ORIGINAL ARTICLE

Pharmacologic suppression of MITF expression via HDAC inhibitors in the melanocyte lineage

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Summary

Melanoma incidence continues to rise at an alarming rate while effective systemic therapies remain very limited. Microphthalmia-associated transcription factor (MITF) is required for development of melanocytes and is an amplified oncogene in a fraction of human melanomas. Microphthalmia-associated transcription factor also plays an oncogenic role in human clear cell sarcomas, which typically exhibit melanoma-like features. Although pharmacologic suppression of MITF is of potential interest in a variety of clinical settings, it is not known to contain intrinsic catalytic activity capable of direct small molecule inhibition. An alternative drug-targeting strategy is to identify and interfere with lineage-restricted mechanisms required for its expression. Here, we report that multiple histone deacetylase (HDAC)-inhibitor drugs potently suppress MITF expression in melanocytes, melanoma and clear cell sarcoma cells. Although HDAC inhibitors may affect numerous cellular targets, we observed suppression of skin pigmentation by topical drug application as well as evidence of anti-melanoma efficacy in vitro and in mouse xenografts. Consequently, HDAC inhibitor drugs are candidates to play therapeutic roles in targeting conditions affecting the melanocyte lineage.

Introduction

Microphthalmia-associated transcription factor (MITF) is a basic-helix/loop/helix-leucine-zipper transcription factor which plays an essential lineage-specific role during melanocyte development (Hodgkinson et al., 1993; Hughes et al., 1993; Steingrimsson et al., 1994). The *MITF* gene has a complex promoter organization, with at least nine distinct isoforms sharing exons 2 to 9. Some of these isoforms exhibit tissue-specific restriction such as M-MITF, which is expressed in melanocytes and melanoma but not most other lineages (Hershey and Fisher, 2005; Udono et al., 2000). The melanocyte-specific promoter is located most proximal to the common downstream exons and is known as the *M-MITF* promoter (Fuse et al., 1996). As a transcription factor, MITF regulates multiple genes related to melanin synthesis, control of apoptosis, and cell cycle progression (Goding, 2000; Levy et al., 2006; Shibahara et al., 2001). Appropriate regulation of MITF is required for cell growth/survival in melanocytes and recent work has implicated MITF as a melanoma oncogene due to its amplification in ∼20% of human metastatic melanomas (Garraway et al., 2005). In addition, human clear cell sarcomas typically contain a chromosomal translocation which produces the EWS-ATF1 fusion protein (Brown et al., 1995; Fujimura et al., 1996; Lessnick et al., 1995). This chimeric oncoprotein directly triggers dysregulated expression of MITF, which in turn appears to play a vital role within this tumor (Davis et al., 2006). Thus, MITF is a potentially attractive molecular target for melanoma and clear cell sarcoma therapy. However being a transcription factor lacking ligand dependency, it is largely considered a difficult drug target by current approaches.

In contrast, histone deacetylase inhibitors (HDACi) have been shown to exhibit anti-cancer effects, and suberoylanilide hydroxamic acid (SAHA) has been FDA approved for treatment of cutaneous T-cell lymphoma (Kelly and Marks, 2005; Kelly et al., 2003, 2005; O'Connor et al., 2006). Cancer-related consequences of HDACi drugs include growth arrest and apoptosis as well as repression of angiogenesis (Liu et al., 2006). An effect of HDACi drugs on growth arrest and apoptosis has been shown in melanoma cells, but the mechanism has been poorly understood (Boyle et al., 2005). Recently, HDACi-treated embryos were observed to exhibit disrupted pigmentation in *zebrafish* and *Xenopus*

(Gurvich et al., 2005), suggesting that HDACi might have effects on melanocyte lineage-specific factor(s).

In this study, we provide evidence that multiple HDAC inhibitor drugs, including sodium butyrate (NaB), Trichostatin A (TSA), SAHA, and LBH589, repress M-MITF expression in melanoma and clear cell sarcoma cells. Although HDACi drugs have numerous effects, we observe this lineage-selective effect in melanocytes, which is associated with transcriptional downregulation of the M-MITF promoter. The resulting data suggest that HDACi drugs may antagonize MITF, with consequent therapeutic applications either alone or perhaps in combination with other MITF suppressing strategies, to control skin pigmentation and/or survival of MITF-dependent cancers.

