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The DNA methylation inhibitor 5-azacytidine decreases melanin synthesis by inhibiting CREB phosphorylation

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Here we examined the effects of a DNA methylation inhibitor, 5-azacytidine, on melanogenesis in Mel-Ab cells. We found that 5-azacytidine decreased the melanin content and tyrosinase activity in these cells in a dose-dependent manner; importantly, 5-azacytidine was not cytotoxic at the concentrations used in these experiments. On the other hand, 5-azacytidine did not affect tyrosinase activity in a cell-free system, indicating that 5-azacytidine is not a direct tyrosinase inhibitor. Instead, 5-azacytidine decreased the protein levels of microphthalmia-associated transcription factor (MITF) and tyrosinase. Thus, we investigated the effects of 5-azacytidine on signal transduction pathways related to melanogenesis. However, 5-azacytidine did not have any effect on either Akt or glycogen synthase kinase 3β (GSK3β) phosphorylation. The phosphorylation of cAMP response element-binding protein (CREB) is well known to regulate MITF expression, thereby also regulating tyrosinase expression. We found that 5-azacytidine decreased the phosphorylation of CREB. Therefore, we propose that 5-azacytidine may decrease melanin synthesis by downregulating MITF and tyrosinase via CREB inactivation.

1. Introduction

Melanin pigment determines skin, hair, and eye color in mammals. Melanin is produced in specialized organelles called melanosomes that contain tyrosinase, which is a key enzyme in melanogenesis that catalyzes the two initial rate-limiting reactions of melanogenesis (Montefiori and Zhou 1991). Melanogenesis can be stimulated by α-melanocyte-stimulating hormone (α-MSH), endothelin-1 (ET-1), cytokines, and growth factors (Costin and Hearing 2007). The binding of α-MSH to the melanocortin 1 receptor (MC1R) generates an increase in the intracellular cyclic AMP (cAMP) concentration, which in turn leads to the phosphorylation of cAMP response element-binding protein (CREB) (Sassone-Corsi 1998). Activated CREB is known to up-regulate the expression of microphthalmia-associated transcription factor (MITF). MITF plays an important role in melanocyte development and differentiation and stimulates tyrosinase expression as a melanocyte-specific transcription factor (Hodgkinson et al. 1993; Steingrimsson et al. 1994). Therefore, increased tyrosinase levels due to CREB phosphorylation may facilitate melanin synthesis.

MITF expression is also regulated by glycogen synthase kinase 3β (GSK3β) phosphorylation (Takeda et al. 2000). In addition, several reports have suggested that the extracellular signal-regulated kinase (ERK) and Akt pathways are involved in the regulation of melanogenesis (Kim et al. 2006; Oka et al. 2000). 5-Azacytidine is a cytidine analogue that was originally developed as an antitumor agent (Fig. 1) and exerts its antitumor effects through its incorporation into RNA and DNA. In addition, 5-azacytidine has been shown to regulate cell growth in hematopoietic cells by DNA hypomethylation through the suppression of methyltransferase activity (Kaminskas et al. 2005). Recently, the microRNA (miRNA) miR-125b was reported to decrease melanin synthesis (Kim et al. 2014). Moreover, hypermethylation of the MIR125B-1 promoter reduced the expression of miR-125b in pigmented cells (Kim et al. 2014). In addition, the level of miR-125b has been shown to be controlled by the cAMP signaling pathway (Kim et al. 2014). From these data, we hypothesized that melanin synthesis may decrease when MIR125B-1 promoter methylation is inhibited by 5-azacytidine. Therefore, we investigated the effects of a DNA methylation inhibitor, 5-azacytidine, on melanin synthesis and tyrosinase activity in Mel-Ab cells. In addition, we examined the effects of 5-azacytidine on MITF expression, tyrosinase expression, and the activation of the cAMP signaling pathway.

2. Investigations and results

2.1. Effects of 5-azacytidine on the viability of Mel-Ab cells

To test the cytotoxicity of 5-azacytidine on Mel-Ab cells, we performed a crystal violet assay. The cells were treated with 5-

Abbreviations: α-MSH, α-melanocyte-stimulating hormone; CREB, cAMP response element-binding protein; ERK, extracellular signal-regulated kinase; GSK3β, glycogen synthase kinase 3β; MITF, microphthalmia-associated transcription factor.

