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Original Article

Diarylpropionitrile inhibits melanogenesis *via* protein kinase A/cAMP-response element-binding protein/microphthalmia-associated transcription factor signaling pathway in α-MSH-stimulated B16F10 melanoma cells

Hyun Jeong Lee#, Sungkwan An#, Seunghee Bae, and Jae Ho Lee*

Department of Cosmetics Engineering, Konkuk University, Seoul 05029, Korea

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***Correspondence**
Jae Ho Lee
E-mail: jaeho390@konkuk.ac.kr

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#These authors contributed equally to this work.

ABSTRACT Diarylpropionitrile (DPN), a selective agonist for estrogen receptor β (ERβ), has been reported to regulate various hormonal responses through activation of ERβ in tissues including the mammary gland and brain. However, the effect of DPN on melanogenesis independent of ERβ has not been studied. The aim of this study is to examine the possibility of anti-melanogenic effect of DPN and its underlying mechanism. Melanin contents and cellular tyrosinase activity assay indicated that DPN inhibited melanin biosynthesis in alpha-melanocyte stimulating hormone-stimulated B16F10 melanoma cell line. However, DPN had no direct influence on *in vitro* tyrosinase catalytic activity. On the other hand, 17β-estradiol had no effect on inhibition of melanogenesis, suggesting that the DPN-mediated suppression of melanin production was not related with estrogen signaling pathway. Immunoblotting analysis showed that DPN down-regulated the expression of microphthalmia-associated transcription factor (MITF), a central transcription factor of melanogenesis and its down-stream genes including tyrosinase, tyrosinase-related protein (TRP)-1, and TRP-2. Also, DPN attenuated the phosphorylation of protein kinase A (PKA) and cAMP-response element-binding protein (CREB). Additionally, DPN suppressed the melanin synthesis in UVB-irradiated HaCaT conditioned media culture system suggesting that DPN has potential as an anti-melanogenic activity in physiological conditions. Collectively, our data show that DPN inhibits melanogenesis via down-regulation of PKA/CREB/MITF signaling pathway.

INTRODUCTION

2,3-bis(4-hydroxyphenyl)propionitrile (diarylpropionitrile, DPN) was firstly discovered by screening a select group of compounds for inducing the transcriptional activity of estrogen receptor beta (ERβ) in human endometrial cancer cells [1]. It has been reported that DPN acts as a selective ERβ agonist having a 70-fold higher relative binding affinity and 170-fold higher relative potency in transcription assays with ERβ than with ERα [1]. Previous studies have reported ERβ-dependent functions of DPN [2-4]. It has been studied that DPN enhanced cognitive performance of female mice in the object recognition and placement tasks but not in the ERβ knock mice [2], and decreased anxiety-like behaviors in mice due to association with ERβ [5]. It was also reported that DPN has an anti-inflammation effect associated with down-regulation of LPS-induced regulated on activation normal T cell expressed and secreted production [4] and growth inhibitory effect on several cancers [3,6]. However, ER-indepen-

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dent bioactivities of DPN are remain unknown. Interestingly, DPN has been reported to include a structure of stilbenoid, which is one of the main classes of phytoestrogens and has been known to have bioactivities including anti-pigmentation independent of ER signaling [7,8]. Therefore, it is considerable to investigate whether DPN has ER signaling-independent activities, especially on melanogenesis.

Melanin, a crucial pigment for determining color of skin, eyes, and hair, plays an essential role in protecting human skin from various stress involving environmental pollutants, drugs, chemicals, and especially protects against UV radiation [9]. Melanin is synthesized in melanocytes through metabolic process called melanogenesis and transferred to adjacent keratinocyte [10]. Tyrosinase, a rate-limiting enzyme in the melanogenic biochemical cascade, catalyzes conversion of L-tyrosinase into L-dihydroxyphenylalanine (DOPA) and L-DOPA into dopaquinone, a precursor of melanin in melanocyte [11]. Tyrosinase related protein (TRP)-2 has a dopachrome tautomerase activity that isomerizes dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) which is a relatively stable intermediate [12]. TRP-1 oxidates DHICA leading to conversion into eumelanin, a subtype of melanin [13]. Tyrosinase, TRP-1, and TRP-2 are major transcriptional genes of a melanocyte-specific transcription factor, M-microphthalmia-associated transcription factor (MITF) [11]. In addition, it has been reported that MITF activates a number of pigmentation-related genes, including melanocortin 1 receptor (*MC1R*), *MART*-1, *PMEL*, and endothelin receptor b [14-17].

In response to UV irradiation, p53 is accumulated in epidermal keratinocytes and transactivates its target genes including pro-opiomelanocortin [18,19], which is a precursor protein of adrenocorticotropic hormone (ACTH) and alpha-melanocyte stimulating hormone (α-MSH) [20,21]. The POMC-derived bioactive products bind to MC1R expressed on membrane of melanocytes, which is the member of G-protein coupled receptor (GPCR), in a paracrine manner and act as important determinants on cutaneous pigmentation [20]. Subsequently, the activation of MC1R accumulates the intracellular cAMPs followed by binding of cAMP to the regulatory subunit of protein kinase A (PKA) [22,23]. The released catalytic subunits of PKA phosphorylate CREB at serine 133 residue, one of the critical transcription factors of MITF along with sex-determining region Y-box 10 and lymphoid enhancer-binding factor 1 [11,24,25]. It has been reported that p38 and extracellular signal-regulated kinase (ERK) 1/2 also mediate the phosphorylation of CREB at serine 133 residue in response to α-MSH stimulation [26,27].

Here, we newly proposed the functional activity of DPN independent of ERβ signaling. In this study we investigated the effect of DPN on melanogenesis in α-MSH-stimulated B16F10 melanoma cells and associated molecular mechanisms.

METHODS

Reagents and antibodies

Diarylpropionitrile, arbutin, 17β-estradiol, α-MSH, L-DOPA, mushroom tyrosinase, and dibutyryl-cAMP were purchased from Sigma Aldrich (St. Louis, MO, USA). The primary antibodies against β-actin, MITF, tyrosinase, TRP-1, and TRP-2 were purchased from Santa Cruz Biotechnology (Dallas, TX, USA) and primary antibodies against CREB, p-CREB (Ser133), PKA C-α, p-PKA C- α (Thr197), ERK1/2, p-ERK1/2 (Thr202/Tyr204), p38, and p-p38 (Thr180/Tyr182) were purchased from Cell signaling Technology (Danvers, MA, USA). Horseradish peroxidase-conjugated secondary anti-rabbit and anti-mouse antibodies were purchased from Cell signaling Technology.

Cell culture

B16F10 murine melanoma cells and HaCaT keratinocytes were purchased from ATCC (Manassas, VA, USA). Cells were cultured in Dulbeco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Biowest, Paris, France) and 1% penicillin/streptomycin (PS; Gibco). All cells were maintained in a humidified 5% CO_2 incubator at 37°C .

Cell viability assay

The effect of DPN on cell viability was assessed with water-soluble tetrazolum salt (WST-1) assay (EZ-Cytox; Dogen, Seoul, Korea). B16F10 cells (4×10^4) were seeded in 96 well plate and cultured for 24 h. Then, cells were further incubated with DPN (1–100 μM) for 24 or 48 h. The cell viability was determined by measuring formation of formazan from tetrazolium salt, absorbance at 450 nm using SYNERGY HTX multi-mode reader (BioTek, Winooski, VT, USA).

