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(-)-Epigallocatechin-3-gallate and Hinokitiol Reduce Melanin Synthesis *via* Decreased MITF Production

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In this study, the effects of (-)-epigallocatechin-3-gallate (EGCG) and/or hinokitiol (β-thujaplicin) on melanogenesis were investigated. Our results showed that both EGCG and hinokitiol significantly inhibited melanin synthesis in a concentration-dependent manner, and that their hypopigmenting effects were stronger than that of kojic acid, which is known to inhibit melanin formation in melanocytes and melanoma cells. Interestingly, EGCG did not show any additive hypopigmenting effect in combination with kojic acid, though EGCG did show a synergistic effect in combination with hinokitiol. Several reports indicate that the activation of extracellular signal-regulated kinase (ERK) induces microphthalmia-associated transcription factor (MITF) degradation. Accordingly, the effects of EGCG and hinokitiol on the ERK signaling pathway were examined. EGCG and hinokitiol induced neither ERK activation nor MITF degradation. On the other hand, both EGCG and hinokitiol reduced the protein levels of MITF and of tyrosinase, the rate limiting melanogenic enzyme, whereas kojic acid had no effect. In addition, hinokitiol strongly downregulated the activity of tyrosinase, whereas EGCG or kojic acid had only a little effect. These results show that both EGCG and hinokitiol reduce MITF production, and suggest that reduced tyrosinase activity by hinokitiol explains their synergistic effect on melanogenesis.

Key words: EGCG, Hinokitiol, Melanogenesis, MITF, Tyrosinase

INTRODUCTION

Melanin pigments are responsible for skin and hair colors and play a major role in the defense against solar UV. However, increased levels of epidermal melanin synthesis can darken the skin and induce cosmetic problems. In mammalian melanocytes, melanins are synthesized within melanosomes that contain tyrosinase, which plays a key role in melanogenesis, as it catalyses the rate-limiting reaction of the melanogenic process (Hearing and Jimenez, 1989; Kobayashi *et al.*, 1994; Yokoyama *et al.*, 1994). Accordingly, melanin production is mainly controlled by the expression and activation of tyrosinase (Hearing and Tsukamoto, 1991).

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(-)-Epigallocatechin-3-gallate (EGCG), a major constituent of green tea, has been found to possess antioxidant, anti-inflammatory, and anti-carcinogenic properties (Katiyar *et al.*, 2001). Hinokitiol (β-thujaplicin), a constituent found in the wood of Chamaecyparis obtuse and Thuja plicata, has antibacterial effects (Arima *et al.*, 2003). However, although it has been reported that both EGCG and hinokitiol inhibit tyrosinase (No *et al.*, 1999; Sakuma *et al.*, 1999), the effects of EGCG or hinokitiol on melanogenesis have received little attention.

Microphthalmia-associated transcription factor (MITF) is involved in the pigmentation, proliferation, and survival of melanocytes (Hodgkinson *et al.*, 1993; Steingrimsson *et al.*, 1994). Furthermore, MITF strongly stimulates tyrosinase promoter activities, indicating that MITF is an important transcriptional regulator of melanogenesis (Bentley *et al.*, 1994; Bertolotto *et al.*, 1998b; Yasumoto *et al.*, 1997; Yavuzer *et al.*, 1995). In humans, mutations in the MITF gene cause Waardenburg Syndrome type IIA, which manifests as an abnormal pigmentation of the skin and

hair (Hughes *et al.*, 1994; Tachibana, 1997; Tassabehji *et al.*, 1994). On the other hand, α-melanocyte-stimulating hormone (α-MSH) potently induces the expression of MITF and increases melanin synthesis (Bertolotto *et al.*, 1998a; Bertolotto *et al.*, 1998b; Price *et al.*, 1998). Because MITF is a critical factor in melanogenesis, we investigated whether EGCG or hinokitiol influences MITF protein production.