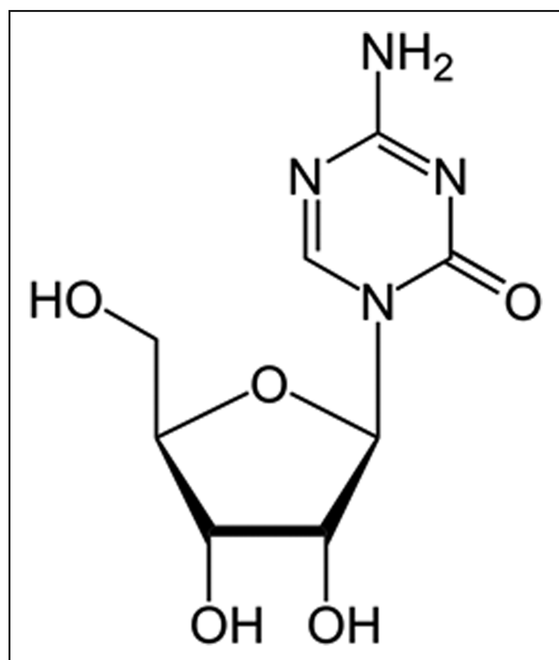

Fig. 1: Structure of 5-azacytidine.

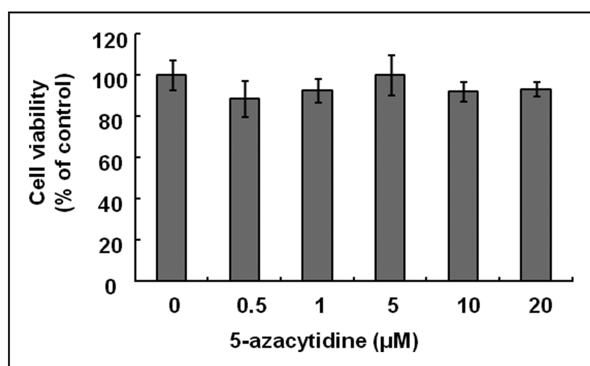

Fig. 2: Effect of 5-azacytidine on cell viability. Mel-Ab cells were seeded at 4×10^4 cells per well and then serum-starved for 24 h. Cells were then treated with different concentrations (0-20 μM) of 5-azacytidine for another 24 h. Cell viability was measured by the crystal violet assay.

azacytidine at concentrations of 0-20 μM for 24 h. As shown in Fig. 2, 5-azacytidine was not cytotoxic at any of the concentrations tested.

2.2. *Effects of 5-azacytidine on melanin synthesis and tyrosinase activity*

To examine the effects of 5-azacytidine on melanin synthesis in Mel-Ab cells, the cells were treated with 5-azacytidine at 0-20 μM for 3 days and the cellular melanin contents were quantified. Before measuring the melanin content, the cells were observed under a phase contrast microscope. As shown in Fig. 3A, melanin pigmentation decreased in 5-azacytidine-treated cells in a dose-dependent manner. Similarly, melanin content and tyrosinase activity were significantly reduced in a dose-dependent manner after treatment with 5-azacytidine (Fig. 3B, C). To determine whether 5-azacytidine directly inhibits tyrosinase, we used a cell-free system to examine tyrosinase activity. However, we found that 5-azacytidine did not directly inhibit tyrosinase (Fig. 3D). These results suggest that 5-azacytidine is not an enzymatic inhibitor of tyrosinase, but that it does regulate the expression of tyrosinase.