Results and discussion

HDACi drugs repress M-MITF levels in melanoma and clear cell sarcoma cell lines

Microphthalmia-associated transcription factor is a lineage survival factor as well as amplified oncogene in ~20% of metastatic melanomas. Because of its vital role in the melanocyte lineage as well as in clear cell sarcoma (Davis et al., 2006), MITF is an attractive molecular target for melanoma and clear cell sarcoma therapy. In searching for small molecules which affect MITF expression, we wanted to explore if HDACi drugs could exhibit a role as MITF antagonists given the observed disruption of pigmentation in *zebrafish* and *Xenopus* (Gurvich et al., 2005). The melanocyte-specific isoform of MITF (M-MITF) migrates as a doublet (Figure 1A, B, arrows) due to serine 73 phosphorylation by MAPK (Hemesath et al., 1998), whereas higher migrating MITF isoforms (asterisk) reflect non-melano-cyte-specific forms of MITF derived from distinct promoters (Hershey and Fisher, 2005; Udono et al., 2000). We subjected several established melanoma and clear cell sarcoma cell lines to HDACi treatments and reproducibly observed suppressed expression of the M-MITF doublet protein species, but not the non-melanocytic isoforms (with some cases even being associated with observable slight upregulation) (Figure 1A, B, and data not shown). The acetylation status of histone H3 by HDACi in human melanoma cells correlated with activity of the HDACi drugs. Other HDACi drugs, suberoylanilide hydroxamic acid (SAHA) and LBH589 (George et al., 2005), also repressed M-MITF expression in melanoma cells (Figure 1A). We detected diminished RNA levels of the *M-MITF* isoform in UACC62 melanoma cells with NaB and TSA (Figure 2A and data not shown), suggesting that the decreased M-MITF protein levels result from reduced mRNA levels. To further examine whether stability of the *MITF* mRNA may contribute to diminished protein levels following HDACi exposure, *MITF* mRNA decay kinetics were tested following actinomycin D treatment, in the presence or absence of
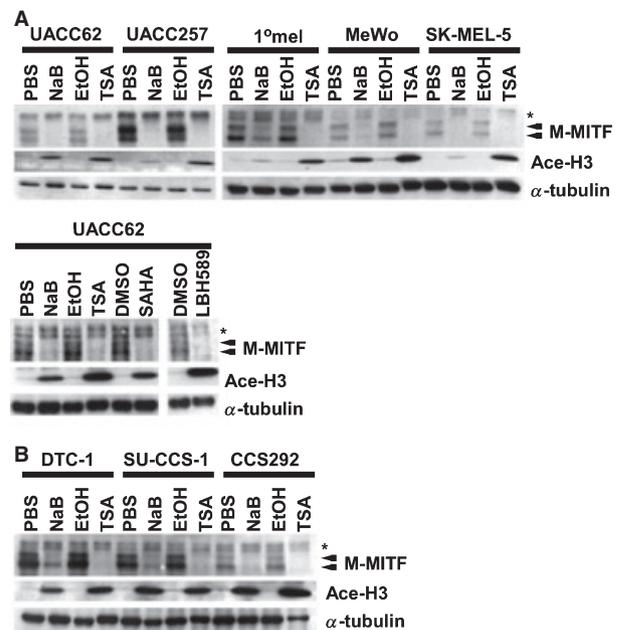

Figure 1. Histone deacetylase inhibitor (HDACi) drugs repress M-microphthalmia-associated transcription factor (MITF) and SOX10 levels in melanoma and clear cell sarcoma cell lines. (A) Western blotting analysis of whole-cell lysates prepared from UACC62, UACC257, MeWo, or SK-MEL-5 human melanoma cells and 1°melanocytes treated with PBS, sodium butylate (NaB), EtOH, or Trichostatin A (TSA) for 24 h (Upper panel) or from UACC62 human melanoma cells treated with PBS, NaB, EtOH, TSA, DMSO, suberoylanilide hydroxamic acid (SAHA), or LBH589 for 24 h (lower panel). Lysates were immunoblotted with antibodies against MITF, acetylated histone 3 (Ace-H3), or α-tubulin. The arrows show the bands of M-MITF protein. Asterisk indicates A-MITF protein. (B) Whole-cell lysates were prepared from DTC-1, SU-CCS-1, or CCS292 human clear cell sarcoma cells treated with PBS, NaB, EtOH, or TSA for 24 h. Other conditions are represented as indicated in Figure 1A.