Extracellular signal-regulated kinase (ERK) plays a crucial role in cell proliferation and differentiation control (Cowley *et al.*, 1994; Marshall, 1995; Sale *et al.*, 1995). In a recent report, inhibition of the ERK pathway was found to induce hyperpigmentation, suggesting that the ERK signaling pathway is also involved in melanogenesis (Englaro *et al.*, 1998). In addition, ERK activation by sphingosine-1-phosphate or by ceramide downregulates melanogenesis (Kim *et al.*, 2003; Kim *et al.*, 2002).

In this study, the effects of EGCG or hinokitiol on melanin synthesis and tyrosinase activity were investigated in a spontaneously immortalized mouse melanocyte cell line, Mel-Ab. In particular, we analyzed changes in MITF and tyrosinase protein production, and examined their effects on the ERK signaling pathway.

MATERIALS AND METHODS

Materials

EGCG, kojic acid, hinokitiol, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), cholera toxin (CT), synthetic melanin, L-DOPA, and mushroom tyrosinase were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Antibodies recognizing phospho-specific ERK1/2 (Thr202/Tyr204, number 9101S) and total (phosphorylated and non-phosphorylated) ERK1/2 (number 9102) were purchased from Cell Signaling Technology. Microphthalmia Ab-1 (C5, MS-771-P0) was from NeoMarkers (Fremont, CA, USA); and tyrosinase (C-19) and actin (I-19) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell cultures

Mel-Ab cell line is a mouse-derived spontaneously immortalized melanocyte cell line that produces large amounts of melanin (Dooley *et al.*, 1994). Mel-Ab cells were incubated in 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.

Cell viability assay

Cell viability was determined by using a crystal violet assay (Dooley *et al.*, 1994). After incubating cells with test substances for 24 h, the culture medium was removed and replaced with 0.1% crystal violet in 10% ethanol. The cells were then stained for 5 min at room temperature and rinsed four times. Crystal violet retained by the adherent cells was then extracted with 95% ethanol, and absorbance was determined at 590 nm using an ELISA reader (TECAN, Salzburg, Austria)

Measurement of melanin contents and microscopy

Melanin contents were measured as described previously (Tsuboi *et al.*, 1998), with slight modification. Briefly, cells were treated with the test substances in DMEM containing 2% FBS for 4 days. Cell pellets were dissolved in 1 mL of 1 N NaOH at 100°C for 30 min and centrifuged for 20 min at 16,000 g. Optical densities (OD) of the supernatants were measured at 400 nm using an ELISA reader. A standard synthetic melanin curve (0 to 300 μg/mL) was prepared in triplicate for each experiment. Before determining the melanin contents, the cells were observed under a phase contrast microscope (Olympus Optical Co., Tokyo, Japan) and photographed using a CoolSNAP$_{cf}$ digital video camera system (Roper Scientific, Inc., Tucson, AZ, USA) supported by RS Image software (Roper Scientific, Inc., Tucson, AZ, USA).

Tyrosinase activity

A cell-free assay system was used to test for direct effects on tyrosinase activity. Seventy microliters of phosphate buffer containing the various test substances (EGCG, kojic acid, or hinokitiol) were mixed either with 20 μL of 10 μg/mL mushroom tyrosinase or with 20 μL of human tyrosinase (as 20 μg of total protein) extracted from primary cultured human melanocytes, and 10 μL of 10 mM L-DOPA was added to each well. Following incubation at 37°C, absorbance was measured at 475 nm.

Western blot analysis

Cells were lysed in cell lysis buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 5% β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, protease inhibitors (Complete™, Roche, Mannheim, Germany), 1 mM Na_3VO_4, 50 mM NaF, and 10 mM EDTA]. Ten micrograms of protein per lane was separated by SDS-polyacrylamide gel electrophoresis and blotted onto PVDF membranes, which were saturated with 5% dried milk in Tris-buffered saline containing 0.4% 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 antibody. Bound antibodies were detected using an enhanced chemiluminescence plus kit (Amersham International, Little Chalfont, U.K.).

Statistics

Differences between results were assessed for significance using the Student's *t*-test. *P* values of <0.01 were taken to be significant.