Determination of melanin contents

B16F10 melanoma cells (2×10^5) were seeded in 60 mm plate and cultured for 24 h. Then, cells were co-treated with DPN (20 and 50 μM) and α-MSH (100 nM) followed by incubation for 48 h. Cells were harvested and lysed with 200 μl of 1 N NaOH, followed by boiling at 100°C for 1 h. The intracellular melanin contents were determined by measuring absorbance at 450 nm using SYNERGY HTX multi-mode reader. Total protein amount was quantified with Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), standardized with bovine serum albumin. 17β-estradiol (1 and 10 μM) was used for determining the effect of ER activation on melanogenesis in α-MSH-treated B16F10 cells. Arbutin (500 μM) was used as positive control.

Measurement of tyrosinase activity

The cellular tyrosinase activity was examined using B16F10 cells. Cells (2×10^5) were seeded in 60 mm plate and further cultured for 24 h. Then, cells were co-treated with DPN (20 and 50 μM) and α-MSH (100 nM) followed by incubation for 48 h. Cells were harvested and lysed with radioimmunoprecipitation assay (RIPA) buffer. Total protein amount was quantified with Pierce BCA Protein Assay Kit according to manufacturer's protocol, standardized with BSA. The obtained supernatant was reacted with L-DOPA (1 mM) and spectrophotometric analysis of dopachrome formation was performed at 450 nm absorbance using SYNERGY HTX multi-mode reader. Arbutin (500 μM) was used as positive control.

To measure *in vitro* mushroom tyrosinase activity, dopachome formation from L-DOPA by mushroom tyrosinase was analyzed. DPN (1–100 μM) was reacted with L-DOPA (1 mM) and mushroom tyrosinase (100 U/ml) in 0.1 M sodium phosphate buffer (pH 6.8), followed by incubation for 10 min at 37°C. The relative tyrosinase activities were assessed by measuring absorbance at 450 nm using SYNERGY HTX multi-mode reader. Arbutin (500 μM) was used as positive control.

RNA analysis

B16F10 cells (2×10^5) were seeded in 60 mm plate and further cultured for 24 h. Then, cells were co-treated with DPN (20 and 50 μM) and α-MSH (100 nM) followed by incubation for 24 h. Total RNAs were extracted from B16F10 melanoma cells using Ribo-EX (Geneall, Seoul, Korean). One microgram of total RNAs were reverse-transcribed into cDNA using Moloney Murine Leukemia Virus reverse transcriptase (M-MLV) (Invitrogen, Waltham, MA, USA) according to the manufacturer's protocol. The transcriptional expression levels of MITF, tyrosinase, TRP-1, and TRP-2 were quantified by quantitative real time PCR (qRT-PCR) using StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). All reactions in qRT-PCR analysis were performed using SYBR green PCR master mix (Thermo Fisher Scientific) according to manufacturer's protocol. The cDNA was amplified with the following primers: *MITF*: forward, 5'-AGAAGCTGGAGCATGCGAACC-3' and reverse, 5'-GTTCCTGGCTGCAGTTCTCAAG-3', *Tyrosinase*: forward, 5'-AGTCGTATCTGGCCATGGCTTCTTG-3', reverse, 5'-GCAAGCTGTGGTAGTCGTCTTTGTC-3', *TRP-1*: forward, 5'-CTGCGATGTCTGCACTG ATGACCTG-3', reverse, 5'-TTTCTCCTGATTGGTCCACCCTCAG-3', *TRP-2*: forward, 5'-CGTGCTGAACAAGGAATGCT-3', reverse, 5'-GCATGTC-CGGTTGAAGAAT-3', *GAPDH*; forward, 5'-GTCTCCTCT-GACTTCAACAGCG-3' and reverse, 5'- ACCACCCTGTTGCT-GTAGCCAA-3'.

Melanin content analysis using UVB-irradiated HaCaT-conditioned medium

UVB-irradiated HaCaT conditioned media system was modified according to previous study [28]. HaCaT keratinocyte cells (1×10^6) were seeded in 100 mm plate and further cultured for 24 h. Cells were washed with phosphate-buffered saline (PBS; Gibco) prior to UVB irradiation. Cells were irradiated with 30 mJ/cm^2 of UVB using BoTeck Super Light–IV UV illuminator (BoTeck, Gunpo, Korea) and further cultured with fresh culture media for 24 h. Conditioned media from UVB-irradiated or nonirradiated HaCaT cells were harvested and co-treated with DPN (20 and 50 μM) to B16F10 cells. After 48 h of incubation, the melanin contents analysis was performed using the same method as mentioned above.

Statistical analysis

All data were analyzed using One-Way ANOVA followed by Tukey's test in Prism version 8.0.1 (GraphPad software, San Diego, CA, USA) and are presented as mean ± SD. p-value < 0.05 was considered statistically significant.

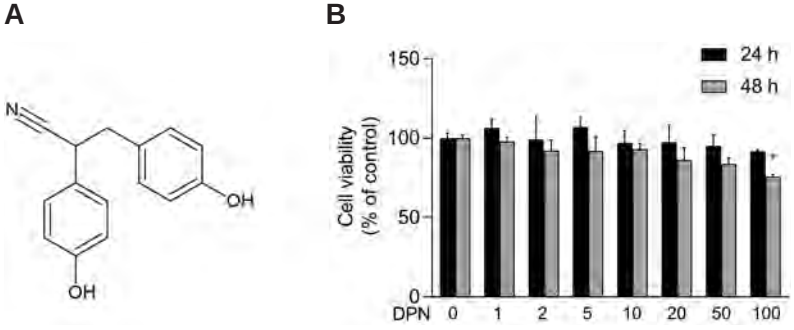

Fig. 1. Cell viability of B16F10 melanoma cells after treatment with DPN. (A) Chemical structure of DPN. (B) B16F10 cells were treated with DPN (1–100 μM). Cells were further cultured for 24 h and 48 h, then the cell viability was determined by WST-1 assay. Data are shown as mean ± SD from three independent experiments. DPN, diarylpropionitrile; WST-1, water-soluble tetrazolum salt. *p < 0.05 were considered as statistically significant.

RESULTS

DPN attenuated melanin synthesis in B16F10 melanoma cells

DPN, an ERβ selective agonist derived from resveratrol, has been known to include a structure of stilbenoid which has high affinity and selectivity for ERs (Fig. 1A) [8]. Prior to investigate whether DPN affects melanogenesis, the cell cytotoxicity of DPN in B16F10 melanoma cell line was examined by WST-1 assay at 24 and 48 h. As shown in Fig. 1B, the cell viability was not affected by DPN treatment (1–100 μM) even at the maximum concentration at 24 h. At 48 h, DPN had no cytotoxicity on B16F10 at low concentration but marginally reduced cell viability at the concentration of 100 μM ($p < 0.05$). To elucidate whether DPN affects melanin synthesis, the cells were co-treated with α-MSH and DPN (20 and 50 μM) for 48 h then the intracellular melanin contents were examined. As a result, DPN significantly blocked the melanin production in α-MSH-stimulated B16F10 cells (Fig. 2A). As tyrosinase is the key enzyme in melanin synthesis [10], we analyzed the cellular tyrosinase activity by measuring conversion of L-DOPA into dopachrome. The cellular tyrosinase activities were assessed with tyrosinase obtained from B16F10 co-treated with α-MSH and DPN for 48 h. DPN significantly inhibited the cellular tyrosinase activities in α-MSH-stimulated B16F10 cells (Fig. 2B). To determine whether DPN directly affects the enzymatic activity of tyrosinase, the effect of DPN (1–100 μM) on *in vitro* mushroom tyrosinase activity was evaluated. As shown in Fig. 2C, DPN did not have significant effect on the *in vitro* mushroom tyrosinase activities relative to the control even at the maximum concentration, indicating that DPN might affect the intracellular tyrosinase expression level. Since it has been known that DPN can induce estrogen signaling by binding ER [29,30], we next investigated whether estrogen signaling affects melanogenesis using 17β-estradiol in α-MSH-treated B16F10. As severe cytotoxicity was observed in B16F10 cells treated with 20 μM