HDACi. No significant difference was observed in the rate of *MITF* mRNA decay by this assay (Figure S1A), suggesting that HDACi exposure is unlikely to affect MITF expression via altered RNA stability.

As HDACi drugs led to *M-MITF*-specific mRNA downregulation and SOX10 and LEF1 are key upstream transcriptional regulators of the *M-MITF* promoter, we also examined the expression of these upstream regulators after the same treatments. Diminished protein and mRNA levels of *SOX10* and *LEF1* were observed in a time-dependent manner (Figure 2A, B) which correlated closely with, or slightly preceded MITF's decreases. Moreover, SOX10 overexpression was able to significantly rescue M-MITF expression and growth upon HDACi treatment in UACC62/SOX10 cells but not in UACC62/GFP (control) cells or UACC62/SOX10/Dom cells (Figure 2C and Figure S1B). Collectively these data suggest that the potent suppression of M-MITF expression by HDACi drugs likely occurs via downregulation of transcription of the MITF gene and might be
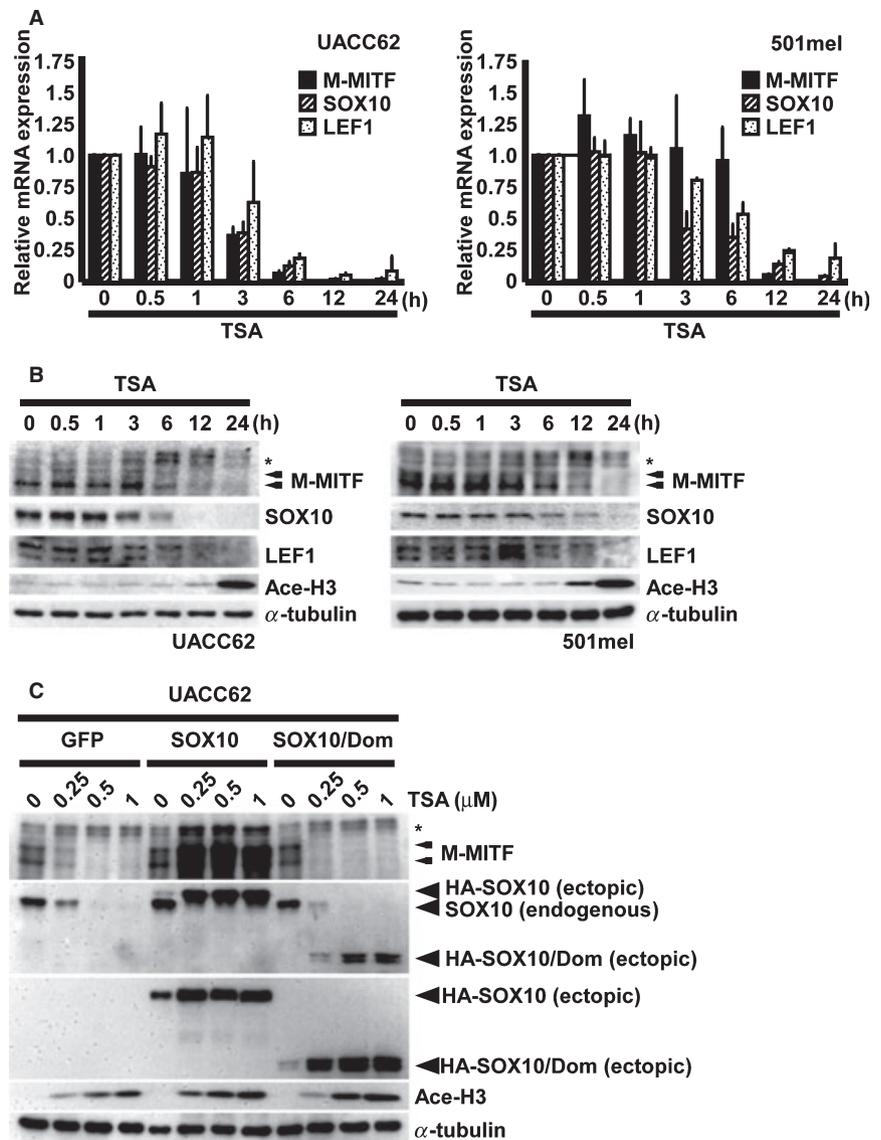
Figure 2. Trichostatin A represses SOX10 and LEF1 expression. (A) Quantitative reverse transcription PCR analysis of total RNA isolated from UACC62 and 501mel treated with 1 μM TSA for the indicated times. *M-MITF* (filled bars), *SOX10* (shadow bars) and *LEF1* (dotted bars) mRNA levels were normalized to glyceraldehyde-3-phosphate dehydrogenase and performed in triplicate. Data are mean ± SD of at least three independent experiments. (B) Western blotting analysis of whole-cell lysates prepared from UACC62 and 501mel treated with 1 μM of TSA for the indicated times. Lysates were immunoblotted with antibodies against MITF, SOX10, LEF1, Ace-H3, or α-tubulin. (C) Western blotting analysis of whole-cell lysates prepared from UACC62 overexpressing GFP, SOX10, or SOX10/ Dom cells. Arrowheads show the ectopic expressing SOX10 or SOX10/Dom and endogenous SOX10. Other conditions are represented as indicated in Figure 1A.