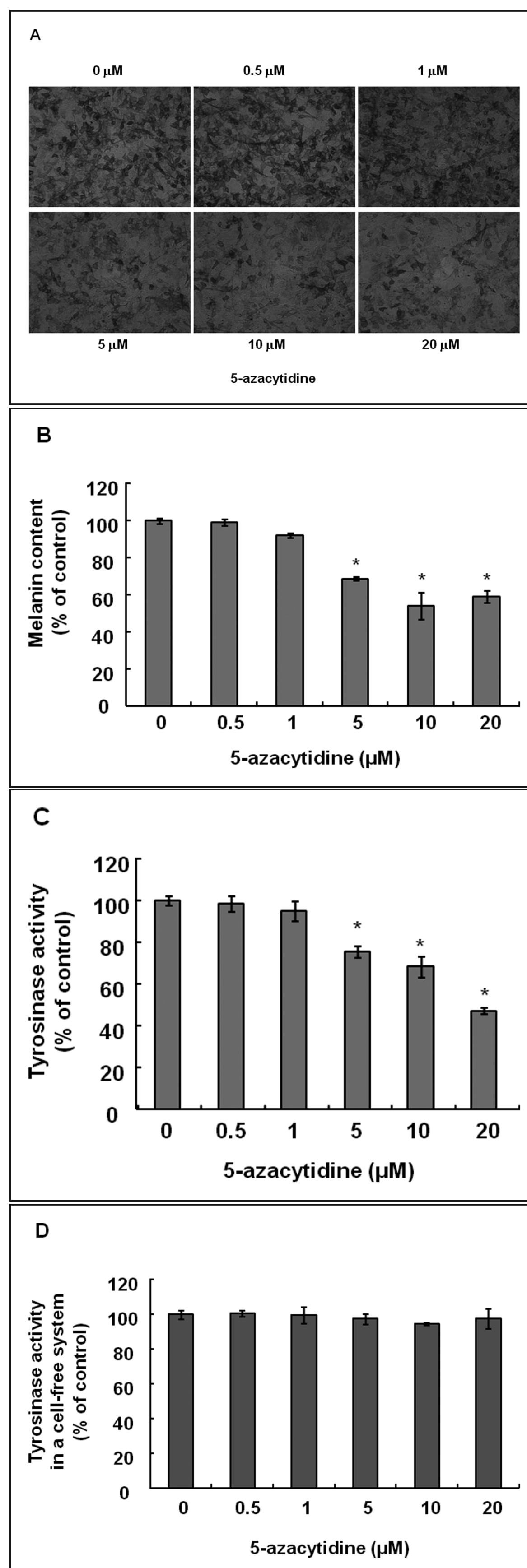

Fig. 3: Effects of 5-azacytidine on melanin content and tyrosinase activity. Mel-Ab cells were seeded at 5×10^5 cells per well and treated with 5-azacytidine (0-20 μM) for 3 days. Images were then captured using a phase contrast microscope (A). The effects of 5-azacytidine on melanin content (B), tyrosinase activity (C), and tyrosinase activity in a cell-free system (D) were assessed as described in the Experimental section.

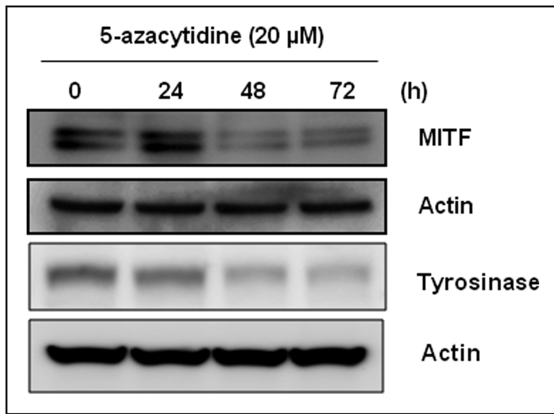

Fig. 4: Effects of 5-azacytidine on MITF and tyrosinase expression. Mel-Ab cells were seeded and incubated with 20 μM 5-azacytidine for 24, 48, and 72 h. After generating cell lysates, the levels of MITF and tyrosinase were analyzed by Western blotting using antibodies against MITF and tyrosinase. Actin was used as a loading control.

2.3. *Effects of 5-azacytidine on MITF and tyrosinase levels*

We hypothesized that down-regulation of melanogenic proteins may explain the reduced melanogenesis in 5-azacytidine-treated cells. Thus, we examined the levels of MITF and tyrosinase in 5-azacytidine-treated cells. We found that 5-azacytidine treatment (20 μM) decreased the levels of both MITF and tyrosinase after 48 h (Fig. 4). These results indicate that 5-azacytidine inhibits the production of MITF and tyrosinase, thereby reducing melanin production.

2.4. *5-Azacytidine inhibits CREB phosphorylation*

Next, we tested the effects of 5-azacytidine on the activation of various melanogenic signaling proteins. As shown in Fig. 5, 5-azacytidine did not affect the phosphorylation status of either Akt or GSK3β. On the other hand, 5-azacytidine treatment resulted in decreased CREB phosphorylation. This result implies that 5-azacytidine inhibits melanin synthesis by inhibiting CREB phosphorylation.

