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Modulation of Microphthalmia-associated Transcription Factor Gene Expression Alters Skin Pigmentation

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The microphthalmia-associated transcription factor is implicated in melanocyte development and in the regulation of melanogenesis. Microphthalmia-associated transcription factor is thought to bind to the M-box promoter elements of tyrosinase, tyrosinase-related protein-1 and dopachrome tautomerase/tyrosinase-related protein-2 and transactivate these genes, resulting in increased pigmentation. Using a luciferase reporter construct driven by the microphthalmia-associated transcription factor promoter, we identified agents that modulate microphthalmia-associated transcription factor promoter activity. Changes in endogenous microphthalmia-associated transcription factor expression levels upon treatment with these agents were confirmed using northern and western blots, and their pigmentary modulating activities were demonstrated. Ultraviolet B irradiation and traditional Chinese medicine-1, a natural extract used in traditional Chinese medicine, upregulated microphthalmia-associated transcription factor gene expression and enhanced tyrosinase activity *in vitro*. Dihydrolipoic acid, lipoic acid, and resveratrol reduced microphthalmia-associated transcription factor and tyrosinase promoter activities. These agents also inhibited the forskolin- and ultraviolet B-stimulated promoter activities of these genes and significantly reduced tyrosinase activity in melanocyte cultures, resulting in depigmentation. Overexpressed microphthalmia-associated transcription factor was capable of rescuing the repressive effects of these compounds on the cotransfected tyrosinase promoter. Dark-skinned Yucatan swine treated with these agents showed visible skin lightening, which was confirmed histologically, whereas ultraviolet B-induced tanning of light-skinned swine was inhibited using these agents. Our findings suggest that modulation of microphthalmia-associated transcription factor expression can alter skin pigmentation and further confirm the central role of microphthalmia-associated transcription factor in melanogenesis.

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Skin pigmentation, resulting from the production and distribution of melanin in the epidermis, is the major physiologic defense against solar irradiation. In mammals, melanogenesis is stimulated by ultraviolet (UV)B irradiation, α-melanocyte-stimulating hormone (α-MSH), and cyclic adenosine monophosphate (cAMP)-elevating agents (e.g., forskolin, isobutylmethylxanthine) (Abdel-Malek *et al*, 1987). Melanin is synthesized in epidermal melanocytes, and then transferred into epidermal keratinocytes via the melanocytes' dendrites (Le Douarin, 1982). Three melanocyte-specific enzymes, tyrosinase, tyrosinase-related proteins-1 (TRP-1/Tyrp1) and dopachrome tautomerase (DCT, also known as tyrosinase related protein-2, TRP-2) are involved in the enzymatic conversion of tyrosine to melanin (Korner and Pawelek, 1982; Barber *et al*, 1984; Hearing and Jimenez, 1987; Prota, 1988; Jimenez-Cervantes *et al*, 1994; Yokoyama *et al*, 1994). The promoter sequences of tyrosinase, Tyrp1, and DCT share a highly conserved motif termed the M-box, which contributes to their melanocyte-specific expression (Yasumoto *et al*, 1997; Aksan and Goding, 1998). The microphthalmia-associated transcription factor (MITF), a basic helix–loop–helix leucine zipper transcription factor, is thought to bind to the M-box and transactivates the tyrosinase, Tyrp1, and DCT promoters (Hemesath *et al*, 1994; Yasumoto *et al*, 1997; Aksan and Goding, 1998). MITF is involved in melanocyte development and survival (Moore, 1995; Fisher, 2000), and overexpression of MITF in NIH/3T3 fibroblasts could induce dendricity and tyrosinase and Tyrp1 expression (Tachibana *et al*, 1996). Homozygous mutations at the mouse microphthalmia (Mi) locus lead to a complete loss of pigmentation due to melanocyte loss (Silvers, 1979; Tachibana *et al*, 1994). Mutations in the human homolog of the mouse Mi gene result in abnormal pigmentation and deafness, as observed in Waardenburg syndrome type IIA (Tachibana *et al*, 1994; Tassabehji *et al*, 1994, 1995; Nobukuni *et al*, 1996; Bondurand *et al*, 2000). The MITF gene contains multiple

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Abbreviations: MITF, microphthalmia-associated transcription factor; Tryp-1, tyrosinase-related protein-1; LA, lipoic acid; DHLA, dihydrolipoic acid; TCM-1, traditional Chinese medicine-1; CRE, cAMP response element; MSH, melanocyte-stimulating hormone; DOPA, 3,4-dihydroxyphenylalanine; F&M, Fontana–Mason; NHEM, normal human epidermal melanocytes.

promoters, which give rise to unique 5′ exons, subsequently spliced on to a common downstream coding region (Udono *et al*, 2000). One of these alternative promoters is melanocyte specific (Fuse *et al*, 1996) and contains a cAMP response element (CRE) consensus motif. The melanocyte-specific MITF promoter CRE is believed to mediate cAMP-induced melanogenesis in response to MSH stimulation (Wong and Pawelek, 1975; Halaban *et al*, 1984; Gordon *et al*, 1989; Takahashi and Parsons, 1990; Hunt *et al*, 1994; Fuse *et al*, 1996; Bertolotto *et al*, 1998; Price *et al*, 1998b).

The significance of MITF as a master regulator of melanogenesis led us to search for agents that modulate MITF expression, and therefore might affect skin pigmentation. Here we show that UVB irradiation and the natural extract traditional Chinese medicine-1 (TCM-1) activate the melanocyte-specific MITF promoter, whereas dihydrolipoic acid (DHLA), lipoic acid (LA), and resveratrol (*trans*-3,4′,5-trihydroxystibene) reduce MITF expression, and induce skin lightening *in vivo*.

MATERIALS AND METHODS

Reagents Chemicals were from Sigma (St Louis, MO). Pycnogenol was from Cogniz Corporation (Cincinnati, OH) and a concentrated green tea polyphenols extract was from Lipton (Grand Rapids, MN). TCM-1, an aqueous extract of the plant *Astragulus membraneus Bunge. Ex Maxim* was kindly provided by Dr Zhi Sheng He (Shanghai Institute of Material Medica, Chinese Academy of Science, Shanghai, China).

Cell cultures Dulbecco's modified Eagle's medium, fetal bovine serum, trypsin, penicillin, and streptomycin were from Life Technologies (Rockville, MD). Primary normal human epidermal melanocytes (neonatal, NHEM) were purchased from Cascade Biologics, Inc. (Portland, OR) and were maintained according to the manufacturer's instructions. Human HaCaT keratinocytes (kind gift of Dr N.E. Fusenig, Heidelberg, Germany) were maintained as described (Boukamp *et al*, 1988). B16 murine melanoma cells were from the American Type Culture Collection (Manassas, VA) and were maintained according to the manufacturer's instructions. The murine melanocyte line melan-a (kind gift of Dr D. Bennett, St George's Hospital Medical School, London, U.K.) was maintained according to Bennett *et al* (1987). Viability assays were performed using alamarBlue™ (Acumed International, West Lake, OH) following manufacturer's instructions. UVB irradiation (typically, 30 mJ per cm^2 for melanocytes and 18.8 mJ per cm^2 for keratinocytes) was performed with a UVB FS light source (Spectronics Corp. Westbury, NY, Ble-1T158 light bulb) in an exposure chamber, with plate covers removed and phosphate-buffered saline (PBS) present in the wells. UVB intensity was measured with a UVX radiometer (UVP Inc., San Gabriel, CA). After irradiation, PBS was immediately replaced with maintenance medium. All studies were repeated at least three times.