mediated by suppression of the upstream factors SOX10 or LEF1.

Skin-lightening effects of topical HDACi treatment

Multiple human pathologic hyperpigmentation conditions exist, including melasma, aging spots (lentigo senilis), post-inflammatory hyperpigmentation, and others. Aside from MITF's requirement for melanocyte viability, it also plays a central role in control of pigmentation. Multiple pathways control expression and activity of MITF, including the action of α-melanocyte stimulating hormone (e.g. in sun/UV-induced pigmentation) where it is thought to activate expression of multiple enzymes in the pigment synthesis cascade and related factors involved in melanin transport/secretion (Levy et al., 2006). Moreover, mutants of MITF exist which alter pigment color, without absence of melanocytes (Steingrímsson et al., 1996) suggesting that suppression of MITF might potentially offer

a topical therapeutic strategy for treatment of hyperpigmentation, if a dose were utilized which affects pigmentation without loss of melanocyte viability. To test this possibility, we used a mouse model of forskolin-induced hyperpigmentation (D'Orazio et al., 2006). The congenic C57BL/6 K14-SCF Mc1r[e/e] mouse strain contains epidermal melanocytes due to the keratin 14 (K14) promoter driving expression of stem cell factor (*Scf*; also known as *Kitl*) (humanized skin model) (Kunisada et al., 1998). Mc1r[e/e] encodes a frameshift mutation in the melanocortin receptor gene, which produces the redhair/fairskin phenotype, and which is susceptible to forkolin-induced hyperpigmentation (D'Orazio et al., 2006). As shown in Figure 3A, dorsal pigmentation induced by topical treatment with forskolin was potently repressed by topical treatment with the HDAC inhibitor TSA. Using Fontana-Masson staining, which detects melanin in tissue sections, substantial suppression of skin pigmentation was
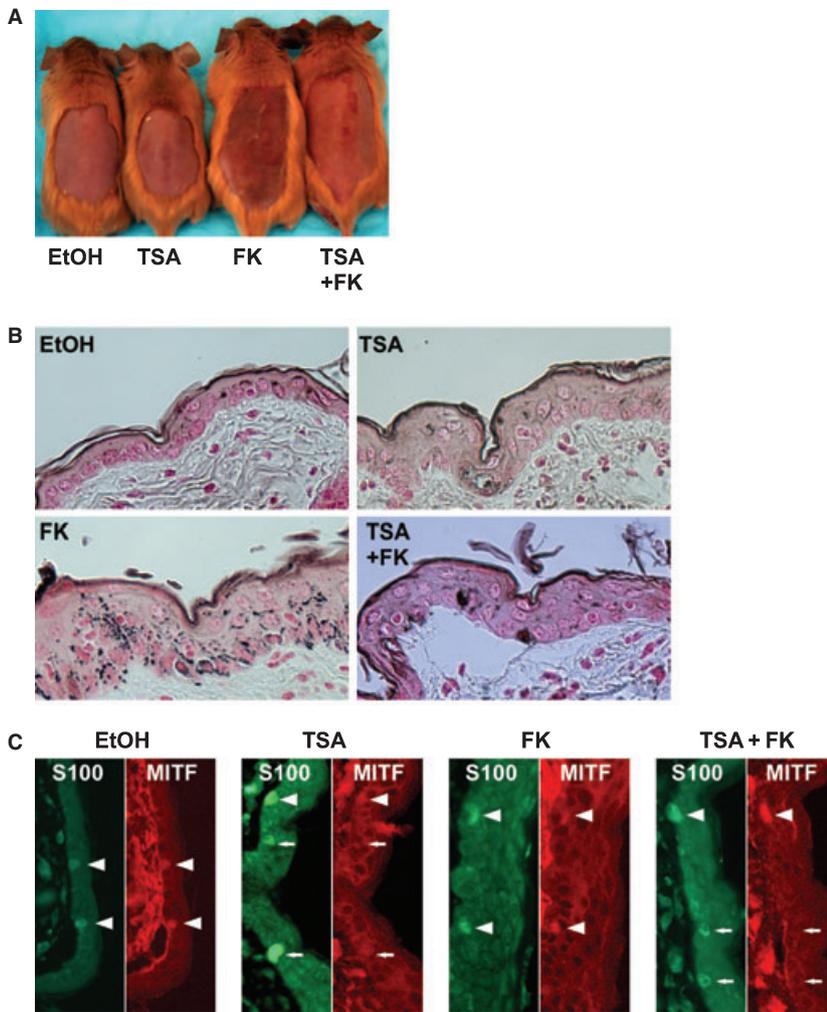
Figure 3. Trichostatin A represses the FK-induced pigmentation in the skin of mice. (A) *K14-SCF* Mc1r$^{e/e}$ mice were treated with vehicle (70% Ethanol/30% polyethylene glycol), crude forskolin, 0.7 mM trichostatin A for 2-weeks. After shaving the hair, photograph was taken. (B) Fontana-Masson staining was performed from paraffin section. Eumelanin was detected by Fontana-Masson staining. (C) MITF and S100 staining were performed from paraffin section. Cy3 (red) or FITC (green) was conjugated to secondary antibody.

