Published in final edited form as:
Int J Cancer. 2012 July 1; 131(1): 18–29. doi:10.1002/ijc.26320.

Epigenetic regulation by decitabine of melanoma differentiation *in vitro* and *in vivo*

Oscar Alcazar, **Susan Achberger**, **Wayne Aldrich**, **Zhenbo Hu**, **Soledad Negrotto**, **Yogen Saunthararajah**[*], and **Pierre Triozzi**[*]
Taussig Cancer Institute, Cleveland Clinic Foundation, Cleveland, Ohio, USA

Abstract

Apoptosis genes, such as *TP53* and *p16/CDKN2A*, that mediate responses to cytotoxic chemotherapy, are frequently non-functional in melanoma. Differentiation may be an alternative to apoptosis for inducing melanoma cell cycle exit. Epigenetic mechanisms regulate differentiation, and DNA methylation alterations are associated with the abnormal differentiation of melanoma cells. The effects of the deoxycytidine analogue decitabine (5-aza-2'-deoxycytidine), which depletes DNA methyl transferase 1 (DNMT1), on melanoma differentiation were examined. Treatment of human and murine melanoma cells *in vitro* with concentrations of decitabine that did not cause apoptosis inhibited proliferation accompanied by cellular differentiation. A decrease in promoter methylation, and increase in expression of the melanocyte late-differentiation driver SOX9, were followed by increases in cyclin dependent kinase inhibitors (CDKN) p27/CDKN1B and p21/CDKN1A that mediate cell cycle exit with differentiation. Effects were independent of the *TP53*, *p16/CDKN2A*, and also the *BRAF* status of the melanoma cells. Resistance, when observed, was pharmacologic, characterized by diminished ability of decitabine to deplete DNMT1. Treatment of murine melanoma models *in vivo* with intermittent, low-dose decitabine, administered sub-cutaneously to limit high peak drug levels that cause cytotoxicity and increase exposure time for DNMT1 depletion, and with tetrahydrouridine to decrease decitabine metabolism and further increase exposure time, inhibited tumor growth and increased molecular and tumor stromal factors implicated in melanocyte differentiation. Modification of decitabine dose, schedule and formulation for differentiation rather than cytotoxic objectives inhibits the growth of melanoma cells *in vitro* and *in vivo*.

Keywords

decitabine; DNA methyl transferase 1; DNMT1; melanoma; differentiation

INTRODUCTION

Melanoma is a refractory malignancy. Systemic therapy rarely results in durable responses, and new treatment approaches are needed. Epigenetic alterations (aberrant DNA methylation, histone modifications and RNA-based regulation) play an important role in melanomagenesis by down-regulating tumor suppressors, apoptosis mediators, DNA repair enzymes, and immune recognition factors, and there has been considerable interest in addressing epigenetic changes therapeutically[1, 2] (reviewed in [3]). Recent clinical trials in melanoma have focused on the deoxycytidine analogue decitabine (5-aza-2'-deoxycytidine)

Correspondence: Yogen Saunthararajah, MD and Pierre L. Triozzi, MD Taussig Cancer Institute, 9500 Euclid Avenue R40, Cleveland, OH 44195, USA. Tel: 216 444 8170, saunthy@ccf.org or triozzp@ccf.org.
[*]equal contribution

which can deplete DNA methyl-transferase 1 (DNMT1) after incorporation into DNA. The objectives in these clinical trials have been diverse, including direct induction of melanoma cell apoptosis through DNA damage and other anti-metabolite effects,[4] direct epigenetic reactivation of tumor suppressor genes to cause apoptosis,[5] or reactivation of genes that enhance sensitivity to immunotherapy.[6] Choice of decitabine dose and schedule is influenced by the biologic objectives of treatment. Hence, a number of different decitabine regimens have been assessed. However, even in mouse melanoma models, the *in vivo* activity of decitabine has been limited.[7, 8]

Another potential treatment objective is to terminate melanoma cell proliferation by restoring an epigenetically repressed differentiation pathway. An important reason to pursue differentiation therapy for melanoma is that it may not require apoptosis genes such as *TP53* and *p16/CDKN2A*, which are frequently not functional in melanoma, and which mediate responses to cytotoxic chemotherapy.[9–11] Using differentiation to terminate malignant proliferation has been most effective with all-trans retinoic acid treatment of acute promyelocytic leukemia (APL). Retinoids also induce differentiation in melanoma models but have not demonstrated significant clinical activity.[12] Possibly, the benefit of retinoids is restricted to APL because the molecular target, the retinoic acid receptor, is translocated in this disease. Alternative molecular targets for differentiation-therapy could include key components of the epigenetic machinery (for example, DNMT1) that may be aberrantly recruited to repress late-differentiation genes.[13]