Reporter and expression constructs The human MITF promoter (–387 to +97 bp, in pGL2.hMIP) (Fuse *et al*, 1996) was cloned upstream of the firefly luciferase gene as described (Price *et al*, 1998a). The human tyrosinase promoter (–300 to +80 bp) luciferase reporter construct is described in (Price *et al*, 1998a). The human Tyrp1 promoter (–344 to +114 bp) luciferase construct (pGL3.HTyrp1) (Yavuzer and Goding, 1994) was kindly provided by Dr C.R. Goding (Marie Curie Research Institute, The Chart, Oxted, U.K.). The dominant negative MITF mutant construct mutCRE-MITF (pGL2.hMiPmutCRE) is described by Price *et al* (1998a). For the rescue experiments, MITF was expressed from the elongation factor 1α promoter (Mizushima and Nagata, 1990) using pEBB MITF as described by Wu *et al* (2000). All studies were repeated at least three times.

Reporter assays Reporter and expression constructs were transfected into HaCaT, NHEM, B16, or melan-a cells using Fugene™ 6 according to the manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN). Forty-eight hours post-transfection, cells were treated with test compounds for 7 h or as otherwise indicated. Cells were harvested using the passive lysis buffer and assayed using the Dual Luciferase assay according to manufacturer's instructions (Promega, Madison, WI). Luciferase values were normalized to constitutive Renilla (sea pansy) luciferase produced from cotransfected pRL-Null plasmid (Promega).

cAMP (low pH) immunoassay cAMP concentration was analyzed using a cAMP (low pH) kit from R&D systems, Inc. (Minneapolis, MN). In brief, B16 cells (7×10^4) were lyzed in 0.1 M of HCl to inhibit phosphodiesterase activity. Supernatants were collected, neutralized, and diluted. Following neutralization and dilution, a fixed amount of cAMP conjugate (alkaline phosphatase-labeled cAMP) was added to compete with cAMP present in the cell lysates for sites on a rabbit polyclonal antibody immobilized on a 96 well plate. After a wash to remove excess conjugated and unbound cell lysate cAMP, a substrate solution was added to the wells to determine the bound enzyme activity. The color development was stopped and the absorbance was read at 405 nm. The intensity of the color was inversely proportional to the concentration of cAMP in the cell lysates. All studies were repeated at least three times.

RNA isolation and northern blot analysis Total RNA was isolated using RNA STAT-60™ (TEL-TEST, Inc., Friendswood, TX) according to manufacturer's instructions. Twenty micrograms of total RNA were separated on a 1% agarose gel, and transferred on to Gene Screen plus membrane (Du Pont-NEN, Boston, MA). Hybridization was performed in ExpressHyb™ Hybridization Solution (Clontech, Palo Alto, CA) according to the manufacturer's instructions, using a human MITF cDNA fragment (base pairs 58–1432) as a probe.

Protein extraction and western blot analysis Western blot analysis was performed on B16 cell extracts as previously described (Sharlow *et al*, 2000). Briefly, B16 cells were lyzed in RIPA buffer containing 1% nonidet-P40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, Complete™ protease inhibitors (Boehringer Mannheim, Indianapolis, IN) and phosphatase inhibitors (1 mM sodium vanadate and 20 mM sodium fluoride) in PBS. RIPA lysates (20 μg per lane) were electrophoresed in 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and proteins were analyzed by enhanced chemiluminescence western blotting (Amersham, Arlington Heights, IL). Antibodies used are monoclonal antibodies against Mi (Hemesath *et al*, 1998), and polyclonal antibody against tyrosinase (αPEP-7 kind gift of Dr V. Hearing, National Cancer Institute, NIH, Bethesda, MD) and against α-tubulin (loading control).

Tyrosinase activity analysis and 3,4-dihydroxyphenylalanine (DOPA) staining For total tyrosinase activity analysis, control and treated cells were trypsinized with 0.25% trypsin and cells (7×10^4) were then lyzed in 1% Triton-X 100 in 0.1 M Na phosphate buffer (pH 6.8) for approximately 20 min. Lysates (100 μl) were incubated with 100 μl of DOPA, 3 mg per ml in 0.1 M Na phosphate buffer (pH 6.8), at 37°C, for 3 h. Tyrosinase activity was determined by measuring absorbance at 490 nm (UV Max Kinetic Microplate Reader, Molecular Device, Sunnyvale, CA). To measure inhibition of tyrosinase enzymatic activity only, aliquots of lysates from untreated cells were incubated with both DOPA and test compounds at 37°C for 3 h and then analyzed for tyrosinase activity as described above. For *in-situ* DOPA staining, cells were fixed in 1.85% formaldehyde and 2 mg glutaraldehyde per ml in PBS for 5 min, washed with PBS, and incubated with 0.1% DOPA in 0.1 M sodium phosphate buffer for 4–5 h at 37°C following by PBS washes. Images of the stained cells were taken using a Leitz-Diaplan microscope (Nurnberg, Germany) and a Polaroid 35 mm camera for Digital Palette (Cambridge, MA). Images were analyzed using Image Pro Plus 4.0 software (Media Cybernetics, Silver Spring, MD). All studies were repeated at least three times.

Swine Yucatan swine (Charles River, Portland, ME) were housed in appropriately sized cages in an environmentally controlled room with a 12 h light/12 h dark photoperiod and supplied with food and water ad libitum. Animal care and use complied with the criteria described in the guide published by the U.S. National Institutes of Health, 1996. Twenty microliters of test material or vehicle (ethanol/propylene glycol, 70 : 30 v/v) was applied topically, twice a day, 5 d per wk, for 8 wk, on the dorsum of swine. Treatments of individual swine were always arranged in a head to tail order on one side, and in a tail to head order on the other side of the animal. Visual observations of lightening were made weekly. Biopsies were taken at the end of the study using standard techniques. Swine experiments were repeated with at least three individual swine. A mean erythema dose of UVB was determined by placing a plastic template with 1×1 $inch^2$ cutouts on the dorsum of the swine. Using a UVB lamp (Model UVM-57, 302 nm lamp, UVP Inc., Upland, CA) placed on the template, sites were exposed to UVB with increasing time points, every other day for 5 d. Unexposed sites were covered with the same material as the template. One mean erythema dose was established as the dose that produces the least amount of visible erythema. Swine were exposed to one mean erythema dose, once per day, on three alternate days (Monday, Wednesday, Friday). Test compounds were applied once daily, for 2 wk, immediately after each UVB exposure, and on non-UVB exposure days. To prevent a

possible sunscreen effect, the treated sites were cleaned with water and allowed to dry before each UVB exposure. Visual observations of the tanning response were made after 2 wk, and biopsies were taken at that time for histologic analysis. All swine studies presented here had no visual irritation, and histologic analyses revealed no markers of irritation or other pathologic signs.

Histology Sections from swine biopsies were stained with Fontana–Mason (F&M) according to Sheehan and Hrapchak (1980). F&M detects silver nitrate reducing molecules, which in skin identifies primarily melanin. At least two biopsies per treatment, three sections per biopsy were processed for each swine.

RESULTS

UVB and TCM-1 induce MITF promoter-luciferase activity To evaluate the effects of various agents on MITF promoter activity, murine B16 melanoma cells, murine melan-a melanocytes, and human HaCaT keratinocytes were transiently transfected with the human MITF promoter-luciferase construct. Validation of the system with forskolin, a known cAMP elevating agent, revealed a concentration- and time-dependent induction of MITF promoter transcription in melanocytes, as previously described (Price *et al*, 1998b), but not in keratinocytes (**Fig 1*a***). Forskolin (100 μM) significantly induced MITF promoter activity (91.0 ± 18% in B16 cells and 92.0 + 0.5% in melan-a) compared with untreated controls (**Fig 1*a***). α-MSH, a known natural inducer of melanogenesis, also stimulated MITF promoter activity in this system, but to a lower level (not shown) as expected (Price *et al*, 1998b). Therefore, forskolin was used as a positive control in this study. To understand better the molecular mechanism of UVB-induced pigmentation, we examined the effect of UVB irradiation on MITF promoter activity. UVB irradiation resulted in an increase in MITF promoter activity, which was slightly lower than that induced by forskolin (67.4 ± 28.6% in B16 cells and 62.2 ± 26.2% in melan-a) (**Fig 1*a***). The regulation of the MITF promoter via UVB irradiation was found to be time dependent (**Fig 1*b***). The maximal response to UVB was observed at 7–8 h, whereas forskolin is known to exert maximal effect at 3–4 h post-treatment (Price *et al*, 1998b). Concentration-dependent upregulation of the MITF promoter activity was documented at lower dose exposures (**Fig 1*c***) as higher UVB exposure resulted in increased cytotoxicity. Combination of UVB and forskolin showed an additive response, suggesting that forskolin and UVB may not share identical induction pathways (**Fig 1*a***). These data indicate a strong correlation between MITF promoter activation and UVB-induced pigmentation. MITF promoter activity was not detectable in HaCaT keratinocytes, when stimulated by either forskolin or UVB radiation (**Fig 1*a***), further indicating that the stimulation of MITF promoter by forskolin and UVB is a melanocyte-specific event.