RESULTS

Effects of EGCG and hinokitiol on melanin synthesis in Mel-Ab cells

To investigate the influences of EGCG or hinokitiol, the melanin contents of the Mel-Ab cells were measured after EGCG or hinokitiol treatment. Kojic acid was used as a positive control, since it is known to reduce melanin synthesis. Cells were exposed to 10 μM EGCG, 10 μM hinokitiol, or 100 μM kojic acid for 4 days and then photographed under a phase contrast microscope (Fig. 1). Our results showed that both EGCG- and kojic acid-treated cells were less pigmented than the control, and that the hinokitiol-treated cells were markedly less pigmented. We also measured the cellular melanin contents after EGCG, hinokitiol, or kojic acid treatment. EGCG treatment inhibited melanin synthesis in a concentration-dependent manner (Fig. 2). Kojic acid also reduced melanin synthesis at 100 μM. However, the combination of EGCG and kojic acid did not show an additive effect (Fig. 2). As shown in Fig. 3, the melanin contents of cells were significantly downregulated by hinokitiol, at concentrations higher than 0.1 μM (Fig. 3). Moreover, melanin downregulation by EGCG was significantly potentiated by hinokitiol treatment (Fig. 4). These results indicate that EGCG and hinokitiol may regulate melanogenesis via different mechanisms.

To examine whether EGCG, hinokitiol, or kojic acid has cytotoxic effects, we treated EGCG, hinokitiol, or kojic acid at the various concentrations used to determine melanin content; cell viability was determined by using the crystal violet assay. However, showed no effect on cell viability at the concentrations used, indicating that they are not cytotoxic to Mel-Ab cells (data not shown).

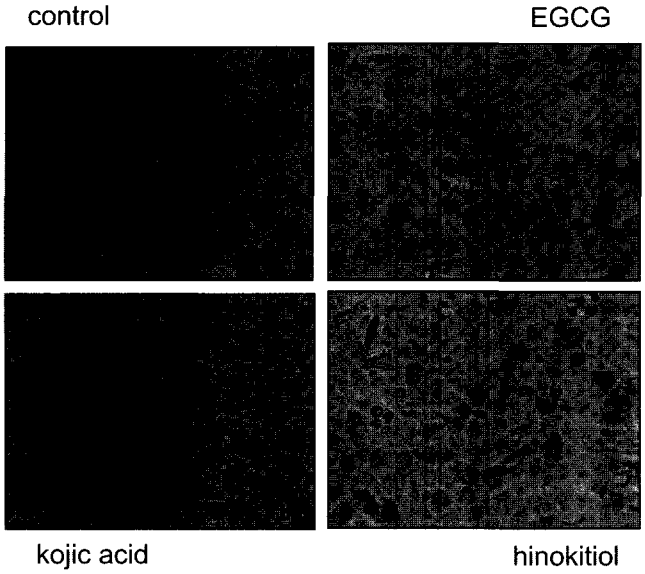

Fig. 1. Effects of EGCG or hinokitiol on melanin synthesis in Mel-Ab cells. Cells were cultured with 10 μM EGCG, 100 μM kojic acid, or 10 μM hinokitiol for 4 days. Phase contrast photomicrographs were taken using a digital video camera, as described in "Materials and Methods".

Both EGCG and hinokitiol downregulate MITF and tyrosinase protein production, but do not activate ERK