Therefore, the present studies evaluated DNMT1 depletion by decitabine as a method to induce melanoma cell cycle exit by differentiation. Differentiation-associated events after treatment of melanoma cells with concentrations of decitabine that do not cause apoptosis were characterized. We focused on key drivers of early (microphthalmia-associated transcription factor, MITF)[14] and late melanocyte differentiation (SOX9[15]), and the cyclin dependent kinase inhibitors (CDKNs) p27/CDKN1B and p21/CDKN1A that are implicated in melanocyte cell cycle exit by differentiation[16, 17]. Experiments included cells that were null for key mediators of cell cycle exit with apoptosis, such as p53 and p16/CDKN2A. For the *in vivo* studies, the objectives were to overcome pharmacologic barriers to effective epigenetic-differentiation therapy with decitabine: decitabine is S-phase specific in its mechanism of action, hence, exposure timings are a major determinant of its activity. However, decitabine is rapidly destroyed *in vivo* by the enzyme cytidine deaminase (CDA), drastically shortening *in vivo* half-life to <20 minutes compared to *in vitro* half-life of 5–16 hours.[18] CDA mediated destruction of cytosine analogues at the cellular level is also a major mechanism by which cancer cells resist the effects of decitabine.[19] Hence, effective *in vivo* translation may require methods to address these aspects of decitabine pharmacology, for example, by manipulating decitabine regimen and metabolism by CDA. The potential role of *in vivo* host-tumor interactions in treatment effects was also examined. Potential regulators of MITF in the tumor stroma, such as Wnt/Frizzle, α-melanocyte stimulating hormone (α-MSH)/melanocortin, endothelin/endothelin receptor, stem cell factor (Scf)/C-Kit, and hepatocyte growth factor (Hgf)/c-Met, were examined as were cytokines implicated in regulating melanoma differentiation, such as IL-1, IL-6, transforming growth factor β (TGF-β), and tumor necrosis factor α (TNF-α). We found that administration of decitabine for differentiation rather than cytotoxic objectives can mediate anti-melanoma activity, and have identified possible pharmacologic and biologic regulators of this treatment approach.

MATERIALS AND METHODS

Cell lines

A panel of human cutaneous melanoma cell lines (Table S1) and the mouse B16 (B16.F10) melanoma cell line were purchased from American Type Culture Collection (Manassas,

VA). Normal human melanocytes (Clonetics Adult Normal Human Epidermal Melanocytes) were purchased from Lonza (Allendale, NJ). Melanoma cells were maintained in Dulbecco's Modified Essential Medium (DMEM) with 10% heat-inactivated fetal calf serum, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin (Mediatech, Herndon, VA). Normal human melanocytes were maintained in Melanocyte Basal Medium MBM-4 supplemented with growth factors ($CaCl_2$, hFGF-B, PMA, rh-insulin, hydrocortisone, BPE, and FBS) from Lonza. The cultures were incubated at 37°C in 5% CO_2.

Animals and reagents

Female C57BL/6 mice and athymic male NCr *nu/nu* mice, four to six weeks of age, were purchased from Taconic Farms (Hudson, NY) and fed with commercial diet and water *ad libitum*. The animal use and care protocol was approved by the Institutional Animal Use and Care Committee. Decitabine was purchased from Tocris (Ellisville, MO). Tetrahydrouridine (THU) was purchased from BioVision Inc (MountainView, CA).

Tumor model

Tumors were established by injecting 2×10^5 melanoma cells in 100 μl of serumless DMEM subcutaneously (s.c.) into a flank. Mice were treated with decitabine at 0.2 mg/kg three times per week (Monday, Tuesday and Friday) with THU at 4 mg/kg twice per week (Monday and Friday) administered 30 minutes before decitabine, either s.c. or intraperitoneally (i.p.). Tumor size was measured bidimensionally with calipers every two to three days, and tumor volume calculated by the formula $(\text{length} \times \text{width}^2) \div 2$. Mice were euthanized when tumors reached the size of 2000 mm^3. Mice were also weighed and blood counts were assayed using a Drew Scientific Hemavet 950FS Hematology Analyzer (Waterbury, CT).