Interestingly, the natural extract TCM-1 induced MITF promoter activation in a concentration-dependent manner. At 0.05 mg per ml, TCM-1 treatment resulted in a stimulation level similar to that of 100 μM of forskolin (**Fig 1*d***). The combined effect of TCM-1 and forskolin was found to be greater than that of each treatment alone (**Fig 1*d***), suggesting the possibility of different induction mechanisms by TCM-1 and forskolin.

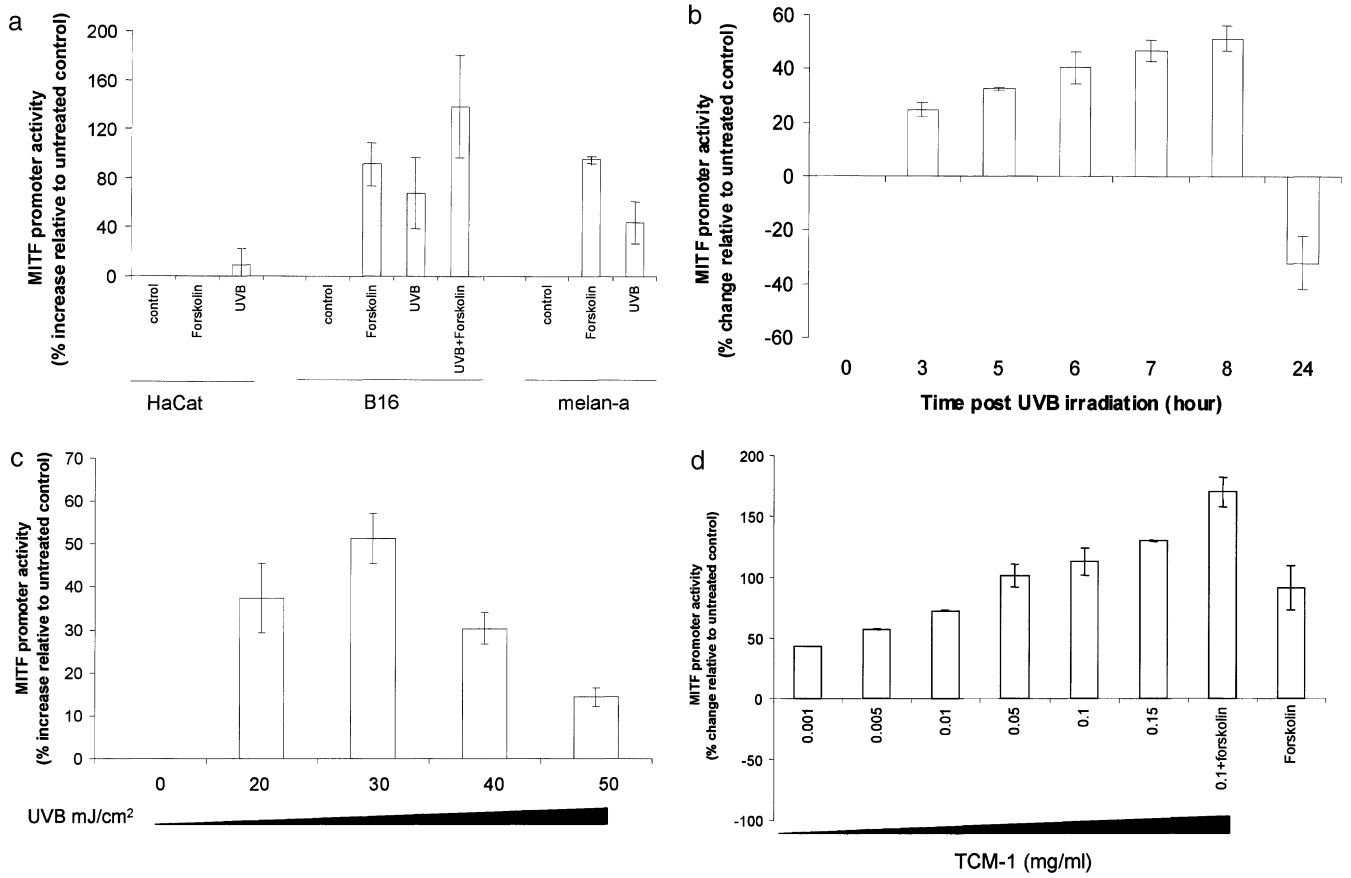

Figure 1. Modulation of MITF promoter activity. HaCaT, B16, and melan-a cells, transfected with the MITF promoter-luciferase construct (pGL2.hMIP) were treated, at 48 h after transfection, for 7 h with (*a*) forskolin (100 μM), UVB (30 mJ per cm^2) and the combination of forskolin and UVB. (*b*) Transfected B16 cells were treated with 30 mJ per cm^2 for the times indicated. (*c*) Transfected B16 cells were treated with increasing doses of UVB for 7 h. (*d*) Transfected B16 cells were treated with increasing concentrations of TCM-1 and combination of TCM-1 (0.1 mg per ml) and forskolin (100 μM) as indicated. Measured luciferase activity is expressed as percent change of MITF promoter activity relative to untreated control (mean ± SEM).