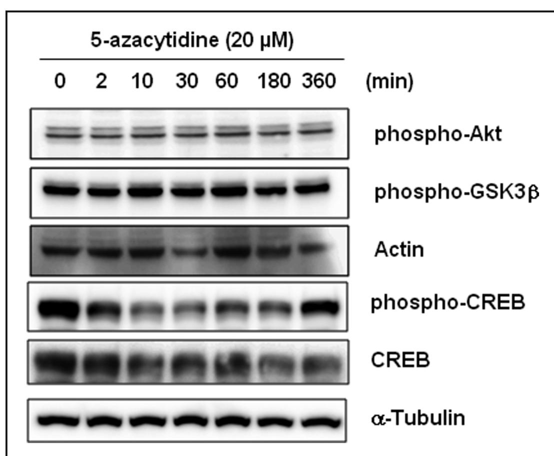

Fig. 5: Effects of 5-azacytidine on melanin-related signaling pathways. After serum starvation for 24 h, Mel-Ab cells were incubated with 20 μM 5-azacytidine for 0-360 min. Cell lysates were generated, and the activation of various melanin-related signaling molecules was analyzed by Western blotting using antibodies against phospho-Akt, phospho-GSK3β, and phospho-CREB. Equal amounts of protein loading were confirmed using anti-actin or anti-α-tubulin antibodies.

3. Discussion

In mammals, DNA methylation plays an important role in the regulation of gene expression and other cellular functions (Jaenisch and Bird 2003). However, the effects of DNA methylation on melanin synthesis are poorly understood. Recently, hypermethylation of the MIR125B-1 promoter was reported to increase melanin synthesis in melanoma cells (Kim et al. 2014). 5-Azacytidine is known to cause DNA hypomethylation by decreasing methyltransferase activity (Kaminskas et al. 2005). However, the effects of 5-azacytidine on melanogenesis had not been examined prior to the present study. Therefore, we investigated the effects of 5-azacytidine on melanin production. We found that 5-azacytidine inhibited melanin production in Mel-Ab cells. Interestingly, the whitening efficacy of most skin lightening agents has been shown to be correlated with their ability to directly inhibit tyrosinase activity or to modulate tyrosinase expression. We investigated the effect of 5-azacytidine on mushroom tyrosinase activity and found that 5-azacytidine did not inhibit tyrosinase in a cell-free system (Fig. 3D). However, 5-azacytidine-treated melanocytes showed a dose-dependent reduction in tyrosinase activity (Fig. 3C). Our results indicate that 5-azacytidine may regulate the protein levels of tyrosinase, thereby inhibiting melanogenesis. Therefore, we hypothesize that inhibition of DNA methylation may contribute to hypopigmentation.

MITF is a critical regulator of melanogenic protein expression (Vachtenheim and Borovansky 2010). Therefore, we examined the levels of MITF after extended treatments with 5-azacytidine. 5-Azacytidine decreased the protein levels of MITF and tyrosinase, the latter of which is transcriptionally modulated by MITF (Fig. 4).

Several signaling pathways are known to regulate melanin synthesis. For instance, the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway has been reported to regulate melanin production and the expression of melanogenic proteins. Furthermore, LY294002, a specific inhibitor of PI3K/Akt, was shown to upregulate MITF mRNA levels in B16/F10 murine melanoma cells (Khaled et al. 2003). Moreover, wortmannin-mediated inhibition of PI3K/Akt increased the melanin content and tyrosinase protein levels in human G361 melanoma cells (Oka et al. 2000). GSK3β is known to regulate melanogenesis (Busca and Ballotti 2000; Khaled et al. 2002). GSK3β phosphorylates MITF at Ser298 and enhances its affinity for the tyrosinase promoter, resulting in increased tyrosinase mRNA expression (Takeda et al. 2000). CREB phosphorylation is another major factor by which melanin synthesis is increased. CREB phosphorylation is related to MITF expression; MITF is a central melanogenic transcription factor (Costin and Hearing 2007). We found that 5-azacytidine treatment resulted in a time-dependent decrease in CREB phosphorylation, whereas no phosphorylation of either Akt or GSK3β was observed (Fig. 5). These findings indicate that 5-azacytidine-induced hypopigmentation may involve CREB dephosphorylation.

Recently, miR-125b was reported to regulate melanin production; moreover, hypermethylation of the MIR125B-1 promoter was shown to decrease the expression of miR-125b in pigmented cells (Kim et al. 2014). Since 5-azacytidine is a DNA methyltransferase inhibitor, it could potentially drive miR-125b expression and thereby inhibit melanogenesis in pigmented cells. Moreover, the level of miR-125b has been shown to be regulated by the cAMP signaling pathway (Kim et al. 2014). Further studies are required to evaluate this possibility.

In summary, the present study examined the effects of 5-azacytidine on melanogenesis. Our results suggest that 5-azacytidine inhibits melanin production. Moreover, we found that 5-azacytidine treatment resulted in reduced levels of MITF

and tyrosinase, in addition to reduced CREB phosphorylation. Based on these data, we propose that 5-azacytidine inhibits melanin synthesis *via* inhibition of CREB phosphorylation.