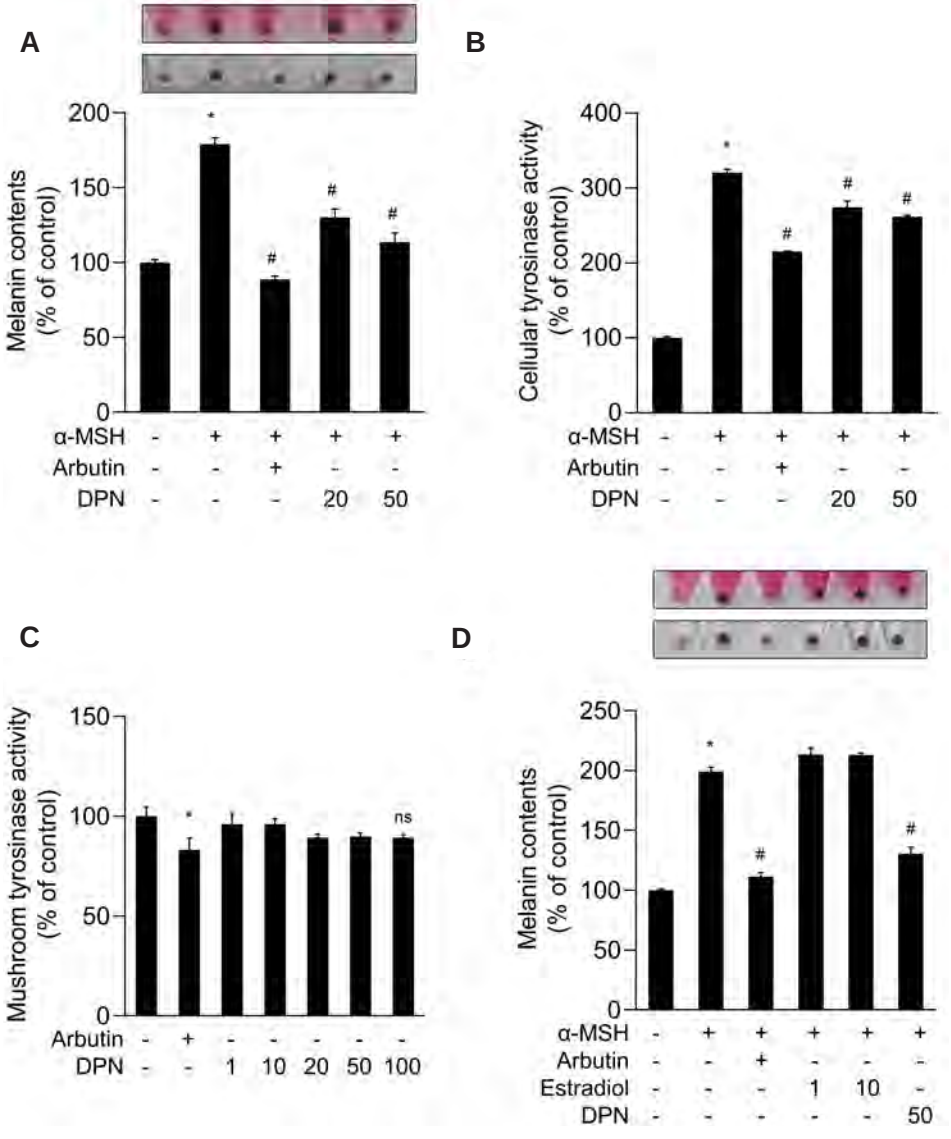

Fig. 2. Effect of DPN on melanogenesis in α-MSH-stimulated B16F10 cells. B16F10 cells were co-treated with α-MSH (100 nM) and DPN (20 and 50 μM). (A) After 48 h, the melanin contents were examined by measuring absorbance at 450 nm. (B) Cellular tyrosinase activities were examined by analyzing conversion of L-DOPA into dopachrome by measuring absorbance at 490 nm. (C) The effects of DPN (1–100 μM) on enzymatic activity of tyrosinase were examined using mushroom tyrosinase in cell free system. Tyrosinase activities were determined by analyzing conversion of L-DOPA into dopachrome. (D) B16F10 cells were co-treated with α-MSH (100 nM) and 17β-estradiol (1 and 10 μM) or α-MSH (100 nM) and DPN (50 μM). Cells were further cultured for 48 h and the melanin contents were determined by measuring absorbance at 450 nm. Arbutin (500 μM) was used as positive control. Data are shown as mean ± SD from three independent experiments. DPN, diarylpropionitrile; α-MSH, alpha-melanocyte stimulating hormone; DOPA, dihydroxyphenylalanine; ns, not significant. *$p < 0.05$ were considered as statistically significant from vehicle treated group. #$p < 0.05$ were considered as statistically significant from α-MSH-treated group.

17β-estradiol through previous experiments (Supplementary Fig. 1), we analyzed the intracellular melanin amounts in B16F10 cells treated with 1 and 10 μM 17β-estradiol. As shown in Fig. 2D, 17β-estradiol did not affect the melanin biosynthesis in α-MSH-stimulated B16F10 cells at both concentrations of 1 and 10 μM, whereas DPN effectively inhibited melanogenesis. These results suggest that DPN can interfere melanin synthesis independently of the ER signaling pathway.

DPN inhibited the expression of tyrosinase and its related genes in B16F10 melanoma cells

Since DPN did not directly influence on tyrosinase enzymatic activity, we examined whether DPN affects the expressions of melanogenic enzymes including tyrosinase and tyrosinase-related proteins (Fig. 3A). Immunoblotting analysis showed that the expressions of tyrosinase, TRP-1, and TRP-2 were down-regulated in α-MSH-stimulated B16F10 cells relative to control. Next, quantitative RT-PCR analysis revealed that DPN down-regulated the transcriptional expressions of tyrosinase, TRP-1, and TRP-2 genes in α-MSH-stimulated B16F10 cells (Fig. 3B–D). Taken together, these data showed that the expressions of tyrosinase, TRP-1, and TRP-2 were inhibited by DPN at the transcription level.