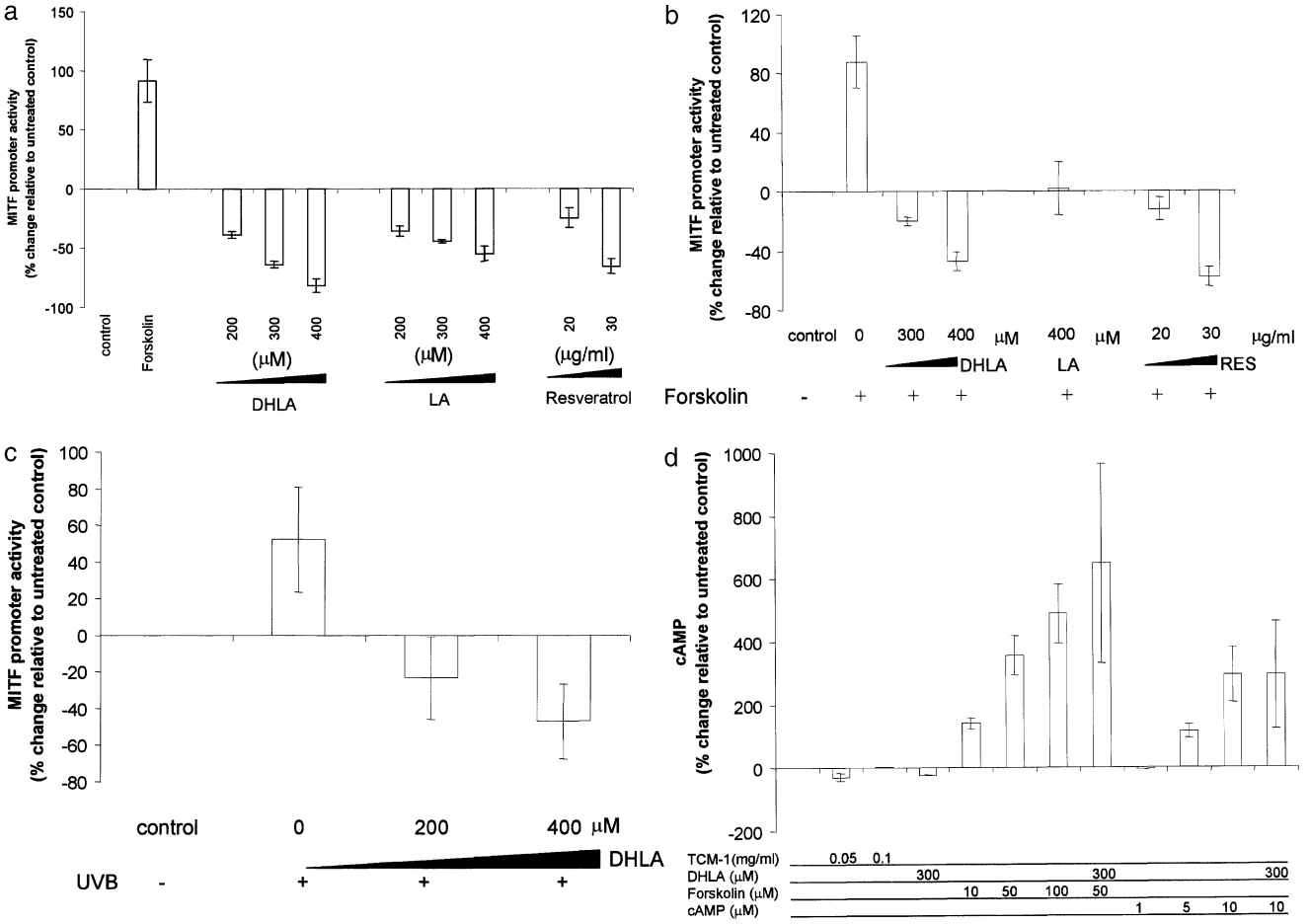

Figure 2. DHLA, LA, and resveratrol inhibit forskolin and UVB-induced MITF promoter activity. B16 cells transfected with the MITF promoter-luciferase construct (pGL2.hMIP) were treated 48 h after transfection, with (*a*) Increasing concentrations of DHLA, LA, and resveratrol. (*b*) Forskolin alone (100 μM), or in combination with DHLA (300 and 400 μM), LA (400 μM), or resveratrol (20 and 30 μg per ml); (*c*) UVB alone (30 mJ per cm^2), or in combination with DHLA (200 and 400 μM). Measured luciferase activity is expressed as percent increase of MITF promoter activity relative to untreated control (mean ± SEM). (*d*) B16 cells were treated with TCM-1, DHLA, forskolin, and cell permeable 8-bromo-cAMP at the concentrations indicated for 7 h. Measured cellular cAMP is expressed as percent increase of cAMP concentration relative to untreated control (mean ± SEM).

Inhibition of MITF promoter-luciferase activity by DHLA, LA, and resveratrol As anti-oxidants are known to affect pigment production (Hamanaka *et al*, 1990; Darr *et al*, 1996; Lee and Youn, 1998; Firkle *et al*, 2000), we tested their possible effect on MITF promoter activity. Evaluation of a battery of anti-oxidants revealed that only DHLA, LA, and resveratrol could inhibit MITF promoter-luciferase activity. As shown in **Fig 2**(***a***), these effects were concentration dependent, with a strong inhibitory activity at 400 μM for both DHLA and LA, and 30 μg per ml for resveratrol. To assure that the observed inhibition was not due to cytotoxicity, these compounds were tested for their effects on cell viability. No detectable changes in cell viability were observed up to 600 μM concentrations of DHLA and LA. In contrast, resveratrol inhibited MITF promoter activity at a narrower range. At 10 μg per ml no inhibitory activity was detected but at concentrations higher than 30 μg per ml, resveratrol had a cytotoxic effect. All other anti-oxidants tested (e.g., ascorbic acid, caffeic acid, glutathione, pycnogenol, and concentrated green tea polyphenols, not shown), did not induce a significant inhibitory effect in the MITF promoter-luciferase assay.

DHLA, LA, and resveratrol inhibit forskolin and UVB-induced MITF promoter activity The induction of MITF promoter-luciferase activity by UVB irradiation (**Fig 1***a*) suggests that UVB-induced skin pigmentation could be mediated via the activation of MITF gene expression. As anti-oxidants can provide photoprotection (Hamanaka *et al*, 1990; Darr *et al*, 1996; Lee and Youn, 1998; Firkle *et al*, 2000), we examined the effects of LA, DHLA, and resveratrol on UVB and forskolin-induced MITF promoter activity. B16 cells transfected with the MITF reporter construct were treated with these agents in combination with either forskolin or UVB irradiation. DHLA, LA, and resveratrol inhibited forskolin-induced MITF promoter-luciferase activity (**Fig 2***b*) and UVB-induced MITF promoter-luciferase activity (**Fig 2***c*) in a concentration-dependent manner. DHLA was found to be less potent in inhibiting UVB-induced MITF stimulation, compared with its inhibition of forskolin-induced MITF activity, further suggesting that UVB and forskolin do not share identical induction pathways. Similar results were obtained using LA and resveratrol (not shown).

The inhibition of either basal or forskolin-induced MITF promoter activity by DHLA did not involve a change in cAMP concentration in the treated melanocytes. As shown in **Fig 2**(***d***), cAMP concentration increased only following treatment with forskolin or a cell permeable cAMP. This increase of cAMP was not inhibited by DHLA. Similarly, TCM-1 treatment had no effect on cAMP concentration, whereas MITF promoter activity was strongly stimulated. Interestingly, a MITF promoter mutated at the CRE lost almost completely its activity in B16 cells and responded minimally to forskolin, TCM-1, or DHLA treatment