observed in the TSA-treated skin (Figure 3B). Direct assessment of MITF expression in skin upon TSA treatment was carried out by co-staining with a monoclonal antibody against MITF as well as staining for S100-antigen (melanocytic marker) as MITF and S100-antigen are co-expressed in epidermal melanocytes (Figure 3C; EtOH and FK). Treatment of mice with topical HDACi drug produced a significant decrease in MITF-staining, whereas S100 appeared unaffected by TSA treatment (Figure 3C; TSA and TSA + FK). As M-MITF appears to be the most abundantly expressed MITF isoform in melanocytes (Figure 1A), decreasing levels of MITF-staining in S100-antigen positive cells likely reflected M-MITF reduction although the MITF antibody could detect other MITF isoforms as well. Importantly using this topical treatment regimen, melanocyte viability did not appear to be affected because the ratio of melanocytes to keratinocytes in the basal layer (number of keratinocytes/number of melanoctyes/field) among each group did not significantly change (EtOH; 5.49 ± 1.03, TSA; 6.47 ± 1.07, FK; 5.81 ± 1.97, FK + TSA; 5.67 ± 0.87). These data demonstrate that HDACi could repress MITF expression as well as pigmentation within skin of topically treated mice under conditions in which melanocyte viability was not compromised.

Melanoma growth suppression in vitro and in vivo

Depletion of MITF induces cell cycle arrest or loss of survival (Carreira et al., 2006; Garraway et al., 2005; Widlund et al., 2002) likely via a variety of mechanisms including induction of p27^{kip1} or alterations of other cell cycle or apoptotic factors in specific settings. In order to examine if HDAC inhibitors (HDACi) influence melanoma cell growth or survival, WST-1 assays were carried out using human melanoma cells treated with HDACi drugs sodium butyrate (NaB) or trichostatin A (TSA) (Figure 4A). Both NaB (2.5 mM) and TSA (1 μM) repressed cell growth at 48 h in UACC62 melanoma cells and UACC257 melanoma cells. The same growth inhibition was observed in other tested melanoma cell lines (data not shown). We also detected apoptosis [propidium iodide (PI) negative/Annexin V positive cells] in UACC62 cells treated with TSA (12.2%) as compared with vehicle (1.7%) by FACS analysis (Figure 4B).

