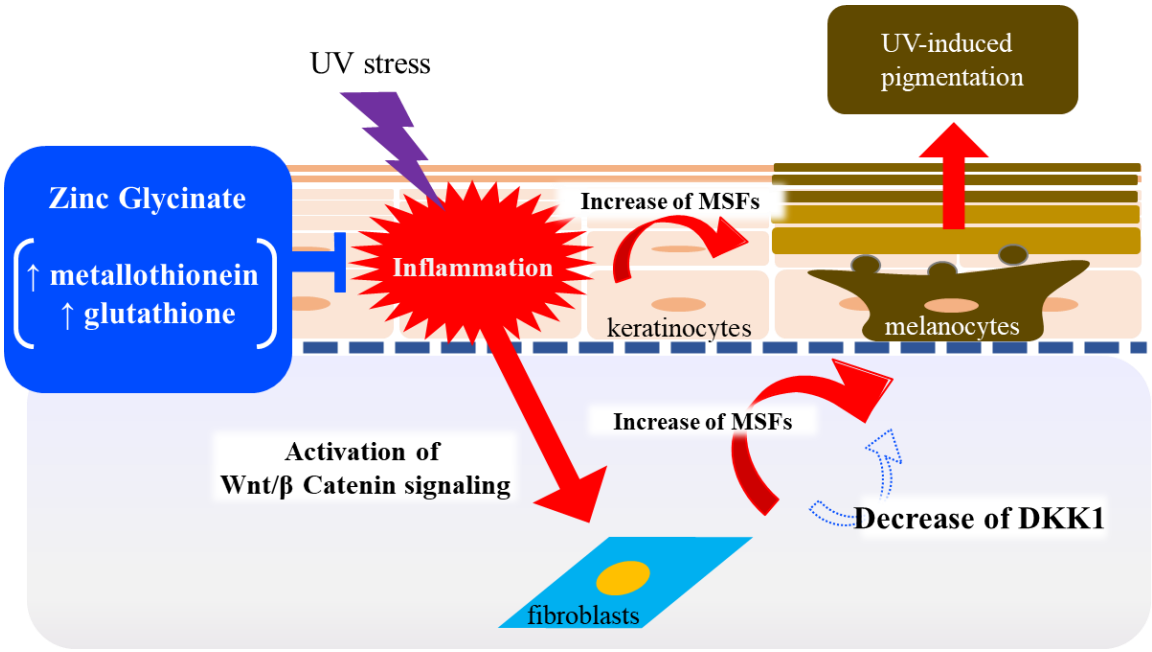

Figure 4. Summary This study and previous studies have shown that inflammatory factors secreted from keratinocytes by UV stimulation can regulate paracrine factors involved in melanin production in fibroblasts. In addition, anti-inflammatory actives such as Zinc Glycinate can suppresst UV-induced pigmentation.