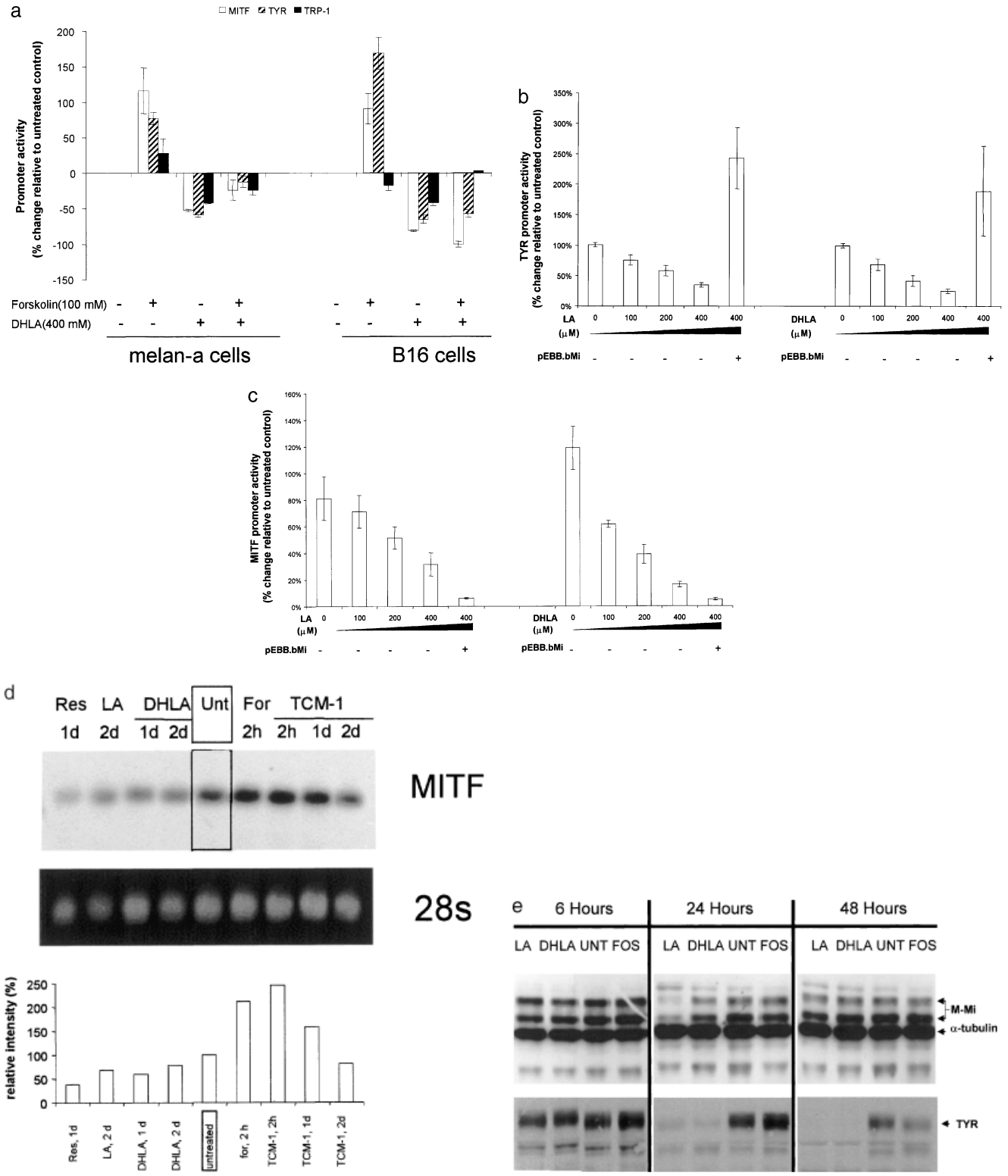

Figure 3. DHLA inhibits tyrosinase and TRP-1 promoter activities via the inhibition of MITF promoter. (*a*) DHLA inhibits MITF, tyrosinase, and TRP-1 promoter activities. Melan-a and B16 cells were transfected with MITF, tyrosinase, or TRP-1 promoter luciferase reporter constructs. At 48 h after transfection, cells were treated with forskolin (100 μM), DHLA (400 μM), or their combination for 7 h. Measured luciferase activity is expressed as percent of the basal luciferase activity relative to untreated control (mean ± SEM). (*b,c*) MITF rescues DHLA and LA-inhibited tyrosinase activity. B16 cells were transfected with either MITF or tyrosinase promoter luciferase reporter constructs and with either vector (pEBB) or MITF expression plasmid (pEBB.bMi). Cell lysates were prepared following 24 h incubation with LA or DHLA. MITF overexpression rescues the tyrosinase promoter activity inhibited by LA or DHLA (*b*), but has no effect on MITF transcription (*c*). (*d*) Modulation of endogenous MITF mRNA levels. NHEM were treated with DHLA (350 μM), LA (350 μM), and resveratrol (20 μg per ml) for 1 or 2 d, and with forskolin (100 μM) or TCM-1 (0.1 mg per ml) for 2 h, 1 d, or 2 d. Total RNA (20 μg) was analyzed by northern blots, probed for MITF expression. The relative intensity of the MITF mRNA band is expressed as percent of MITF signal intensity relative to untreated control, normalized to the 28S rRNA ethidium bromide stain. (*e*) Modulation of endogenous MITF and tyrosinase protein levels. Protein extracts from B16 cells treated with DHLA (350 μM), LA (350 μM), and forskolin (100 μM) for indicated times, were western blotted and probed with anti-Mi antibodies followed by anti-tubulin and with αPEP-7 (anti-tyrosinase) antibodies.

(data not shown). These data suggest that DHLA and TCM-1 may possibly target transcription factors/elements, which may function cooperatively with the CRE binding protein(s) to transactivate the MITF promoter.

Melanogenic gene expression is affected by MITF modulation As MITF regulates tyrosinase and Tryp1 expression (Yasumoto *et al*, 1994; Bentley *et al*, 1994; Hemesath *et al*, 1994) we studied the effect of DHLA, LA, and resveratrol on the expression levels of these genes. B16 and melan-a cells were transfected with the human MITF, tyrosinase, or Tryp1 promoter-luciferase reporter constructs. Following transfection, cells were treated with forskolin, DHLA, or their combination, and their relative luciferase activities were measured. As shown in **Fig 3**(***a***), incubation with forskolin for 7 h increased both MITF and tyrosinase promoter activities, but had no effect, at that time point, on Tryp1 promoter-driven transcription. DHLA treatment significantly reduced the activity of all three promoters, relative to their baseline transcription levels. Moreover, DHLA was able to inhibit the forskolin-induced expression of MITF and tyrosinase (**Fig 3*a***). Similar inhibitory activities were also observed using LA and resveratrol (not shown).

To decipher whether the inhibitory effect of DHLA and LA on the tyrosinase promoter was mediated by the downregulation of endogenous MITF gene expression, a rescue study with overexpressed MITF was performed. A MITF expression plasmid (pEBB.MITF) was cotransfected with MITF or tyrosinase-luciferase constructs, and luciferase activity was measured in the presence of DHLA and LA. Cells transfected with vector alone (pEBB) served as a control. In B16 cells, the inhibition of tyrosinase promoter activity by LA and DHLA was rescued by the overexpressed MITF (**Fig 3*b***) and further increased (150%) relative to untreated cells. As a control, the cotransfected MITF promoter, which was inhibited by LA or DHLA, was not rescued by the overexpressed MITF (**Fig 3*c***). A further decrease in the MITF promoter-luciferase activity was documented. This is possibly due to a feedback mechanism, as the overexpressed MITF protein competes with the cotransfected MITF promoter construct for endogenous transcription factors; however, this hypothesis needs to be further studied. These results indicate that the overexpressed MITF protein could activate the tyrosinase promoter, whereas the endogenous MITF promoter is still inhibited by LA or DHLA. These data support a model in which the inhibition of tyrosinase promoter activity by LA and DHLA is mediated by downregulation of MITF gene expression.

Northern blot analysis was performed to confirm the modulatory effect of these agents on the endogenous levels of MITF gene expression. NHEM cells were treated with test compounds for different times, based on the time course of their effect on MITF promoter-luciferase activity. No significant changes in cell numbers were observed following these treatments. In agreement with the promoter studies, treatment with DHLA, LA, and resveratrol reduced endogenous MITF mRNA levels by 35%, 35%, and 63%, respectively, relative to untreated control (normalized to gel loading by image analysis; **Fig 3*d***). TCM-1 treatment enhanced endogenous MITF expression in normal melanocytes in a time-dependent manner, with a maximum at 2 h, and a complete return to basal level by 2 d (**Fig 3*d***). Similar results were obtained using melan-a or B16 cells (not shown). These data indicate that the modulation of MITF promoter activity, as demonstrated by the luciferase studies, correlates with the modulation of the endogenous MITF expression pattern.

We then further investigated the effect of DHLA and LA on MITF and tyrosinase protein expression levels. B16 cells, treated with LA (350 μM), DHLA (350 μM), and forskolin (100 μM) for 6, 24, and 48 h, were lyzed for western blot analysis. Probing with Mi-specific monoclonal antibody demonstrated that total Mi protein levels [including phosphorylated and nonphosphorylated melanocyte-specific microphthalmia (M-Mi) bands] were significantly reduced at 24 h post-treatment and returned to almost basal levels at 48 h post-treatment (**Fig 3*e***). Unlike forskolin, LA and DHLA treatments did not produce an obvious mobility shift of Mi (due to phosphorylation on serine 73) at the time points tested (Hemesath *et al*, 1998). The ratio of phosphorylated to nonphosphorylated Mi was retained, whereas the total protein levels were changed. It is possible that LA or DHLA could affect phosphorylation of Mi at earlier time points. Interestingly, the effects of LA and DHLA treatments on tyrosinase protein levels were found to be more significant than their effects on Mi. As shown in **Fig 3**(***e***), tyrosinase levels were unaffected at 6 h, reduced significantly at 24 h and continued to decrease at 48 h, relative to untreated control. Forskolin treatment increased tyrosinase protein expression level at 24 h and reduced to a level even lower than untreated control by 48 h.