The inhibitory effect of DPN on melanogenesis was mediated by cAMP/PKA/CREB signaling pathway

It has been reported that MITF acts as a master transcription factor for the expression of tyrosinase and tyrosinase related proteins including TRP-1 and TRP-2 in the process of melanin biosynthesis [31]. So, we examined whether DPN affects the expression of MITF. Immunoblot analysis indicated that the expression of MITF was significantly inhibited by DPN in a dose-dependent manner (Fig. 4A). Subsequently, qRT-PCR analysis showed that DPN down-regulated the transcriptional expression level of MITF (Fig. 4B). It has been well known that α-MSH can induce the expression of MITF through PKA/CREB signaling pathway in melanocyte [24,25]. Therefore, down-regulation of MITF by

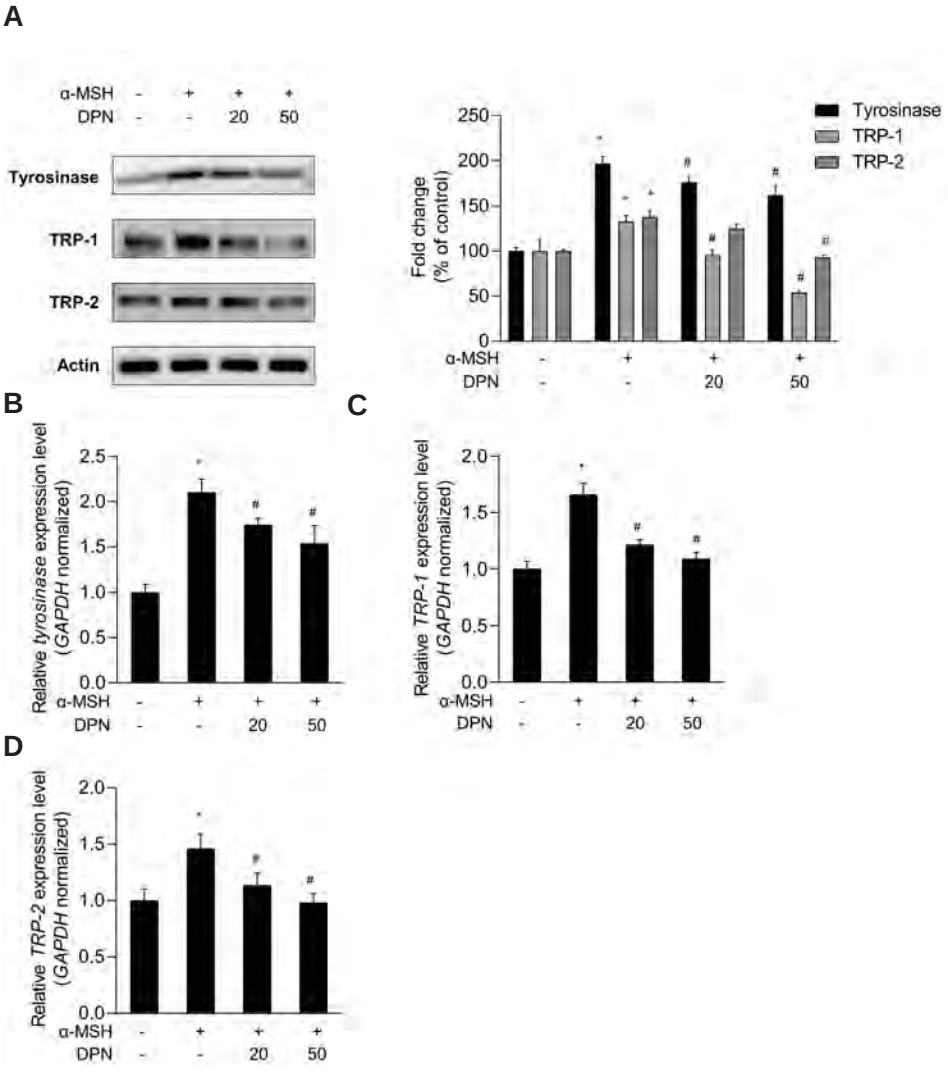

Fig. 3. Effect of DPN on the expression of tyrosinase and its related genes in α-MSH-stimulated B16F10 cells. (A) B16F10 cells were co-treated with α-MSH (100 nM) and DPN (20 and 50 μM) for 24 h. The expression levels of tyrosinase, TRP-1 and TRP-2 were examined by immunoblotting with their specific antibodies. Actin was used as a loading control. Indicated immunoblot intensities were analyzed by Image-J. (B–D) The transcriptional expression levels of tyrosinase, TRP-1 and TRP-2 were examined by qRT-PCR. GAPDH was used for normalizing mRNA expression level. Data are shown as mean ± SD from three independent experiments. DPN, diarylpropionitrile; α-MSH, alpha-melanocyte stimulating hormone; TRP, tyrosinase-related protein. *$p < 0.05$ were considered as statistically significant from vehicle treated group. #$p < 0.05$ were considered as statistically significant from α-MSH-treated group.

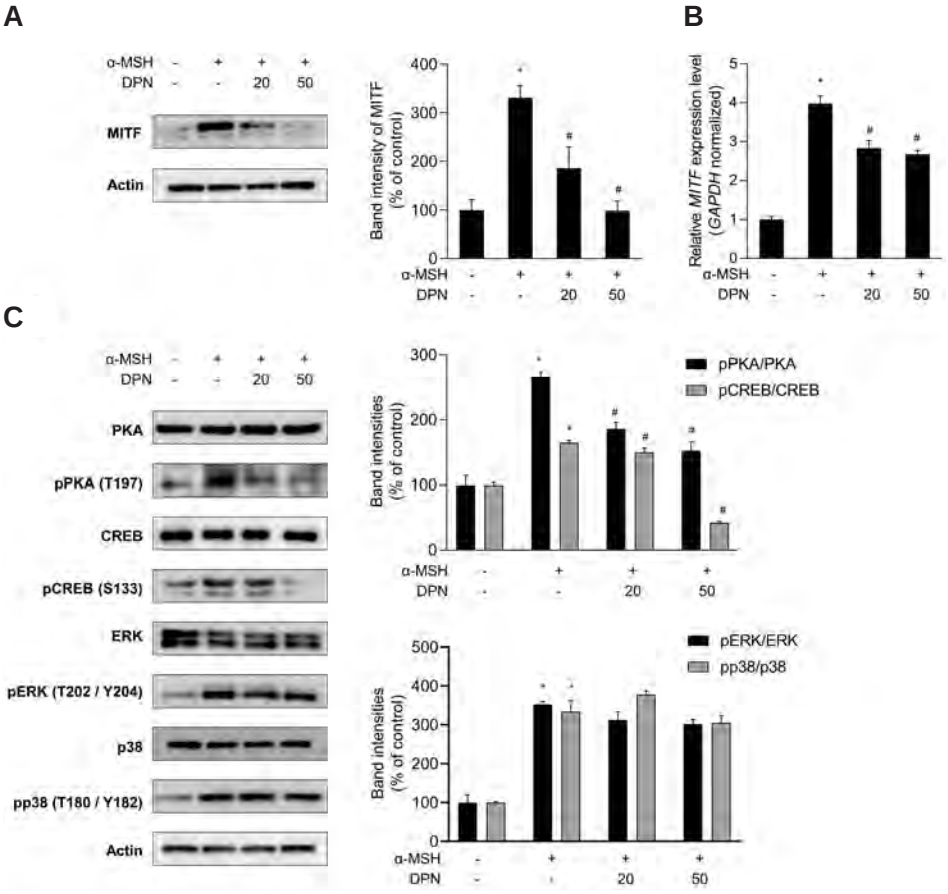

Fig. 4. Effect of DPN on the cAMP/PKA/CREB signaling pathway in α-MSH-stimulated B16F10 cells. (A) B16F10 cells were co-treated with α-MSH (100 nM) and DPN (20 and 50 μM). After 24 h, the expression level of MITF was examined by immunoblotting. (B) Cells were co-treated with α-MSH (100 nM) and DPN (20 and 50 μM). The cells were further cultured for 24 h and mRNA expression level of MITF was examined by qRT-PCR. Data are shown as mean ± SD from three independent experiments. (C) The expressions of PKA, pPKA, CREB, pCREB, ERK, pERK, p38, and pp38 were examined by immunoblotting with their specific antibodies. Indicated immunoblot intensities were analyzed by ImageJ. DPN, diarylpropionitrile; PKA, protein kinase A; CREB, cAMP-response element-binding protein; MITF, microphthalmia-associated transcription factor; α-MSH, alpha-melanocyte stimulating hormone; ERK, extracellular signal-regulated kinase. *$p < 0.05$ were considered as statistically significant from vehicle treated group. #$p < 0.05$ were considered as statistically significant from α-MSH-treated group.