It has been reported that ERK phosphorylation induces

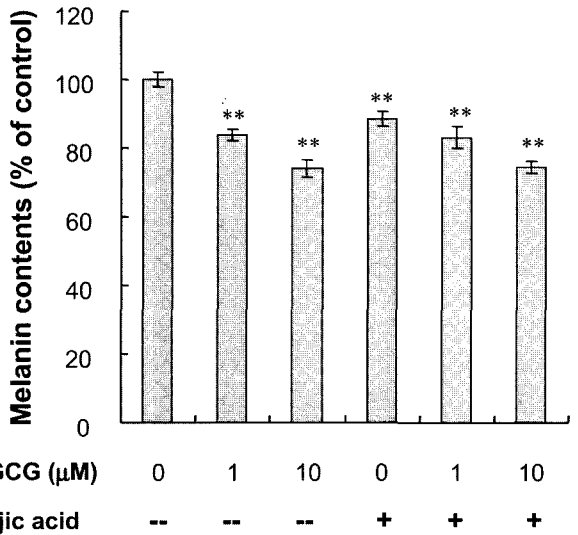

Fig. 2. Effects of EGCG and kojic acid on melanin synthesis in Mel-Ab cells. Cells were cultured with 1 or 10 μM EGCG and/or 100 μM kojic acid for 4 days, and melanin contents were measured, as described in "Materials and Methods". The results shown are the averages of three independent experiments ± SD. ** P< 0.01 compared to control.

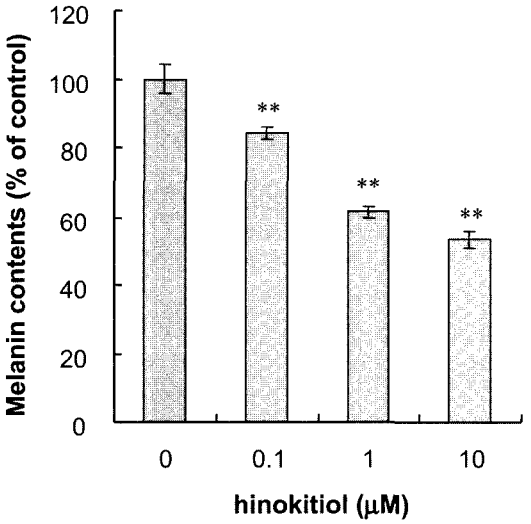

Fig. 3. Effects of hinokitiol on melanin synthesis in Mel-Ab cells. Cells were cultured with 0.1-10 μM hinokitiol for 4 days, and their melanin contents were then measured, as described in "Materials and Methods". Results are the averages of three independent experiments ± SD. **P< 0.01 compared to control.

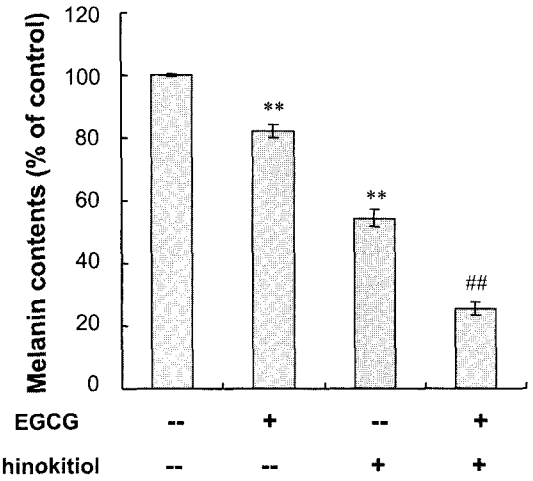

Fig. 4. Effects of EGCG and hinokitiol on melanin synthesis in Mel-Ab cells. Cells were cultured with 1 μM EGCG and/or 1 μM hinokitiol for 4 days, and the melanin contents were measured, as described in "Materials and Methods". Results are the averages of three independent experiments± SD. **P<0.01 compared to control and ##P<0.01 compared to hinokitiol-treated group.