DHLA, LA, and TCM-1 treatments affect tyrosinase activity To investigate further the effect of MITF transcription modulation on melanogenesis, we studied the effect of LA, DHLA, and TCM-1 on tyrosinase activity in B16 cells. B16 cells were treated with DHLA (400 μM), LA (400 μM), forskolin (100 μM), and TCM-1 (0.1 mg per ml) for 48 h, cells were trypsinized, and treated cells (7×10^4) were used for tyrosinase analysis. As shown in **Fig 4**(***a***), DHLA (400 μM) and LA (400 μM) treatments reduced tyrosinase activity by 41.9% and 35.5, whereas forskolin and TCM-1 increased tyrosinase activity by 17.0% and 132%, respectively. These data indicate that MITF transcription modulators can regulate tyrosinase activity. Antioxidants are also expected to inhibit directly tyrosinase enzymatic activity. Therefore, untreated B16 cells were lyzed, and aliquots of lysates were incubated with DOPA in the presence or absence of DHLA, to measure directly the effect of DHLA on tyrosinase enzymatic activity. In parallel, B16 cells were treated with DHLA in culture to measure the combined effect of DHLA on MITF transcription and directly on tyrosinase enzymatic activity. As shown in **Fig 4**(***b***), the enzymatic activity of tyrosinase was indeed inhibited by DHLA. Treatment of the cells in culture with DHLA, however, further decreased tyrosinase activity demonstrating a combined effect on MITF/tyrosinase gene expressions and on tyrosinase enzymatic activity during melanogenesis.

Tyrosinase activity was also analyzed in treated NHEM cells *in situ* by DOPA staining. As shown in **Fig 4**(***c–k***), forskolin and TCM-1 treatments increased tyrosinase activity (**Fig 4*d*,*e*** relative to untreated control ***c***), whereas DHLA, LA, or resveratrol significantly reduced tyrosinase activity *in situ* (**Fig 4*f–h***). The loss of DOPA staining following DHLA, LA, and resveratrol treatments for 48 h correlates well with the greatest reduction of tyrosinase protein expression level at this time point (see **Fig 3*e***). The tyrosinase activity induced by either TCM-1 or forskolin was inhibited by DHLA (**Fig 4*i*** and **Fig 4*j***, respectively) and LA (**Fig 4*k***, forskolin plus LA), further confirming the ability of these agents to modulate pigmentation.

DHLA, LA, and resveratrol reduce skin pigmentation In order to demonstrate the effect of MITF modulating agents *in vivo*, dark-skinned Yucatan swine were topically treated with LA, DHLA, and resveratrol (1%) or with vehicle alone twice a day, 5 d per wk, for 8 wk. These treatments (with the exception of the vehicle alone) resulted in visible skin lightening (e.g., DHLA, **Fig 5*a***) with no signs of irritation or other undesired effects. Histologic analysis using F&M staining demonstrates reduced pigment deposition at the treated sites (DHLA, **Fig 5*c***) relative to the untreated control (**Fig 5*b***). Image analysis of F&M-stained sections quantifies this effect (**Fig 5*d***), further supporting the notion that negative modulators of MITF could be used as skin lightening agents.

DHLA, LA, and resveratrol inhibit UVB-induced tanning DHLA was shown to inhibit UVB-induced MITF

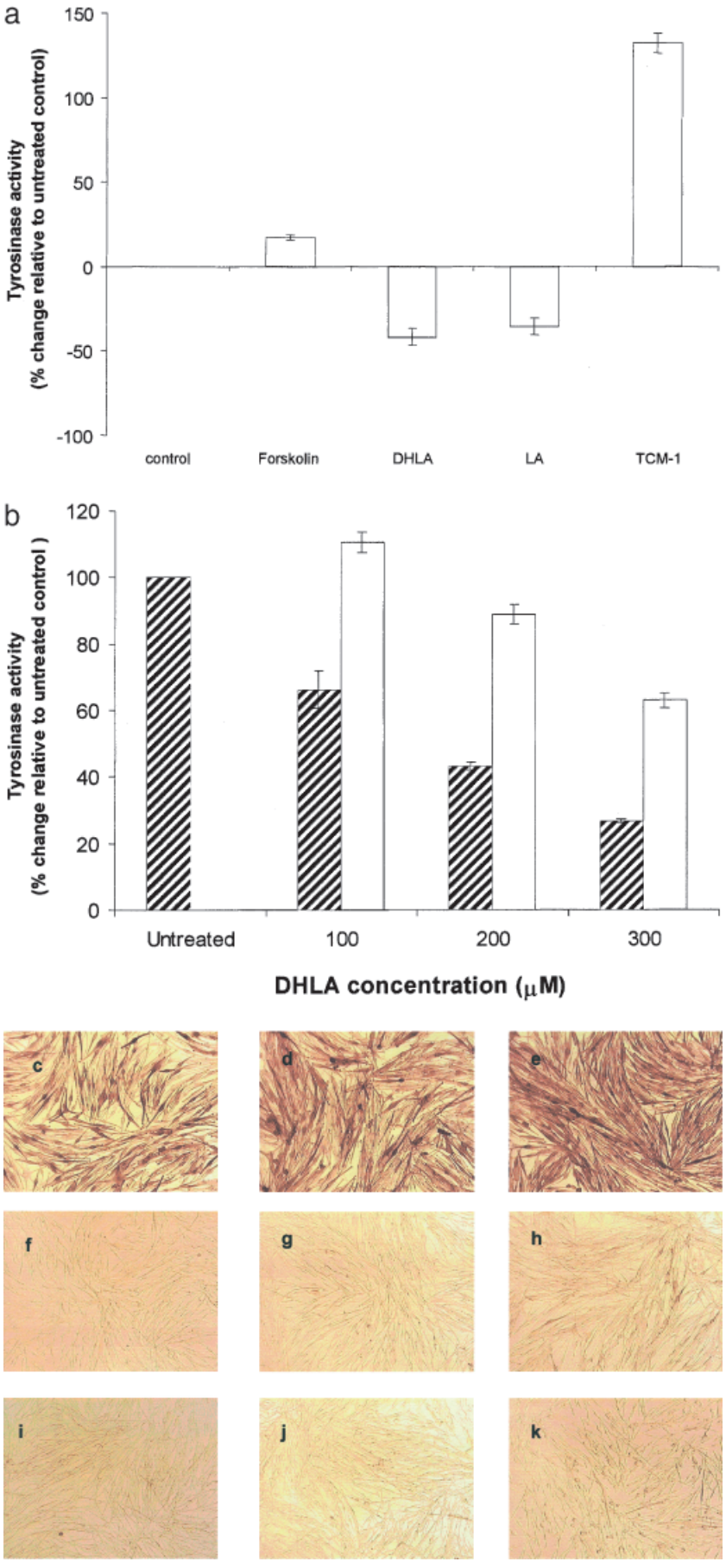

Figure 4. DHLA, LA, and TCM-1 treatments affect tyrosinase activity. (*a*) Tyrosinase activity analysis of B16 cells, treated with test compounds. B16 cells treated for 24 h with forskolin (100 μM), DHLA (400 μM), LA (400 μM), and TCM-1 (0.1 mg per ml) were lyzed and incubated with 100 μl of 0.1% DOPA for 3 h. Tyrosinase activity was determined by measuring absorbance at 490 nm. Data expressed as percent change of the tyrosinase activity relative to untreated control (mean ± SEM). (*b*) Analysis of tyrosinase enzymatic activity in DHLA-treated cells and lysates. B16 cells were either lyzed and then incubated with DHLA and DOPA for 3 h (*open bars*) or treated with DHLA in culture and then lyzed (*hatched bars*). The effect of DHLA on the enzymatic activity of tyrosinase in the lysate (*open bars*) was compared with the effect of DHLA on live cells (*hatched bars*). (*c–k*) Modulation of tyrosinase activity *in situ*. NHEM were treated with 100 μM forskolin (*d*), 0.1 mg TCM-1 per ml (*e*), 350 μM DHLA (*f*), 350 μM LA (*g*), 20 μg resveratrol per ml (*h*), forskolin and DHLA (*i*), forskolin and LA (*j*), and TCM-1 and DHLA (*k*). Untreated control is shown in (*c*). Cells were DOPA stained following 48 h incubation with test agents.