To determine whether HDAC inhibition would attenuate melanoma growth in vivo, we assessed the effects

Figure 4. Histone deacetylase inhibitor drugs repress melanoma growth in vitro and in vivo. (A) UACC62, UACC257, and primary human melanocytes (1°mel) were treated with HDAC inhibitors, sodium butylate (NaB) (shadow bars) and trichostatin A (TSA) (dotted bars) for 48 h. Relative growth rate of NaB- or TSA-treated cells was normalized by PBS- or ethanol (EtOH)-treated cells (filled bars), respectively. Data are mean ± SD of at least three independent experiments. (B) UACC62 human melanoma cells were treated with TSA for 24 h. The cells were stained by Annexin-V and propidium iodide. (C) UACC62 human melanoma cells were injected in nude mice. In cohorts of mice with established tumors (volume 100–200 mm^3), animals were treated with either LBH589 or vehicle via intraperitoneal injection. Data are represented as mean ± SEM, with n = 9 per group. (D) Animals weights were determined every 2 days, and no significant differences were observed between the treatment groups. Data after 12 days of treatment are shown as mean ± SEM. (E) Quantitative reverse transcription PCR analysis of total RNA isolated from injected UACC62 treated with LBH589 or vehicle. Human *M-MITF* mRNA levels were normalized to glyceraldehyde-3-phosphate dehydrogenase and performed in triplicate. Relative M-MITF expression is shown as the ratio to the average obtained from vehicle samples. Data are mean ± SD of each experiment.
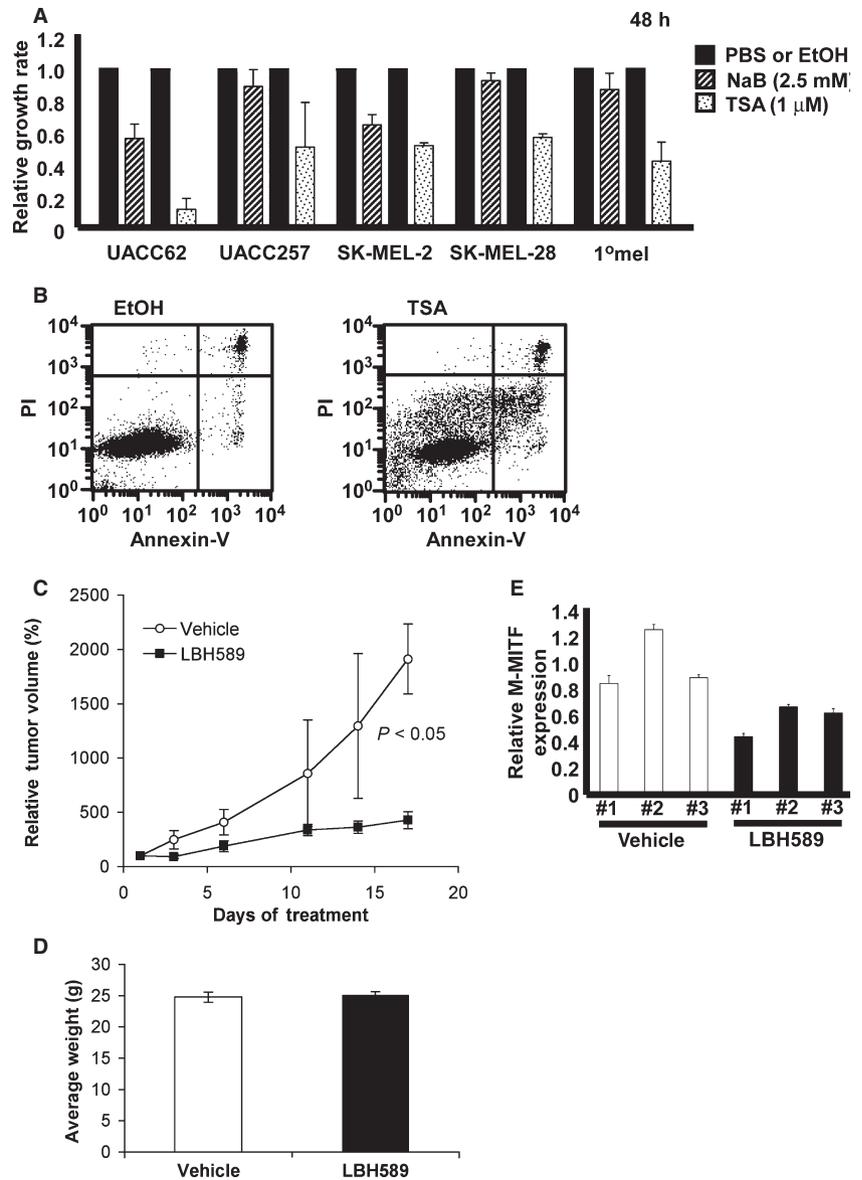
of systemic administration of LBH589 on the growth of UACC62 xenograft tumors. Mice with established tumors were treated with either vehicle or LBH589 at a dose of 10 mg/kg administered daily via intraperitoneal injection. Treatment with LBH589 significantly (P < 0.05) suppressed tumor growth, such that the average tumor volume of LBH589-treated animals was 22% of vehicle-treated animals after 17 days of treatment (Figure 4C). There was no appreciable difference in body weights between the two treatment groups throughout the study (Figure 4D), demonstrating that treatment with LBH589 was well tolerated and not overtly toxic. To determine whether LBH589 treatment altered MITF expression, RNA was isolated from tumors and isoform-specific quantitative RT-PCR was used to determine M-MITF expression. Tumors from animals treated with LBH589 had reduced abundance of