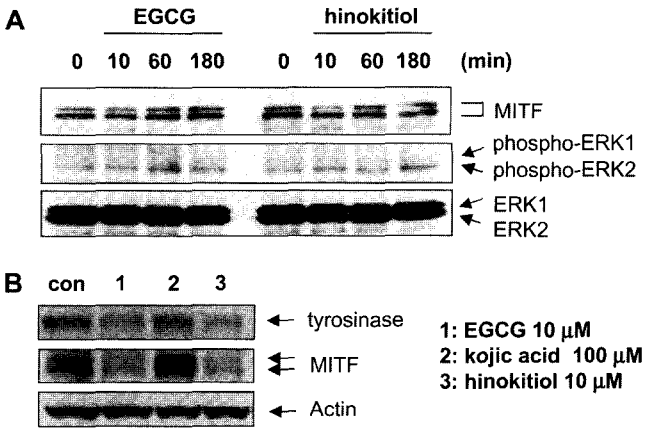

Fig. 5. EGCG or hinokitiol reduced MITF and tyrosinase protein production. (A) After serum starvation, Mel-Ab cells were stimulated with 10 μM EGCG or 10 μM hinokitiol at the times indicated. Whole cell lysates were then subjected to Western blot analysis with antibodies against phospho-specific ERK and MITF. Equal protein loading was confirmed using phosphorylation-independent ERK antibody. (B) Mel-Ab cells were cultured with 10 μM EGCG, 100 μM kojic acid, or 10 μM hinokitiol for 3 days. Whole cell lysates were then subjected to Western blot analysis with antibodies against MITF and tyrosinase. Equal protein loading was confirmed using actin antibody.

MITF degradation (Hemesath *et al.*, 1998; Wu *et al.*, 2000). Therefore, it was examined whether EGCG or hinokitiol influences the ERK pathway. As shown in Fig. 5A, neither EGCG nor hinokitiol activated ERK, and thus did not trigger a MITF mobility shift or degradation, which can be induced by ERK phosphorylation (Kim *et al.*, 2003; Wu *et al.*, 2000). However, the treatment with EGCG or hinokitiol for 3 days reduced the protein level of MITF, whereas kojic acid had no effect (Fig. 5B). Additionally, the protein levels of tyrosinase were examined after EGCG or hinokitiol treatment. Our results show that tyrosinase protein levels dropped after EGCG or hinokitiol treatment in accord with the reduction in MITF protein level. On the other hand, kojic acid also had no effect on the tyrosinase level (Fig. 5B). Thus, reduced MITF and tyrosinase protein levels may be responsible for the hypopigmentary effects of both EGCG and hinokitiol.

The direct effects of EGCG or hinokitiol on tyrosinase activity were also investigated by using human and mushroom tyrosinase. Hinokitiol showed a strong inhibitory effect on tyrosinase activity, whereas EGCG and kojic acid only moderately inhibited tyrosinase (Fig. 6).

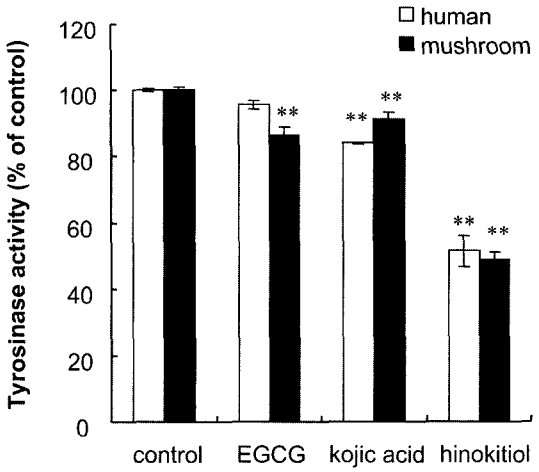

Fig. 6. Effects of EGCG or hinokitiol on the activities of human and mushroom tyrosinase. To test their direct effects on tyrosinase, tyrosinase activity was measured in a cell-free system, as described in "Materials and Methods". We added 10 μM EGCG, 100 μM kojic acid, or 10 μM hinokitiol to each well. Results are the averages of three independent experiments ± SD. **P< 0.01 compared to control.

DISCUSSION

EGCG is a major active constituent of green tea, which is consumed commonly in Korea, China, and Japan. In addition, to its antioxidative effects, EGCG has been reported to inhibit tyrosinase activity, which is known to regulate the rate-limiting step of melanin synthesis (No *et al.*, 1999). However, the mechanism of EGCG-induced hypopigmentation has received little attention. In the present study, we also found that EGCG inhibits mushroom tyrosinase activity directly, and that it inhibits human tyrosinase only slightly. In addition, EGCG significantly suppresses melanin synthesis. Therefore, it appears that reduced tyrosinase activity may contribute to the lower pigment content of EGCG-treated cells. Since the tyrosinase inhibitory effect of EGCG was weak, it was

hypothesized that other mechanisms are involved in EGCG-induced hypopigmentation.