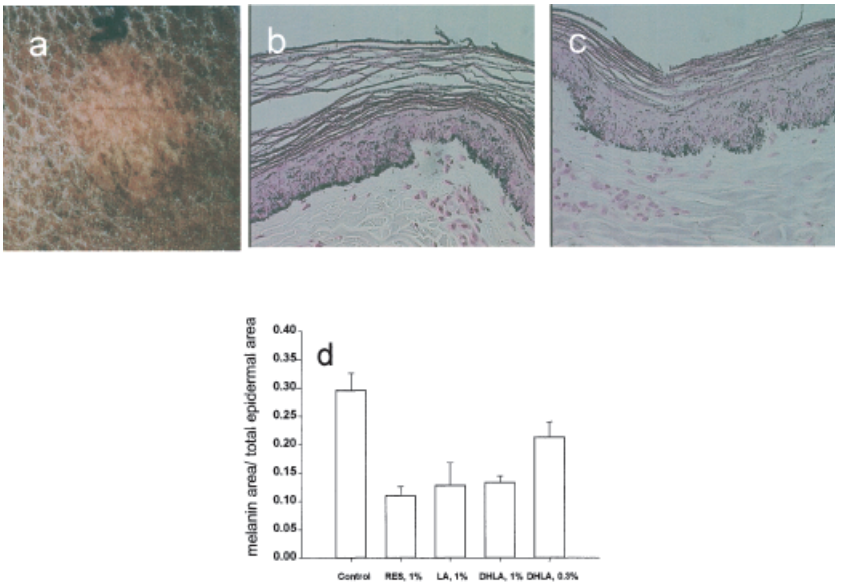

Figure 5. DHLA reduces skin pigmentation *in vivo*. Dark-skinned Yucatan swine were topically treated with 1% LA, 1% resveratrol per ml, 1% DHLA or vehicle, twice a day, 5 d per wk, for 8 wk. (*a*) Picture of a treated site, taken at 8 wk of treatment, demonstrating DHLA-induced skin lightening. (*b,c*) F&M staining of histologic sections of untreated (*b*) and DHLA-treated (*c*) sites. (*d*) Relative pigmentation (melanin area divided by total epidermis area), as analyzed using computerized imaging.

promoter activity (**Fig 2*c***). Therefore, the ability of DHLA to inhibit UVB-induced pigmentation by MITF transcription modulation was also tested *in vivo*. Light-skinned Yucatan swine were UVB irradiated as described in *Materials and Methods*, to create a visible tanning response. UVB-irradiated sites were treated with test compounds or controls once daily, for 10 d, either immediately after UVB exposure or on non-UVB exposure days, and the level of tanning was monitored visually and histologically. Treatment with UVB alone or with UVB and vehicle induced visible darkening, which was confirmed histologically using F&M staining (compare **Fig 6*b***, UVB and vehicle treatment, to the untreated skin shown in **Fig 6*a***). Treatments with DHLA, LA, and resveratrol (**Fig 6*c–e***, respectively), reduced the UVB-induced pigment deposition. Image analysis of F&M-stained sections quantifies this effect (**Fig 6*f***), further confirming that modulation of MITF transcription by these agents could reduce UVB-induced tanning.

DISCUSSION

Solar irradiation is a major source of environmental damage to skin, and UV-induced skin pigmentation has been widely recognized as a defense mechanism against such damage (Ortonne, 1990; Kollias *et al*, 1991; Gilchrest, 1998; Sturm, 1998; Chakraborty *et al*, 1999; Gilchrest and Eller, 1999). The transcription factor MITF plays a key role in pigmentation, but has not been associated with the effect of UVB on pigment production. To better understand the molecular mechanism of UVB-induced pigmentation, we examined the effect of UVB irradiation on MITF gene expression. This study demonstrates that UVB can induce MITF promoter activity *in vitro* in a time- and concentration-dependent manner. Furthermore, the induction of MITF promoter activity by UVB was not identical to that of forskolin and was delayed relative to that of forskolin-induced MITF transcription. It has been shown previously that UVB-mediated events could be mimicked or enhanced by MSH (Im *et al*, 1998), and that exposure to sunlight results in increased levels of circulating MSH and adrenocorticotropic hormone in both horse and human (Bolognia *et al*, 1994; Im *et al*, 1998; Suzuki *et al*, 1999). Therefore, we hypothesized that MITF promoter activity may be indirectly mediated by UVB and that the activation step may involve the release of MSH and/or adrenocorticotropic hormone. The

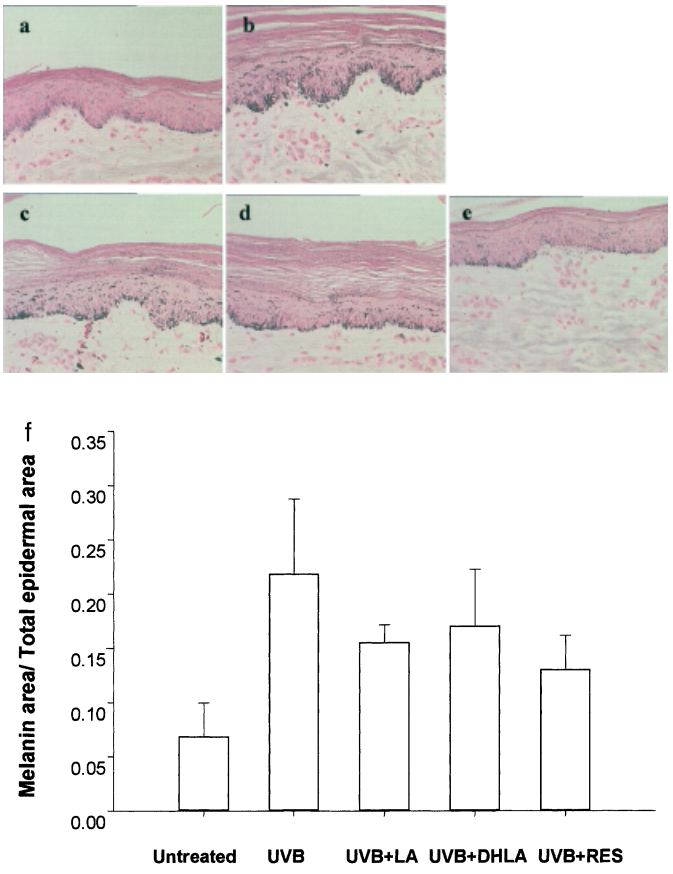

Figure 6. Prevention of UVB-induced tanning. Light-skinned Yucatan swine were UVB treated with 1 mean erythema dose to create visible tanning as described in *Materials and Methods*, with or without daily treatment of test material. Tanning was observed in UVB alone and in UVB and vehicle-treated sites, and was reduced or prevented with LA, DHLA, and resveratrol treatments. F&M staining of histologic sections confirm this observation: (*a*) untreated control; (*b*) UVB plus vehicle; (*c*) UVB plus LA (1%); (*d*) UVB plus DHLA (1%); (*e*) UVB plus resveratrol (1%). Relative pigmentation (melanin area divided by total epidermis area), as analyzed using computerized imaging, is shown in (*f*).

delayed activation of MITF by UVB (relative to that of 3–4 h by forskolin) is in agreement with the time frame required for the processing and release of MSH and adrenocorticotropic hormone (Bolognia *et al*, 1994; Im *et al*, 1998;reviewed in Suzuki *et al*, 1999). In turn, released MSH and/or adrenocorticotropic hormone could transactivate the MITF promoter through the CRE and/or other transcription factors (Busca and Ballotti, 2000). Indeed, MSH was shown to transactivate the MITF promoter activity, although with relative lower activity compared with forskolin and to increase MITF protein levels in B16 cells as described by Price *et al* (Price *et al*, 1998b). MITF is regulated by two signaling pathways, the MSH and c-Kit pathways (reviewed in Fisher, 2000; Tachibana, 2000). As the c-Kit and the MSH signal transduction pathways have different kinetics (minutes *vs* hours) (Price *et al*, 1998a), we suggest that UVB exerts its effect on MITF promoter via the MSH, and not via the c-Kit pathway.