SDS-PAGE and Western blotting

Approximately 50 μg of protein extracts, together with molecular weight markers, were subjected to 1D SDS-PAGE on 4–12% gradient gels (Invitrogen, Carlsbad, CA). After electrophoresis per manufacturer's manual (Invitrogen), proteins were transferred to PVDF membranes (Millipore, Billerica, MA) at 35 constant voltage (35V) for 1 hour using Invitrogen's semidry blotting apparatus. Western analyses of PVDF membranes utilized established protocols and antibodies for p15 (#4822, Cell Signaling, Beverly MA), p21 (#2946, Cell Signaling), p27 (#3686, Cell Signaling), p53 (#2524, Cell Signaling), p16 (#4824, Cell Signaling), Phospho-serine15p-53 (#9286, Cell Signaling), SOX9 (#AB5335, Miilipore), DNMT1 (#5119, Cell Signaling) and anti-β-actin peroxidase (#A3854, Sigma-Aldrich, St. Louis, MO).

Quantitative real-time polymerase chain reaction (QRT-PCR)

RNA from cell lines *in vitro* was obtained using the RNeasy method (Qiagen, Valencia, CA) according to the manufacturer's direction. Dissected tumors grown *in vivo* were placed in RNA Later (Ambion, Austin, TX) and stored at 4°C. RNA was then extracted with RNeasy and stored at −80°C. An ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) was used with pre-standardized primers and TaqMan probes for human and mouse MITF, dopachrome tautomerase (DCT), S100A4, TGF-β, and BCL2, human S100B, and mouse α-MSH/pro-opiomelanocortin (POMC), Wnt3, Wnt5, endothelin, Scf, Hgf, IL-1β, IL-6, Tgf-β, and Tnf-α. Human/mouse glyceraldehyde-3-phosphate dehydrogenase was used as the endogenous control (Applied Biosystems). The reverse transcription and PCR was accomplished using a one-step protocol and TaqMan Universal Master Mix (Applied Biosystems). C_t values were determined, and the relative number of copies of mRNA (RQ) was calculated using the $\Delta\Delta C_t$ method (Relative Quantitation of Gene

Expression, User Bulletin #2, ABI Prism 7700 Sequence Detection System, Applied Biosystems). Expression values were normalized by the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the untreated state as calibrator.

Pyrosequencing assay for SOX9 promoter and LINE-1 methylation

Genomic DNA was isolated using the Wizard Genomic DNA Purification kit (Promega; #A1125), and bisulfite converted using the EZ DNA Methylation kit (Zymo Research; #D5001) per the manufacturer's protocols. CpG methylation status was determined by pyrosequencing on the Qiagen PyroMark Q24 using PyroMark Gold Q24 reagents (Qiagen). Sequence and methylation status analyses used PyroMark Q24 version 1.0.10 software in the CpG (methylation) analysis mode. Human *SOX9* promoter and primer sequences are in figure S3. Annealing temperature: 58°C. Human LINE-1 forward primer: TTTTTTGAGTTAGGTGTGGG; reverse primer: TCTCACTAAAAAATACCAAACAA (amplicon size 203 bp), annealing temperature: 60°C; sequencing primer: GGGTGGGAGTGAT.

DNMT1 measurement by immunofluorescence

Cells on cytospin slides were fixed and permeabilized with 10% formalin and 0.25% triton. Non specific binding sites were blocked with 10% normal goat serum and 6% BSA. Slides were incubated overnight with mouse anti-DNMT1 antibody (ab13537, Abcam, Cambridge, MA) diluted 1:500 in blocking solution, followed by a 655 nm Quantum Dots™-conjugated goat anti-mouse antibody (Q11022MP, Invitrogen) diluted 1:500. Finally, cells were stained with 3 μM DAPI for 5 min before dehydration in graded alcohols and xylene. Image files were loaded into the Image-Pro Plus (Leeds Precision Instruments, Inc. Minneapolis, MN) environment and individual cells were segmented for quantification of fluorescence signal with the "count/size" function, which were integrated into a mean intensity fluorescence (MIF) score.