M-MITF mRNA compared with vehicle-treated tumors (Figure 4E, P < 0.05).

In this study, we provide evidence that the HDACi drugs NaB, TSA, SAHA, and LBH589 repress MITF expression in melanoma and clear cell sarcoma cells. Moreover, the repression of MITF with HDACi was also observed in vivo using a humanized mouse skin model in which HDACi topical treatment inhibited forskolin-induced pigmentation without loss of melanocyte viability. In addition, systemic HDACi treatment exhibited significant growth suppression in a human melanoma xenograft model. The mechanism of MITF suppression by HDACi is incompletely understood, but appears likely to involve suppression of one or several required upstream transcriptional regulators of MITF. The mechanism through which HDACs affect their expression awaits further analysis.

Collectively these data identify HDACi drugs as small molecule inhibitors of MITF expression, which may be worthy of clinical study in a variety of settings associated with MITF over-activity, including aberrant pigmentation or melanocytic neoplasia. As MITF small molecule targeting strategies are identified, it is plausible that combinations of such agents (including HDACi) may hold therapeutic promise for systemic treatment of melanoma.

Materials and methods

Cell cultures
UACC62, UACC257, SK-MEL-2, and SK-MEL-28 human melanoma cells were cultured in RPMI-1640 medium (Mediatech Inc., Herndon, VA, USA) containing 10% fetal bovine serum (FBS) and penicillin/streptomycin/L-glutamine. MeWo and SK-MEL-5 human melanoma cells were cultured in DMEM medium (Mediatech Inc.) containing 10% FBS and penicillin/streptomycin/L-glutamine. 501mel human melanoma cells were cultured in Ham's F-10 medium (Mediatech Inc.) containing 10% FBS and penicillin/streptomycin/L-glutamine. Primary human melanocytes between passages 2 and 5 were derived from neonatal foreskins and grown in TIVA media (Ham's F12; Mediatech Inc.), 7% fetal bovine serum, penicillin/streptomycin/glutamine (Invitrogen, Carlsbad, CA, USA), 1×10^{-4} M 3-isobutyl-1-methyl xanthine (IBMX; Sigma, St Louis, MO, USA), 50 ng/ml 12-O-tetradecanoyl phorbol-13-acetate (TPA; Sigma), 1 μM Na_3VO_4, and 1×10^{-3} M N^6,2'-O-dibutyryladenosine 3:5-cyclic monophosphate (dbcAMP; Sigma). Clear cell sarcoma cell lines, DTC-1, SU-CCS-1, and CCS292, were cultured in DMEM medium containing 15% FBS and penicillin/streptomycin/L-glutamine. The stable UACC62 transformed cells containing pLNCX-GFP, pLNCX-SOX10 (full-length SOX10), and pLNCX-SOX10/Dom (truncated SOX10) were selected with G418 (500 μg/ml) (Mediatech Inc.), and referred to as UACC62/GFP (control), UACC62/SOX10, UACC62/SOX10/Dom. These stable transformed lines were grown in RPMI-1640 medium containing 10% FBS, 500 μg/ml G418, and penicillin/streptomycin/L-glutamine. The cells were treated with sodium butyrate (NaB) (2.5 mM) (Sigma-Aldrich, St Louis, MO, USA), Trichostatin A (TSA) (1 μM) (Sigma-Aldrich), suberoylanilide hydroxamic acid (SAHA) (10 μM) (BIOMOL, Plymouth Meeting, PA, USA), or LBH589 (10 μM) (Novartis, New York, NY, USA) for various times as indicated.