UV irradiation is known to induce the production of reactive oxygen species in human skin, resulting in oxidative stress and damage (Dalle and Pathak, 1992; Tyrrell, 1995; de Gruijl, 2000). Anti-oxidants have been widely used in skin-care products to provide photoprotection (Hamanaka *et al*, 1990; Darr *et al*, 1996; Lee and Youn, 1998; Firkle *et al*, 2000). Of the many anti-oxidants tested in this study, however, only LA, DHLA, and resveratrol were found to affect MITF promoter activity and reduce endogenous MITF mRNA and protein levels. Vitamin C, caffeic acid, pycnogenol, glutathione, and concentrated green tea polyphenols, which are known strong anti-oxidants, had no effect on MITF promoter activity, at either baseline or following UVB or forskolin induction. These finding suggests that the anti-oxidant activity *per se* is not involved in the regulation of MITF transcription.

DHLA and LA are strong anti-oxidants, known for their ability to prevent UV-induced photo-oxidative damage (Packer *et al*, 1995; Saliou *et al*, 1999; Alvarez and Boveris, 2000). LA plays an important part in mitochondrial dehydrogenase actions. Its reduced form, DHLA, exhibits a slightly higher anti-oxidant activity than LA, by reacting with superoxide and hydroxyl radicals (Packer *et al*, 1995). resveratrol is a polyphenolic compound found in edible plants such as grapes and peanuts. Resveratrol contributes to the anti-oxidant potential of red wine by acting as a reactive oxygen species scavenger and is believed to prevent cardiovascular diseases, and to have anti-inflammatory and chemopreventive activities (Fremont, 2000). Interestingly, resveratrol has no chemical structure similarity to DHLA and LA, whereas it also exhibited strong inhibition of MITF and tyrosinase promoter activities in our studies. TCM-1, an extract of the plant *Astragulus membraneus*, has been used orally as traditional Chinese medicine for many years. TCM-1 is believed to enhance immunity, to strengthen the body, to have a diuretic effect, and to affect cardiovascular function by reducing blood viscosity (Terry, 1996). To our surprise, TCM-1 induced MITF expression and tyrosinase activity in normal human melanocytes to the same or even higher extent than forskolin. A combination of TCM-1 and forskolin resulted in less than additive stimulation, suggesting either a saturation of transcription inducibility, or a pathway shared by these agents. Furthermore, the kinetics of MITF induction by TCM-1 was similar to that of forskolin, and LA, DHLA, and resveratrol inhibited both forskolin- and TCM-1 induced pigmentation.

Forskolin is known to elevate cAMP levels (Seamon and Daly, 1981, 1986; Seamon *et al*, 1981), and the melanocyte-specific MITF promoter contains a CRE (Fuse *et al*, 1996; Bertolotto *et al*, 1998; Price *et al*, 1998b). Forskolin-induced MITF and tyrosinase expression is melanocyte specific (Price *et al*, 1998b), providing an example of tissue restricted CRE responsiveness. The mechanistic basis for this restriction is not yet understood. Whereas forskolin upregulates cAMP levels in melanocytes, however, no changes in cAMP production were identified following either TCM-1, UVB (not shown), or DHLA treatment. Moreover, DHLA was able to inhibit completely the forskolin-induced MITF promoter activity without decreasing the cAMP levels induced by forskolin. Our preliminary data demonstrate that a mutated MITF CRE sequence, known to inhibit MITF promoter responsiveness to forskolin (Bertolotto *et al*, 1998; Price *et al*, 1998b), is not responsive to TCM-1 induction. These data suggest that DHLA and TCM-1 possibly target other transcription factor elements on the MITF promoter that may function cooperatively with CRE binding protein(s) to transactivate MITF. Several transcription factors (e.g., Sox 10, Pax 3, and α-catenin/TCF-LEF-1) have been shown to regulate the MITF promoter (Watanabe *et al*, 1998; Lee *et al*, 2000; Potterf *et al*, 2000; Takeda *et al*, 2000; Verastegui *et al*, 2000b). These factors might potentially cooperate with CRE binding protein(s) to enable the MITF promoter activity only in melanocytes. The mutated CRE therefore might not only lose cAMP responsiveness, but could also affect interactions with other transcription factors involved in MITF activation. DHLA, LA, and resveratrol could inhibit the MITF promoter activity by a mechanism that may interfere with these interactions. Alternatively, it is possible that cAMP would re-localize within the melanocytes upon DHLA and TCM-1 treatments without changes in total cAMP concentration. Whereas such a mechanism has not yet been documented, it could not be ruled out as tools are not available for such an analysis. More interestingly, recent studies demonstrate that MITF interacts with LEF-1, a mediator of Wnt signaling, and activates its own gene expression (Takeda *et al*, 2000; Shibahara *et al*, 2001; Saito *et al*, 2002), which could be affected by our test agents (Shibahara *et al*, 2001; Saito *et al*, 2002).

In addition to the inhibition of MITF promoter activity, DHLA, LA, and resveratrol affected tyrosinase and Tyrp1 promoter activities and tyrosinase activity. The inhibitory effect of these agents on tyrosinase promoter activity could be rescued by MITF overexpression, further supporting the notion that these agents affect MITF transcription. Furthermore, northern and western blot analyses confirmed the modulation of MITF gene expression by DHLA and LA. The inhibition of tyrosinase protein expression levels by LA and DHLA is greater than the inhibition of MITF protein levels (especially at 24 h post-treatment). As MITF binds to and activates tyrosinase, one would expect to see the delayed change of tyrosinase gene expression relative to that of MITF followed MITF modulators. Indeed, the inhibition of tyrosinase expression was found to be delayed relative to that of MITF by DHLA and LA in this study (**Fig 3*e***), and the induction of tyrosinase expression was also delayed in the B16 cells treated with α-MSH (Verastegui *et al*, 2000a). Based on the differential kinetic regulations of MITF and tyrosinase, it is possible that the profound effect of these treatments on MITF protein expression may be seen between 6 and 24 h. This also suggests that these agents might affect other transcription factors (e.g., Usf-1), which may directly affect tyrosinase expression. Usf-1 is a transcription factor involved in UV-induced tyrosinase expression (Galibert *et al*, 2001). Further studies are required to better understand the role of Usf-1 and of other MITF transcription factors in the DHLA and LA induced depigmentation. Finally, DHLA, LA, and resveratrol inhibited both basal and forskolin- or TCM-1-induced tyrosinase activities *in situ* without further affecting cell density (**Fig 4*c*–*k***). These agents also inhibited tyrosinase enzymatic activity in a test-tube assay, but to a lower level (even though their effective concentration in culture was much lower). This indicates a dual mechanism affected by these agents, of both decreased gene expression and inhibited enzymatic activity in live cells.

DHLA was found to depigment dark-skinned swine (**Fig 5**) and to prevent UVB-induced tanning *in vivo*. This suggests that modulation of MITF transcription by exogenous agents could be used to modulate skin pigmentation. Due to the lack of swine molecular tools, however, we were unable to provide direct *in vivo* evidence for the modulation of MITF expression. Topical application of TCM-1 did not result in a noticeable increase in skin color using the same animal model (not shown). This might be due to ineffective or insufficient delivery of the active agents within the TCM-1 extract to the basal layer of the epidermis. Alternatively, this might be due to the intrinsically limited capacity

of light-skinned swine to respond to pigmentary stimulation, as forskolin (24 mM) did not induce visible darkening in this animal model.

We show that UVB irradiation results in increased MITF transcription *in vitro*. Agents that reduce MITF transcription *in vitro* are shown to depigment swine skin, and to prevent UVB-induced tanning. These agents are shown to inhibit tyrosinase expression and activity via their MITF transcription modulating effect. This study suggests that such agents could be used to alter skin pigmentation. The precise mechanism of modulation of MITF promoter activity by these agents should be further investigated.

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