DPN led us to explore whether DPN can affect the phosphorylations of CREB and PKA. As shown in Fig. 4C, the phosphorylations of PKA at threonine 197 residue and CREB at serine 133 residue were reduced by treatment with DPN in α-MSH-stimulated B16F10 cells. These results indicate that DPN down-regulates PKA and CREB phosphorylation, thereby reducing MITF gene transcription. Since ERK and p38 have been reported to affect CREB phosphorylation at serine 133 residue [17,32-34], we further investigated whether the phosphorylation levels of ERK and p38 were affected by DPN. In Fig. 4C, immunoblotting showed that the phosphorylations of ERK and p38 were not affected by DPN in α-MSH-stimulated B16F10 cells. These findings indicated that DPN does not affect ERK and p38 signaling, but mediates PKA signaling, thereby interfering with melanin synthesis.

DPN has anti-melanogenic activity in UVB-irradiated HaCaT conditioned media system

In the skin under physiological conditions, keratinocytes generate a series of substances by UVB irradiation, which are transferred to neighboring melanocytes with paracrine action to induce melanogenesis [35]. To investigate whether DPN inhibits melanin synthesis under UV irradiation conditions mimicking physiological skin environment, B16F10 cells were cultured with UVB-irradiated HaCaT keratinocyte conditioned media (UV-CM) and then anti-melanogenic effect of DPN was examined (Fig. 5). As shown Fig. 5, the melanin contents of B16F10 cells were increased by culturing with UV-CM relative to conditioned media not irradiated with UVB (NUV-CM) and DPN effectively down-regulated the melanin contents of B16F10 cells in a dose-dependent manner. This observation suggests the potential of DPN to inhibit melanin synthesis in a physiological environment.

DISCUSSION

DPN is an ERβ agonist with high binding affinity, and it has been specifically studied for its hormonal bioactivities associated with ERβ [1,2,5]. However, potential bioactivities of DPN independent of the ERβ signaling pathway remain unclear. DPN was first synthesized from a derivative of resveratrol that acts as a partial agonist on ERα and has a chemical structure of stilbenoid (Fig. 1A) [8]. Stilbenoids have been reported to belong to phytoestrogens similar to endogenously produced mammalian estrogens [8]. Glyceollin II and resveratrol were also reported to have the same stilbenoid structure as DPN [8] and interestingly, these chemicals have also been reported to inhibit melanogenesis [36-39]. These studies led us to predict that DPN can affect the melanin biosynthesis mechanism independently of the ERβ signaling pathway through the stilbenoid structure. In this study

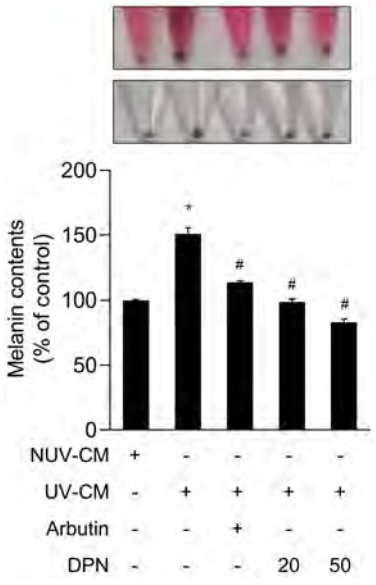

Fig. 5. Effect of DPN on melanogenesis using UVB-irradiated HaCaT conditioned media system. HaCaT keratinocytes were irradiated with 30 mJ/cm^2 of UVB and further cultured for 24 h, then cultured media which referred to as conditioned media (UV-CM) were harvested. B16F10 cells were treated with DPN (20 and 50 μM) and UV-CM and further cultured for 48 h. The melanin contents were examined by measuring the absorbance at 450 nm. HaCaT cultured media not irradiated with UVB were used as a negative control (NUV-CM). Arbutin was used as a positive control. *$p < 0.05$ were considered as statistically significant from vehicle treated group. Data are shown as mean ± SD from three independent experiments. DPN, diarylpropionitrile. #$p < 0.05$ were considered as statistically significant from the conditioned media-treated group.

we analyzed for the first time the inhibitory effect of DPN on melanogenesis in α-MSH-stimulated B16F10 melanoma cells. In addition, we demonstrated that DPN also exerts an inhibitory effect on UVB-induced melanogenesis using a UV-irradiated keratinocyte conditioned medium model that mimics the physiological skin melanogenesis environment.

It has been reported that melanin biosynthesis can be regulated in various ways [10,40]. The most important process in melanin biosynthesis depends on the activity of tyrosinase, a melanocyte-specific enzyme [41]. Several studies have indicated a regulation that can inhibit melanin biosynthesis by directly inhibiting the enzymatic activity of tyrosinase [42,43]. In addition, several reports suggested that tyrosinase can affect melanin synthesis by regulating the amount of intracellular expression [44-48]. It has been reported that the cellular expression of tyrosinase can be affected by transcriptional regulation [44-46] or by post-transcriptional regulation by protein ubiquitination [47,48]. Fig. 2C indicated that DPN does not directly affect the tyrosinase enzymatic activity. Furthermore, Fig. 3 showed that DPN can inhibit melanin biosynthesis by inhibiting the intracellular expression of tyrosinase at the transcriptional level. In Fig. 3, the transcriptional expressions of tyrosinase, TRP-1, and TRP-2 proteins were down-regulated by DPN in α-MSH treated B16F10 cells indicating that the expression of MITF could be affected by DPN. MITF has been reported as a transcription factor for multiple cellular processes including melanogenesis, differentiation, proliferation, and cell survival [49]. Previous studies have demonstrated that the expressions of key melanogenic enzymes including tyrosinase and TRP-1, and TRP-2 are regulated by MITF during melanogenesis [46,50,51]. Fig. 4A indicated that DPN dramatically inhibits the expression of cellular MITF at the transcriptional level. We further investigated whether CREB, an up-stream regulator of MITF [25], and protein kinases of which activate CREB were affected by DPN. Several studies have reported protein kinases that phosphorylate CREB at serine 133 residue [26,27,40]. It has been demonstrated that PKA phosphorylates CREB in a cAMP-dependent pathway, an important second messenger during melanogenesis [24,40,52]. It has been reported that p38 MAPK is activated by α-MSH stimulation to induce phosphorylation at serine 133 residue in CREB [26,34,53]. It has also been reported that ERK is activated in cAMP-dependent manner involving the Ras signaling pathway and can induce the phosphorylation at CREB serine 133 residue independently of PKA in melanocytes [54]. Fig. 4C indicated that phosphorylations of PKA and CREB were inhibited by DPN, whereas p38 MAPK was not affected. There have been conflicting studies on the effect of ERK on melanogenesis. Several reports indicate that ERK directly phosphorylates MITF at serine 73 residue, leading to ubiquitin-dependent degradation of MITF [55,56]. However, these studies were performed in the presence of ROS accumulation or in c-kit-induced melanogenesis. Interestingly, previous study has demonstrated that cAMP was increased by DPN in rat aortic smooth muscle and this is opposite phenomenon from our data [57]. This difference is expected to be due to different origins of the samples, tissues, and cultured cells, or mismatch of cell type. Moreover, our data suggests that DPN did not affect the intracellular level of cAMP because cAMP-dependent phosphorylation of ERK was not affected by DPN (Supplementary Fig. 2) [54,58]. Nevertheless, further investigations are needed to identify the precise regulatory mechanisms of DPN in PKA phosphorylation.