Measurement of apoptosis and necrosis by Annexin/PI-staining

Melanoma cells were incubated for 24 hours with medium alone, decitabine (0.5 μM) or camptothecin (1 μM). Cells were harvested using the enzyme-free cell dissociation solution Cellstripper (Cellgro). Cell membrane phosphatidylserine exposure was detected by Annexin-V, with co-staining with Propidium Iodide (PI) to detect necrosis, using the FITC Annexin-V Apoptosis Detection kit (BD Pharmingen): cells (5×10^5) per the manufacturer's protocol. Annexin-staining was measured by a Coulter Epics XL-MCL flow cytometer equipped with CXP software (Beckman-Coulter).

Analysis of NCI60 melanoma cell line responses to decitabine

Doubling time and decitabine concentrations that produced 50% inhibition of growth at 48 and 144 hours were downloaded from the National Cancer Institute Developmental Therapeutics Program (NCI-DTP) website (http://dtp.nci.nih.gov/docs/cancer/searches/cancer_open_compounds.html). Melanoma cell lines were LOXIMVI, MALME3M, M14, MDAMB435, SKMEL2, SKMEL28, SKMEL5, UACC257, UACC62.

Statistical analysis

SEM or SD for each set of measurements were calculated and represented as y-axis error bars on each graph. Tumor volume data were analyzed using ANOVA. Differences in other parameters between specific groups of replicates were analyzed by two-sided Student's *t* test. Changes were considered significant if $P < 0.05$.

RESULTS

Decitabine inhibited proliferation and induced differentiation of melanoma cells

A panel of human melanoma cell lines containing various mutations (Table S1), the mouse B16 melanoma cell line, and normal human melanocytes were treated *in vitro* with the same concentration and intermittent schedule of decitabine that maintained or increased self-renewal of normal hematopoietic stem cells.[20–22] This treatment diminished proliferation in all melanoma cell lines, but not normal human melanocytes (Fig. 1A). In some, *e.g.*, SKMEL3 and SKMEL28, the antiproliferative effects were not apparent until day 8. Growth inhibition that is greater at later (144 hours) compared to earlier time-points (48 hours) was also noted in decitabine treated melanoma cell lines from the NCI60 panel (Fig. S1A). Melanoma cell lines with shorter doubling time were more sensitive to growth inhibition by decitabine, consistent with the known S-phase specific mechanism of action (Fig. S1B, C). Morphological changes consistent with differentiation were observed in 5/6 melanoma cell lines: these changes were major increases in cell size accompanied by decreases in the nuclear-cytoplasmic ratio (evident on Giemsa-stained cytospin preparations), and increased dendritic morphology and increases in melanin (apparent in phase-contrast microscope images) (Fig. 1B). In contrast, the morphology of normal human melanocytes was minimally altered (Fig. 1B).

Decitabine increased late differentiation driver and CDKN protein levels

Upregulation of total p53, p53 serine-15 phosphorylation, and upregulation of p16/CDKN2A, are key events in cell cycle exit by apoptosis. To evaluate the role of apoptosis-mediated cell cycle exit in the anti-proliferative effects of decitabine, flow cytometry was used to evaluate for cell membrane phosphatidylserine exposure (an early event in apoptosis), and Western blot was used to evaluate for changes in the expression and post-translational modification of p53 and p16/CDKN2A. Decitabine treatment did not induce early apoptosis in human and murine melanoma cells (A375 and B16) measured by cell membrane phosphatidylserine exposure and PI-staining (Fig. 2A). Decitabine treatment did not increase total p53 in either cell line (Fig. 2B, C). p53 phosphorylation increased in B16 cells (Fig. 2C) but not in A375 cells (Fig. 2B). Neither cell line expressed the apoptosis-regulating protein p16/CDKN2A (Fig. 2B, C).

To identify melanocyte differentiation-drivers that might be aberrantly repressed in melanoma, and that might be reactivated by decitabine, gene expression in normal skin, primary benign nevi and primary melanoma cells was compared using a public database of gene expression (Geo DataSets GDS1375).[23] Striking decreases in expression of the late-differentiation driver *SOX9* and the late-differentiation marker *DCT* were evident in the melanoma cells compared to benign nevi (Fig. S2) (aberrant SOX9 repression has been previously implicated in melanoma pathogenesis).[15, 24] Therefore, the effects of the above concentrations of decitabine on SOX9 protein expression were evaluated. Decitabine treatment increased SOX9 protein expression in both the human and murine melanoma cells by 36 hours after treatment (Fig. 2B, C). Melanocyte cell cycle exit by differentiation is associated with upregulation of p27/CDKN1B and p21/CDKN1A protein.[16, 17] p15/CDKN2B is another protein with a possible role in cell cycle exit with differentiation.[25] Decitabine treatment increased p27/CDKN1B and p21/CDKN1A protein levels, with the increases most prominent at late time points (96–120 hours after treatment) (Fig. 2B, C). In B16 cells, decitabine also produced a late increase in p15/CDKN2B (Fig. 2C).