4. Experimental

4.1. Materials

Cholera toxin (CT), 12-O-tetradecanoylphorbol-13-acetate (TPA), and mushroom tyrosinase were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Antibodies specific for phospho-Akt (#9271), phospho-GSK3β (#9336), phospho-CREB (#9198S), and total CREB (#9197) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against tyrosinase (C-19), α-tubulin (DM1A), and actin (I-19) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-microphthalmia Ab-1 antibodies (C5, MS-771-P0) were from NeoMarkers (Fremont, CA, USA). Secondary anti-goat IgG (PI-9500), anti-mouse IgG (PI-2000), and anti-rabbit IgG (PI-1000) antibodies were purchased from Vector Laboratories (Burlingame, CA, USA).

4.2. Cell culture

The Mel-Ab cell line is a mouse-derived spontaneously immortalized melanocyte cell line that synthesizes large quantities of melanin. Mel-Ab cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 nM TPA, 1 nM CT, 50 μg/mL streptomycin, and 50 U/mL penicillin at 37 °C in 5% CO_2.

4.3. Cell viability assay

Cell viability was measured using a crystal violet assay. After incubation with 5-azacytidine for 24 h, the culture medium was removed. Cells were stained with 0.1% crystal violet in 10% ethanol for 5 min at room temperature and then rinsed 4 times with distilled water. The crystal violet retained by live cells was extracted with 95% ethanol, and the absorbances at 590 nm were determined using an ELISA reader (VERSAMax; Molecular Devices, Sunnyvale, CA, USA).

4.4. Measurement of melanin contents and microscopy

Melanin contents were measured essentially as described previously (Kim et al. 2003). Briefly, cells were treated with 5-azacytidine in DMEM containing 10% FBS for 3 days. Cells were then dissolved in 550 μL of 1 N NaOH at 100 °C for 30 min and centrifuged at 13,000 rpm for 5 min. The optical densities (ODs) of the supernatants at 400 nm were measured with an ELISA reader. Before measuring their melanin contents, cells were observed under a phase contrast microscope (Olympus IX50, Tokyo, Japan) and photographed using a DCM300 digital microscope camera (Scopetek, Inc., Hangzhou, China). The camera was supported by ScopePhoto software (Scopetek, Inc.).

4.5. Tyrosinase activity

Tyrosinase activity was analyzed as described previously (Busca et al. 1996). Briefly, Mel-Ab cells were seeded in 6-well plates and incubated with 5-azacytidine for 3 days. The cells were then washed with ice-cold PBS and lysed by several freeze-thaw cycles in phosphate buffer (pH 6.8) containing 1% Triton X-100. The resultant lysates were clarified by centrifugation at 15,000 rpm for 10 min. After quantifying the protein levels of the lysates and adjusting their concentrations with lysis buffer, the lysates were transferred to a 96-well plate (90 μL of lysate per well). Next, 10 μL of 10 mM L-DOPA was added to each well. The control wells contained 90 μL of lysis buffer and 10 μL of 10 mM L-DOPA. Following incubation at 37 °C, absorbance was measured at 475 nm every 10 min for at least 1 h using an ELISA reader. A cell-free assay system was used to examine the effects of 5-azacytidine on tyrosinase activity. For this assay, 60 μL of phosphate buffer containing 5-azacytidine was mixed with 20 μL of mushroom tyrosinase (53.7 units/mL), and 20 μL of 10 mM L-DOPA was then added. Following incubation at 37 °C, absorbance was measured at 475 nm.

4.6. Western blot analysis

Cells were lysed in lysis buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 5% β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, a protease inhibitor cocktail (Complete^{TM}; Roche, Mannheim, Germany), 1 mM Na_3VO_4, 50 mM NaF, and 10 mM EDTA]. A total of 20 μg of protein per lane was separated by SDS-polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene fluoride (PVDF) membranes. Nonspecific binding sites were then blocked with 5% skim milk in Tris-buffered saline containing 0.5% Tween 20. Blots were incubated with the appropriate primary antibodies at a dilution of 1:1000, and then further incubated with horseradish peroxidase-conjugated secondary antibodies. Immunoreactive bands were detected using an enhanced chemiluminescence plus kit (Amersham International, Little Chalfont, UK). Images were obtained using a LAS-1000 lumino-image analyzer (Fuji Film, Tokyo, Japan).

4.7. Statistics

The statistical significances of the differences between groups were assessed by analysis of variance (ANOVA), followed by Student's *t*-test. *P* values < 0.01 were considered significant.

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