Estrogen signaling has been reported to positively regulate melanin biosynthesis and tyrosinase activity in cultured melanocytes [59]. It has also been reported that the production of estrogen is relate to the physiological environment including melasma in pregnancy [60]. Estrogen exerts its biological effects through its specific receptors, ERα and ERβ which are nuclear receptors that form homo- or heterodimer and act as transcription factor [61]. Our data showed that DPN, which can activate ER signaling with binding affinity to ERβ, inhibited α-MSH-induced melanin production (Fig. 2A). This result led us to examine the relationship between ER signaling and melanogenesis in α-MSH-stimulated B16F10 cells. It has been reported that 17β-estradiol, the most abundant estrogen in a physiological condition, binds to ERα and ERβ [29,30]. Therefore, the effect of 17β-estradiol on

melanin synthesis in α-MSH-stimulated B16F10 was analyzed to examine whether the activation of ER signaling has an effect on α-MSH-induced melanogenesis. In Fig. 2D, DPN inhibited melanin biosynthesis, but 17β-estradiol (1 and 10 μM) did not inhibit melanin biosynthesis and even slightly promoted melanin production. This result corresponds to previous studies which have reported that 17β-estradiol enhances tyrosinase activity and melanogenesis [59,62]. Interestingly, there are also reports that 17β-estradiol also binds to ERα and G protein-coupled estrogen receptor (GPER) [30,63]. GPER is partially responsible for inducing melanogenesis in melanoma. It has also been reported that silymarin, known as phytoestrogen, is a selective ERβ agonist and reported to inhibit melanin synthesis [64,65]. Our experimental results did not rule out whether DPN inhibits melanin biosynthesis through GPER signaling or selective ERβ signaling mechanisms. Additional experiments are needed to clarify the inhibitory mechanism of melanin biosynthesis by DPN.

To date, the general model for analyzing the effect on melanin biosynthesis in cells has been used the α-MSH-treated B16F10 mouse melanoma cell line [66,67]. In a physiological environment, skin pigmentation is mainly initiated by UV irradiation. After UV irradiation, keratinocytes release proopiomelanocortin (POMC) precursor peptide, which is then cleaved with α-MSH and ACTH [21,68]. These POMC-derived peptide hormones start to induce melanin biosynthesis by acting as ligands of MC1R expressed on neighboring melanocytes [66,68]. We introduced a UV-irradiated keratinocyte conditioned media system that mimics the physiological skin environment and determined whether DPN affects melanin biosynthesis using this model (Fig. 6). Our results demonstrate the potential of DPN as an anti-pigmentation agent that can be applied to the skin. Further experiment using 3D-reconstucted human pigmented epidermis of skin model is required to validate the anti-pigmentation effect of DPN in a physiological environment.

In conclusion, we demonstrated that DPN inhibited the melanin biosynthesis and it was mediated by modulation of PKA/CREB signaling axis leading to down-regulation of MITF, tyrosinase, and tyrosinase-related proteins via an estrogen receptor-independent pathway (Fig. 6). We also confirmed anti-melanogenetic effect of DPN using a UVB-irradiated HaCaT conditioned medium model that mimics the physiological skin environment. Collectively, our data indicated that DPN could be a potential agent for the treatment of hyper-pigmentation.

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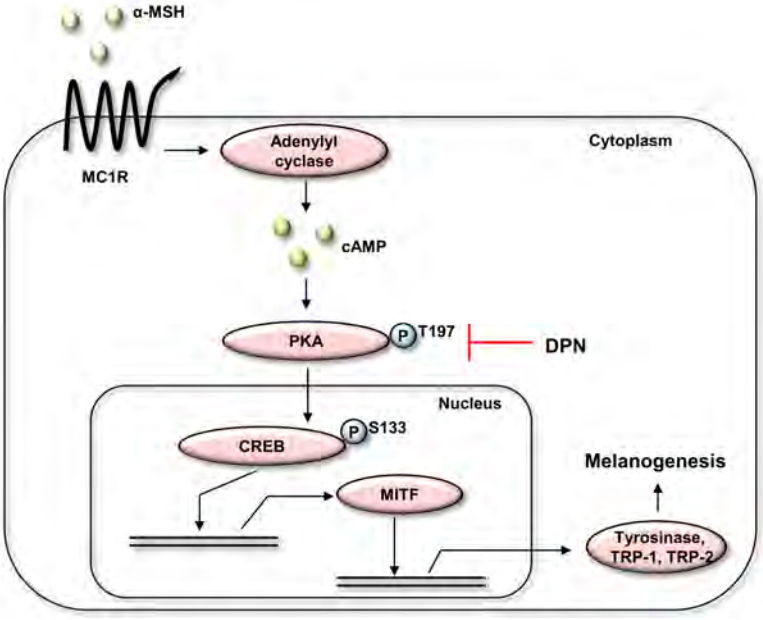

Fig. 6. Schematic illustration of inhibitory effect on melanogenesis *via* PKA/CREB/MITF axis by DPN. DPN inhibits melanin synthesis by down-regulating the activation of PKA and CREB in α-MSH-stimulated B16F10. DPN, diarylpropionitrile; PKA, protein kinase A; CREB, cAMP-response element-binding protein; MITF, microphthalmia-associated transcription factor; α-MSH, alpha-melanocyte stimulating hormone; TRP, tyrosinase-related protein; MC1R, melanocortin 1 receptor.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary data including two figures can be found with this article online at https://doi.org/10.4196/kjpp.2022.26.2.113.