MITF is one of the major transcriptional regulators of tyrosinase, and thus plays a critical role in melanin synthesis (Bentley *et al.*, 1994; Busca and Ballotti, 2000; Tachibana, 2000). Recent reports indicate that the ERK signaling pathway is important in the regulation of melanogenesis, since ERK activation induces MITF phosphorylation and subsequent degradation (Hemesath *et al.*, 1998; Wu *et al.*, 2000; Xu *et al.*, 2000). We also found that ERK activation induces MITF phosphorylation and degradation, which leads to a reduced tyrosinase level and decreased melanogenesis (Kim *et al.*, 2003). However, in the present study, EGCG does not activate the ERK pathway, and thus EGCG induced neither MITF phosphorylation nor degradation (Fig. 5A). Interestingly, it was observed that tyrosinase protein production was reduced, following the attenuation of MITF protein production by melanocytes treated with EGCG for 3 days. Therefore, reduced MITF production by EGCG may be unrelated to the ERK pathway. Based on reports about the importance of the Akt pathway in melanogenesis (Khaled *et al.*, 2002; Oka *et al.*, 2000), it was examined whether EGCG activates the Akt signaling pathway, but it did not (data not shown). In addition, kojic acid neither reduced MITF nor tyrosinase production. Further studies are required to elucidate the mechanisms of the downregulation of MITF production by EGCG.

Hinokitiol was reported to strongly inhibit mushroom tyrosinase (Sakuma *et al.*, 1999), however, its role in melanogenesis is comparatively unknown. Our results also show that hinokitiol suppresses both human and mushroom tyrosinase activity. Furthermore, hinokitiol reduced MITF and subsequently tyrosinase protein production, which resulted in a marked reduction in melanin synthesis. Thus, reduced levels of MITF and direct tyrosinase inhibition by hinokitiol may dramatically reduce melanin synthesis. Moreover, the combination of EGCG and hinokitiol acted synergistically, indicating that the strong tyrosinase inhibitory activity of hinokitiol works in combination with the reduced level of MITF induced by EGCG & hinokitiol. We also examined EGCG & kojic acid, but found no additive effect.

In summary, this study demonstrates that both EGCG and hinokitiol reduce melanin synthesis in Mel-Ab cells. In addition, both EGCG and hinokitiol inhibit MITF and tyrosinase protein production, which in turn reduce melanin synthesis. Hinokitiol also strongly inhibited tyrosinase, thus explaining the effect of EGCG and hinokitiol in combination.

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REFERENCES

Arima, Y., Nakai, Y., Hayakawa, R., and Nishino, T., Anti-bacterial effect of beta-thujaplicin on staphylococci isolated from atopic dermatitis: relationship between changes in the number of viable bacterial cells and clinical improvement in an eczematous lesion of atopic dermatitis. *J. Antimicrob Chemother.*, 51, 113-122 (2003).

Bentley, N. J., Eisen, T., and Goding, C. R., Melanocyte-specific expression of the human tyrosinase promoter: activation by the microphthalmia gene product and role of the initiator. *Mol. Cell Biol.*, 14, 7996-8006 (1994).

Bertolotto, C., Abbe, P., Hemesath, T. J., Bille, K., Fisher, D. E., Ortonne, J. P., and Ballotti, R., Microphthalmia gene product as a signal transducer in cAMP-induced differentiation of melanocytes. *J. Cell Biol.*, 142, 827-835 (1998a).

Bertolotto, C., Busca, R., Abbe, P., Bille, K., Aberdam, E., Ortonne, J. P., and Ballotti, R., Different *cis*-acting elements are involved in the regulation of TRP1 and TRP2 promoter activities by cyclic AMP: pivotal role of M boxes (GTCATGTGCT) and of microphthalmia. *Mol. Cell Biol.*, 18, 694-702 (1998b).

Busca, R. and Ballotti, R., Cyclic AMP a key messenger in the regulation of skin pigmentation. *Pigment. Cell Res.*, 13, 60-69 (2000).