Western blotting analysis
Whole cell extracts (10 μg/lane) were prepared using the method of Schreiber et al. (1989) and then subjected to Western blotting analysis using anti-MITF monoclonal antibody (Weilbaecher et al., 1998), anti-acetyled histone 3 (Ace-H3) polyclonal antibody (Upstate Biotechnology, Lake Placid, NY, USA), anti-SOX10 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-LEF1 antibody (REMB6) (Exalpha Biologicals, Maynard, MA, USA), anti-HA monoclonal antibody (Roche, Indianapolis, IN, USA) or anti-α-tubulin monoclonal antibody (Sigma).

Real-time RT-PCR
The total RNAs were prepared by using Trizol (Invitrogen) from UACC62 and 501mel human melanoma cells with TSA for each indicated time. Real-time RT-PCR was performed by using iQ SYBR Green supermix (Bio-Rad, Hercules, CA, USA) from cDNAs. The primers used were 5'-cat tgt tat gct gga aat gct aga a-3' (sense) and 5'-ggc ttg ctg tat gtg gta ctt gg-3' (antisense) for M-MITF, 5'-gct gct gaa cga aag tga ca-3' (sense) and 5'-gcc tgg gct ggt act tgt

ag-3' (antisense) for SOX10, and 5'-tca tcc cga aga gga agg cga ttt-3' (sense) and 5'-tcc tga gag gtt tgt gct tgt ctg-3' (antisense) for LEF1. All reactions were run in triplicate on an iCycler instrument (Bio-Rad), and *MITF-M* message levels were normalized to glyceraldehyde-3-phosphate dehydrogenase expression.

In vivo de-pigmentation assay
C57BL/6JJ *Mc1r*$^{e/e}$ mice were crossed with *K14-Scf* transgenic mice (D'Orazio et al., 2006). Depilated animals were exposed to topical forskolin, TSA, or vehicle control. Skin biopsies were harvested and processed for Fontana–Masson (eumelanin) staining and immunostaining by S100 antibody (DakoCytomation, Carpinteria, CA, USA), or MITF antibody (23).

In vitro growth assays
Cell metabolic activity was assessed by Cell Proliferation Reagent WST-1 (Roche Diagnostics) according to the manufacturer's instruction. Briefly, the UACC62, UACC257, SK-MEL-2, SK-MEL-28, and primary human melanocytes (1×10^3 cells/well) were cultured for 24 h after plating in 96-well dishes and then the HDAC inhibitors, NaB (2.5 mM) or TSA (1 μM), were added to each well. After 48 h, the WST-1 solution was added. The cells were incubated for an additional 1 h with the WST-1 solution. Finally, the absorbance of 460 nm was measured using a microplate reader. Absorbance of cells treated with HDAC inhibitors was normarized to cells treated with the appropriate control, either PBS or ethanol vehicle.

Apoptosis analysis
UACC62 cells were incubated with TSA (1 μM) or EtOH for 24 h. After incubation, the cells were collected and washed by PBS twice. The cells were resuspended in Annexin V Binding buffer (10 mM Hepes, 140 mM NaCl, and 2.5 mM $CaCl_2$, pH7.4). The suspensions were incubated with Annexin V Alexa647 (Invitrogen) conjugates and propidium iodide (PI).

In vivo melanoma growth assay
UACC62 xenografts were generated by subcutaneous injection of 5×10^6 cells into NCr/Nude mice. After 1 week of growth, mice with measurable tumors were segregated into groups that were treated with either LBH589 10 mg/kg i.p. daily or vehicle control (n = 9 per group). Tumors were measured with calipers, and the tumor volume was calculated as $0.5 \times$ length $\times$ width^2. For each animal, relative tumor volume over the treatment course was determined by normalizing to the tumor volume at the start of treatment (day 1 of treatment). Statistical significance between the two treatment arms was determined by two-tailed Student's *t*-test. All animal studies were performed under the auspices of protocols approved by the Dana-Farber Cancer Institutional Animal Care and Use Committee.

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Supplementary material

The following supplementary material is available for this article online from http://www.blackwellsynergy.com/doi/full/10.1111/j.1755-148X.2008.00480.x

Figure S1. M/MITF mRNA decay and rescue of growth suppression by SOX10 overexpression.

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