REFERENCES

1. Meyers MJ, Sun J, Carlson KE, Marriner GA, Katzenellenbogen BS, Katzenellenbogen JA. Estrogen receptor-beta potency-selective ligands: structure-activity relationship studies of diarylpropionitriles and their acetylene and polar analogues. *J Med Chem.* 2001;44:4230-4251.
2. Walf AA, Koonce CJ, Frye CA. Estradiol or diarylpropionitrile administration to wild type, but not estrogen receptor beta knockout, mice enhances performance in the object recognition and object placement tasks. *Neurobiol Learn Mem.* 2008;89:513-521.
3. Motylewska E, Stasikowska O, Mełeń-Mucha G. The inhibitory effect of diarylpropionitrile, a selective agonist of estrogen receptor beta, on the growth of MC38 colon cancer line. *Cancer Lett.* 2009;276:68-73.
4. Huang SY, Xin H, Sun J, Li R, Zhang XM, Zhao D. Estrogen receptor β agonist diarylpropionitrile inhibits lipopolysaccharide-induced regulated on activation normal T cell expressed and secreted (RANTES) production in macrophages by repressing nuclear factor κB activation. *Fertil Steril.* 2013;100:234-240.
5. Weiser MJ, Wu TJ, Handa RJ. Estrogen receptor-beta agonist diarylpropionitrile: biological activities of R- and S-enantiomers on behavior and hormonal response to stress. *Endocrinology.* 2009;150:1817-1825.
6. Mancuso M, Leonardi S, Giardullo P, Pasquali E, Borra F, Stefano ID, Prisco MG, Tanori M, Scambia G, Majo VD, Pazzaglia S, Saran A, Gallo D. The estrogen receptor beta agonist diarylpropionitrile (DPN) inhibits medulloblastoma development via anti-proliferative and pro-apototic pathways. *Cancer Lett.* 2011;308:197-202.
7. Michel T, Halabalaki M, Skaltsounis AL. New concepts, experimental approaches, and dereplication strategies for the discovery of novel phytoestrogens from natural sources. *Planta Med.* 2013;79:514-532.
8. Yang J, Wen L, Jiang Y, Yang B. Natural estrogen receptor modulators and their heterologous biosynthesis. *Trends Endocrinol Metab.* 2019;30:66-76.
9. Pillaiyar T, Namasivayam V, Manickam M, Jung SH. Inhibitors of melanogenesis: an updated review. *J Med Chem.* 2018;61:7395-7418.
10. D'Mello SA, Finlay GJ, Baguley BC, Askarian-Amiri ME. Signaling pathways in melanogenesis. *Int J Mol Sci.* 2016;17:1144.
11. Vachtenheim J, Borovanský J. "Transcription physiology" of pigment formation in melanocytes: central role of MITF. *Exp Dermatol.* 2010;19:617-627.
12. Tsukamoto K, Jackson IJ, Urabe K, Montague PM, Hearing VJ. A second tyrosinase-related protein, TRP-2, is a melanogenic enzyme termed DOPAchrome tautomerase. *EMBO J.* 1992;11:519-526.
13. Kobayashi T, Urabe K, Winder A, Jiménez-Cervantes C, Imokawa G, Brewington T, Solano F, García-Borrón JC, Hearing VJ. Tyrosinase related protein 1 (TRP1) functions as a DHICA oxidase in melanin biosynthesis. *EMBO J.* 1994;13:5818-5825.
14. Chen T, Zhao B, Liu Y, Wang R, Yang Y, Yang L, Dong C. MITF-M regulates melanogenesis in mouse melanocytes. *J Dermatol Sci.* 2018;90:253-262.
15. Du J, Miller AJ, Widlund HR, Horstmann MA, Ramaswamy S, Fisher DE. MLANA/MART1 and SILV/PMEL17/GP100 are transcriptionally regulated by MITF in melanocytes and melanoma. *Am J Pathol.* 2003;163:333-343.
16. Aoki H, Moro O. Involvement of microphthalmia-associated transcription factor (MITF) in expression of human melanocortin-1 receptor (MC1R). *Life Sci.* 2002;71:2171-2179.
17. Tagashira H, Miyamoto A, Kitamura S, Tsubata M, Yamaguchi K, Takagaki K, Imokawa G. UVB stimulates the expression of endothelin B receptor in human melanocytes via a sequential activation of the p38/MSK1/CREB/MITF pathway which can be interrupted by a French maritime pine bark extract through a direct inactivation of MSK1. *PLoS One.* 2015;10:e0128678.
18. Box NF, Terzian T. The role of p53 in pigmentation, tanning and melanoma. *Pigment Cell Melanoma Res.* 2008;21:525-533.
19. Yamaguchi Y, Coelho SG, Zmudzka BZ, Takahashi K, Beer JZ, Hearing VJ, Miller SA. Cyclobutane pyrimidine dimer formation and p53 production in human skin after repeated UV irradiation. *Exp Dermatol.* 2008;17:916-924.
20. Cui R, Widlund HR, Feige E, Lin JY, Wilensky DL, Igras VE, D'Orazio J, Fung CY, Schanbacher CF, Granter SR, Fisher DE. Central role of p53 in the suntan response and pathologic hyperpigmentation. *Cell.* 2007;128:853-864.
21. Yamamoto H, Yamane T, Iguchi K, Tanaka K, Iddamalgoda A, Unno K, Hoshino M, Takeda A. Melanin production through novel processing of proopiomelanocortin in the extracellular compartment of the auricular skin of C57BL/6 mice after UV-irradiation. *Sci Rep.* 2015;5:14579.
22. Neary CL, Cho-Chung YS. Nuclear translocation of the catalytic subunit of protein kinase A induced by an antisense oligonucleotide directed against the RIalpha regulatory subunit. *Oncogene.* 2001;20:8019-8024.
23. Yun CY, You ST, Kim JH, Chung JH, Han SB, Shin EY, Kim EG. p21-activated kinase 4 critically regulates melanogenesis via activation of the CREB/MITF and β-catenin/MITF pathways. *J Invest Dermatol.* 2015;135:1385-1394.
24. Buscà R, Ballotti R. Cyclic AMP a key messenger in the regulation of skin pigmentation. *Pigment Cell Res.* 2000;13:60-69.
25. Hartman ML, Czyz M. MITF in melanoma: mechanisms behind its expression and activity. *Cell Mol Life Sci.* 2015;72:1249-1260.
26. Smalley K, Eisen T. The involvement of p38 mitogen-activated protein kinase in the alpha-melanocyte stimulating hormone (alpha-MSH)-induced melanogenic and anti-proliferative effects in B16