Cowley, S., Paterson, H., Kemp, P., and Marshall, C. J., Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. *Cell*, 77, 841-852 (1994).

Dooley, T. P., Gadwood, R. C., Kilgore, K., and Thomasco, L. M., Development of an *in vitro* primary screen for skin depigmenta-tion and antimelanoma agents. *Skin Pharmacol*, 7, 188-200 (1994).

Englaro, W., Bertolotto, C., Busca, R., Brunet, A., Pages, G., Ortonne, J. P., and Ballotti, R., Inhibition of the mitogen-activated protein kinase pathway triggers B16 melanoma cell differentia-tion. *J. Biol. Chem.*, 273, 9966-9970 (1998).

Hearing, V. J. and Jimenez, M., Analysis of mammalian pigmenta-tion at the molecular level. *Pigment. Cell Res.*, 2, 75-85 (1989).

Hearing, V. J. and Tsukamoto, K., Enzymatic control of pigmenta-tion in mammals. *FASEB J.*, 5, 2902-2909 (1991).

Hemesath, T. J., Price, E. R., Takemoto, C., Badalian, T. and Fisher, D. E., MAP kinase links the transcription factor Microphthalmia to c-Kit signalling in melanocytes. *Nature*, 391, 298-301 (1998).

Hodgkinson, C. A., Moore, K. J., Nakayama, A., Steingrimsson,

E., Copeland, N. G., Jenkins, N. A., and Arnheiter, H., Mutations at the mouse microphthalmia locus are associated with defects in a gene encoding a novel basic-helix-loop-helix-zipper protein. *Cell*, 74, 395-404 (1993).

Hughes, A. E., Newton, V. E., Liu, X. Z., and Read, A. P., A gene for Waardenburg syndrome type 2 maps close to the human homologue of the microphthalmia gene at chromosome 3p12-p14.1. *Nat. Genet.*, 7, 509-512 (1994).

Katiyar, S. K., Afaq, F., Azizuddin, K., and Mukhtar, H., Inhibition of UVB-induced oxidative stress-mediated phosphorylation of mitogen-activated protein kinase signaling pathways in cultured human epidermal keratinocytes by green tea polyphenol (-)-epigallocatechin-3-gallate. *Toxicol. Appl. Pharmacol.*, 176, 110-117 (2001).

Khaled, M., Larribere, L., Bille, K., Aberdam, E., Ortonne, J. P., Ballotti, R., and Bertolotto, C., Glycogen synthase kinase 3beta is activated by cAMP and plays an active role in the regulation of melanogenesis. *J. Biol. Chem.*, 277, 33690-36907 (2002).

Kim, D. S., Hwang, E. S., Lee, J. E., Kim, S. Y., Kwon, S. B., and Park, K. C., Sphingosine-1-phosphate decreases melanin synthesis *via* sustained ERK activation and subsequent MITF degradation. *J. Cell Sci.*, 116, 1699-1706 (2003).

Kim, D. S., Kim, S. Y., Chung, J. H., Kim, K. H., Eun, H. C., and Park, K. C., Delayed ERK activation by ceramide reduces melanin synthesis in human melanocytes. *Cell Signal.*, 14, 779-785 (2002).

Kobayashi, T., Urabe, K., Winder, A., Jimenez-Cervantes, C., Imokawa, G., Brewington, T., Solano, F., Garcia-Borron, J. C., and Hearing, V. J., Tyrosinase related protein 1 (TRP1) functions as a DHICA oxidase in melanin biosynthesis. *EMBO J.*, 13, 5818-5825 (1994).

Marshall, C. J., Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell*, 80, 179-185 (1995).

No, J. K., Soung, D. Y., Kim, Y. J., Shim, K. H., Jun, Y. S., Rhee, S. H., Yokozawa, T., and Chung, H. Y., Inhibition of tyrosinase by green tea components. *Life Sci.*, 65, PL241-PL246 (1999).

Oka, M., Nagai, H., Ando, H., Fukunaga, M., Matsumura, M., Araki, K., Ogawa, W., Miki, T., Sakaue, M., Tsukamoto, K., Konishi, H., Kikkawa, U., and Ichihashi, M., Regulation of melano-genesis through phosphatidylinositol 3-kinase-Akt pathway in human G361 melanoma cells. *J. Invest Dermatol*, 115, 699-703 (2000).

Price, E. R., Horstmann, M. A., Wells, A. G., Weilbaecher, K. N., Takemoto, C. M., Landis, M. W., and Fisher, D. E., alpha-Melanocyte-stimulating hormone signaling regulates expression of micro-phthalmia, a gene deficient in Waardenburg syndrome. *J. Biol. Chem.*, 273, 33042-33047 (1998).

Sakuma, K., Ogawa, M., Sugibayashi, K., Yamada, K., and Yamamoto, K., Relationship between tyrosinase inhibitory action and oxidation-reduction potential of cosmetic whitening ingredients and phenol derivatives. *Arch. Pharm. Res.*, 22, 335-339 (1999).

Sale, E. M., Atkinson, P. G., and Sale, G. J., Requirement of MAP kinase for differentiation of fibroblasts to adipocytes, for insulin activation of p90 S6 kinase and for insulin or serum stimulation of DNA synthesis. *EMBO J.*, 14, 674-684 (1995).

Steingrimsson, E., Moore, K. J., Lamoreux, M. L., Ferre-D'Amare, A. R., Burley, S. K., Zimring, D. C., Skow, L. C., Hodgkinson, C. A., Arnheiter, H., and Copeland, N. G. *et al.*, Molecular basis of mouse microphthalmia (mi) mutations helps explain their developmental and phenotypic consequences. *Nat. Genet.*, 8, 256-263 (1994).

Tachibana, M., Evidence to suggest that expression of MITF induces melanocyte differentiation and haploinsufficiency of MITF causes Waardenburg syndrome type 2A. *Pigment. Cell Res.*, 10, 25-33 (1997).

Tachibana, M., MITF: a stream flowing for pigment cells. *Pigment. Cell Res.*, 13, 230-40 (2000).

Tassabehji, M., Newton, V. E., and Read, A. P., Waardenburg syndrome type 2 caused by mutations in the human microphthalmia (MITF) gene. *Nat. Genet.*, 8, 251-255 (1994).

Tsuboi, T., Kondoh, H., Hiratsuka, J. and Mishima, Y., Enhanced melanogenesis induced by tyrosinase gene-transfer increases boron-uptake and killing effect of boron neutron capture therapy for amelanotic melanoma. *Pigment. Cell Res.*, 11, 275-282 (1998).

Wu, M., Hemesath, T. J., Takemoto, C. M., Horstmann, M. A., Wells, A. G., Price, E. R., Fisher, D. Z., and Fisher, D. E., c-Kit triggers dual phosphorylations, which couple activation and degradation of the essential melanocyte factor Mi. *Genes Dev.*, 14, 301-312 (2000).

Xu, W., Gong, L., Haddad, M. M., Bischof, O., Campisi, J., Yeh, E. T., and Medrano, E. E., Regulation of microphthalmia-associated transcription factor MITF protein levels by association with the ubiquitin-conjugating enzyme hUBC9. *Exp. Cell Res.*, 255, 135-143 (2000).

Yasumoto, K., Yokoyama, K., Takahashi, K., Tomita, Y., and Shibahara, S., Functional analysis of microphthalmia-associated transcription factor in pigment cell-specific transcription of the human tyrosinase family genes. *J. Biol. Chem.*, 272, 503-509 (1997).

Yavuzer, U., Keenan, E., Lowings, P., Vachtenheim, J., Currie, G., and Goding, C. R., The Microphthalmia gene product interacts with the retinoblastoma protein in vitro and is a target for deregulation of melanocyte-specific transcription. *Oncogene*, 10, 123-134 (1995).

Yokoyama, K., Suzuki, H., Yasumoto, K., Tomita, Y., and Shibahara, S., Molecular cloning and functional analysis of a cDNA coding for human DOPAchrome tautomerase/tyrosinase-related protein-2. *Biochim. Biophys. Acta*, 1217, 317-321 (1994).