murine melanoma cells. *FEBS Lett.* 2000;476:198-202.
27. Bae IH, Lee ES, Yoo JW, Lee SH, Ko JY, Kim YJ, Lee TR, Kim DY, Lee CS. Mannosylerythritol lipids inhibit melanogenesis via suppressing ERK-CREB-MiTF-tyrosinase signalling in normal human melanocytes and a three-dimensional human skin equivalent. *Exp Dermatol.* 2019;28:738-741.
28. Jung JM, Noh TK, Jo SY, Kim SY, Song Y, Kim YH, Chang SE. Guanine deaminase in human epidermal keratinocytes contributes to skin pigmentation. *Molecules.* 2020;25:2637.
29. Marzagalli M, Casati L, Moretti RM, Montagnani Marelli M, Limonta P. Estrogen receptor β agonists differentially affect the growth of human melanoma cell lines. *PLoS One.* 2015;10:e0134396.
30. Marzagalli M, Montagnani Marelli M, Casati L, Fontana F, Moretti RM, Limonta P. Estrogen receptor β in melanoma: from molecular insights to potential clinical utility. *Front Endocrinol (Lausanne).* 2016;7:140.
31. Levy C, Khaled M, Fisher DE. MITF: master regulator of melanocyte development and melanoma oncogene. *Trends Mol Med.* 2006;12:406-414.
32. Niwano T, Terazawa S, Nakajima H, Imokawa G. The stem cell factor-stimulated melanogenesis in human melanocytes can be abrogated by interrupting the phosphorylation of MSK1: evidence for involvement of the p38/MSK1/CREB/MITF axis. *Arch Dermatol Res.* 2018;310:187-196.
33. Cheng X, Ma Y, Moore M, Hemmings BA, Taylor SS. Phosphorylation and activation of cAMP-dependent protein kinase by phosphoinositide-dependent protein kinase. *Proc Natl Acad Sci U S A.* 1998;95:9849-9854.
34. Saha B, Singh SK, Sarkar C, Bera R, Ratha J, Tobin DJ, Bhadra R. Activation of the Mitf promoter by lipid-stimulated activation of p38-stress signalling to CREB. *Pigment Cell Res.* 2006;19:595-605.
35. Yuan XH, Jin ZH. Paracrine regulation of melanogenesis. *Br J Dermatol.* 2018;178:632-639.
36. Lei MJ, Dong Y, Sun CX, Zhang XH. Resveratrol inhibits proliferation, promotes differentiation and melanogenesis in HT-144 melanoma cells through inhibition of MEK/ERK kinase pathway. *Microb Pathog.* 2017;111:410-413.
37. Park J, Park JH, Suh HJ, Lee IC, Koh J, Boo YC. Effects of resveratrol, oxyresveratrol, and their acetylated derivatives on cellular melanogenesis. *Arch Dermatol Res.* 2014;306:475-487.
38. Lee YS, Kim HK, Lee KJ, Jeon HW, Cui S, Lee YM, Moon BJ, Kim YH, Lee YS. Inhibitory effect of glyceollin isolated from soybean against melanogenesis in B16 melanoma cells. *BMB Rep.* 2010;43:461-467.
39. Shin SH, Lee YM. Glyceollins, a novel class of soybean phytoalexins, inhibit SCF-induced melanogenesis through attenuation of SCF/c-kit downstream signaling pathways. *Exp Mol Med.* 2013;45:e17.
40. Lee AY, Noh M. The regulation of epidermal melanogenesis via cAMP and/or PKC signaling pathways: insights for the development of hypopigmenting agents. *Arch Pharm Res.* 2013;36:792-801.
41. Bae-Harboe YS, Park HY. Tyrosinase: a central regulatory protein for cutaneous pigmentation. *J Invest Dermatol.* 2012;132:2678-2680.
42. Fan M, Zhang G, Hu X, Xu X, Gong D. Quercetin as a tyrosinase inhibitor: inhibitory activity, conformational change and mechanism. *Food Res Int.* 2017;100(Pt 1):226-233.
43. Chen QX, Kubo I. Kinetics of mushroom tyrosinase inhibition by quercetin. *J Agric Food Chem.* 2002;50:4108-4112.
44. Ferguson CA, Kidson SH. The regulation of tyrosinase gene transcription. *Pigment Cell Res.* 1997;10:127-138.
45. Verastegui C, Bertolotto C, Bille K, Abbe P, Ortonne JP, Ballotti R. TFE3, a transcription factor homologous to Microphthalmia, is a potential transcriptional activator of tyrosinase and TyrpI genes. *Mol Endocrinol.* 2000;14:449-456.
46. Murisier F, Guichard S, Beermann F. The tyrosinase enhancer is activated by Sox10 and Mitf in mouse melanocytes. *Pigment Cell Res.* 2007;20:173-184.
47. Ando H, Ichihashi M, Hearing VJ. Role of the ubiquitin proteasome system in regulating skin pigmentation. *Int J Mol Sci.* 2009;10:4428-4434.
48. Kageyama A, Oka M, Okada T, Nakamura S, Ueyama T, Saito N, Hearing VJ, Ichihashi M, Nishigori C. Down-regulation of melanogenesis by phospholipase D2 through ubiquitin proteasome-mediated degradation of tyrosinase. *J Biol Chem.* 2004;279:27774-27780.
49. Widlund HR, Fisher DE. Microphthalamia-associated transcription factor: a critical regulator of pigment cell development and survival. *Oncogene.* 2003;22:3035-3041.
50. Fang D, Tsuji Y, Setaluri V. Selective down-regulation of tyrosinase family gene TYRP1 by inhibition of the activity of melanocyte transcription factor, MITF. *Nucleic Acids Res.* 2002;30:3096-3106.
51. Ludwig A, Rehberg S, Wegner M. Melanocyte-specific expression of dopachrome tautomerase is dependent on synergistic gene activation by the Sox10 and Mitf transcription factors. *FEBS Lett.* 2004;556:236-244.
52. Roh E, Yun CY, Yun JY, Park D, Kim ND, Hwang BY, Jung SH, Park SK, Kim YB, Han SB, Kim Y. cAMP-binding site of PKA as a molecular target of bisabolangelone against melanocyte-specific hyperpigmented disorder. *J Invest Dermatol.* 2013;133:1072-1079.
53. Lee HS, Goh MJ, Kim J, Choi TJ, Lee HK, Na YJ, Cho KH. A systems-biological study on the identification of safe and effective molecular targets for the reduction of ultraviolet B-induced skin pigmentation. *Sci Rep.* 2015;5:10305.
54. Buscà R, Abbe P, Mantoux F, Aberdam E, Peyssonnaux C, Eychène A, Ortonne JP, Ballotti R. Ras mediates the cAMP-dependent activation of extracellular signal-regulated kinases (ERKs) in melanocytes. *EMBO J.* 2000;19:2900-2910.
55. Ko GA, Cho SK. Phytol suppresses melanogenesis through proteasomal degradation of MITF via the ROS-ERK signaling pathway. *Chem Biol Interact.* 2018;286:132-140.
56. Wu M, Hemesath TJ, Takemoto CM, Horstmann MA, Wells AG, Price ER, Fisher DZ, Fisher DE. c-Kit triggers dual phosphorylations, which couple activation and degradation of the essential melanocyte factor Mi. *Genes Dev.* 2000;14:301-312.
57. Valero MS, Pereboom D, Barcelo-Batllory S, Brines L, Garay RP, Alda JO. Protein kinase A signalling is involved in the relaxant responses to the selective β-oestrogen receptor agonist diarylpropionitrile in rat aortic smooth muscle in vitro. *J Pharm Pharmacol.* 2011;63:222-229.
58. Dumaz N, Marais R. Integrating signals between cAMP and the RAS/RAF/MEK/ERK signalling pathways. Based on the anniversary prize of the Gesellschaft für Biochemie und Molekularbiologie Lecture delivered on 5 July 2003 at the Special FEBS Meeting in Brussels. *FEBS J.* 2005;272:3491-3504.
59. Poletini MO, de Assis LV, Moraes MN, Castrucci AM. Estradiol dif-

ferently affects melanin synthesis of malignant and normal melanocytes: a relationship with clock and clock-controlled genes. *Mol Cell Biochem.* 2016;421:29-39.
60. Martin AG, Leal-Khouri S. Physiologic skin changes associated with pregnancy. *Int J Dermatol.* 1992;31:375-378.
61. Dika E, Patrizi A, Lambertini M, Manuelpillai N, Fiorentino M, Altimari A, Ferracin M, Lauriola M, Fabbri E, Campione E, Veronesi G, Scarfì F. Estrogen receptors and melanoma: a review. *Cells.* 2019;8:1463.
62. Ranson M, Posen S, Mason RS. Human melanocytes as a target tissue for hormones: in vitro studies with 1 alpha-25, dihydroxyvitamin D3, alpha-melanocyte stimulating hormone, and beta-estradiol. *J Invest Dermatol.* 1988;91:593-598.
63. Sun M, Xie HF, Tang Y, Lin SQ, Li JM, Sun SN, Hu XL, Huang YX, Shi W, Jian D. G protein-coupled estrogen receptor enhances melanogenesis via cAMP-protein kinase (PKA) by upregulating microphthalmia-related transcription factor-tyrosinase in melanoma. *J Steroid Biochem Mol Biol.* 2017;165(Pt B):236-246.
64. Seidlová-Wuttke D, Becker T, Christoffel V, Jarry H, Wuttke W. Silymarin is a selective estrogen receptor beta (ERbeta) agonist and has estrogenic effects in the metaphysis of the femur but no or antiestrogenic effects in the uterus of ovariectomized (ovx) rats. *J Steroid Biochem Mol Biol.* 2003;86:179-188.
65. Choo SJ, Ryoo IJ, Kim YH, Xu GH, Kim WG, Kim KH, Moon SJ, Son ED, Bae K, Yoo ID. Silymarin inhibits melanin synthesis in melanocyte cells. *J Pharm Pharmacol.* 2009;61:663-667.
66. Rouzaud F, Kadekaro AL, Abdel-Malek ZA, Hearing VJ. MC1R and the response of melanocytes to ultraviolet radiation. *Mutat Res.* 2005;571:133-152.
67. Chung S, Lim GJ, Lee JY. Quantitative analysis of melanin content in a three-dimensional melanoma cell culture. *Sci Rep.* 2019;9:780.
68. Abdel-Malek ZA, Kadekaro AL, Swope VB. Stepping up melanocytes to the challenge of UV exposure. *Pigment Cell Melanoma Res.* 2010;23:171-186.