The absence of recurrent genetic deletions at the *SOX9* locus (located on chromosome 17) in cutaneous melanoma, and decitabine-induced upregulation of SOX9, suggest that its aberrant repression is by epigenetic means. Therefore, *SOX9* promoter methylation, and the

effects of decitabine on this methylation, were examined by pyrosequencing analysis of human melanoma cells (MeWo cells, selected for double p16/CDKN2A and p53-null status, further reducing the possibility that decitabine effects were related to apoptosis), and normal human melanocytes. Conserved CpG in the proximal *SOX9* promoter (Fig. S3) were 6.5-fold more methylated in MeWo cells than in normal human melanocytes (39 versus 6%) (Fig. 3A, B). Decitabine treatment of the melanoma cells decreased *SOX9* promoter CpG methylation by up to 63% (average methylation decrease in 7 CpG was 43%) (Fig. 3B). In contrast to *SOX9* promoter CpG, methylation of LINE-1 repetitive element CpG (as an index of global DNA methylation) was decreased in MeWo compared to normal human melanocytes (17 versus 37%)(Fig. 3A, B). Although decitabine treatment of MeWo also decreased LINE-1 CpG methylation (by up to 38%, with an average methylation decrease 32% in 4 LINE-1 CpG), this decrease was substantially smaller than the hypomethylation produced at *SOX9* promoter CpG (Fig. 3B).

Decitabine upregulation of other melanocyte late-differentiation genes

The expression of a key driver of melanocyte lineage-commitment and early differentiation, MITF, was similar between benign nevi and primary melanoma (Fig. S2).[26] However, in the melanoma cells, the expression of the late-differentiation marker DCT (a key enzyme in melanin synthesis) was significantly decreased (Fig. S2). Hence, the DCT/MITF expression ratio, used as an index of progressive differentiation, was inverted in primary melanoma cells (Fig. S2). Decitabine treatment produced a >0.5 log (> 5-fold) increase in DCT expression in 3/5 melanoma cell lines (Fig. 4A). In 3/3 melanoma cell lines in which the DCT/MITF expression ratio was inverted to begin with, it was reverted (Fig. 4B).

Decitabine treatment of melanoma cell lines has been reported to increase expression of CDKN1A, S100A4 (implicated in neuroectodermal, mesenchymal and epithelial cell differentiation[27]) and TGFB1.[28] Consistent with these previous observations, decitabine treatment produced >0.5 log increases in expression of S100A4 and TGFB1 in 5/5 melanoma cell lines and CDKN1A in 4/5 melanoma cell-lines (Fig. 4C). In some of the cell lines, CDKN1A and S100A4 expression increased beyond day 4 to day 8; in some cell lines TGFB1 increases were more evident on day 4 than 8 (Fig. 4C). In contrast to the above genes, BCL2 and S100B have been reported to not undergo major changes in expression in response to decitabine treatment.[28] Similar to the previous report, the increases in expression of BCL2 and S100B were generally smaller than for the other genes examined (Fig. 4D).

Resistance to decitabine *in vitro* is pharmacologic

Resistance to decitabine could occur because decitabine fails to deplete DNMT1 (‘pharmacologic’ resistance) or because melanoma cells tolerate DNMT1 depletion (‘biologic’ resistance). The human and mouse melanoma cell cultures depicted in Figure 1 were maintained for two weeks (with media changes) to confirm that there was no re-growth of cells. Only mouse B16 cells demonstrated re-growth. These emerging B16 melanoma cells had a substantially lower rate of growth (~20%) than wild-type B16 cells. Resistant B16 cells were expanded in culture with decitabine for 90 days then were cultured without decitabine for 21 days. Melanocyte differentiation gene expression was examined in wild-type and decitabine-resistant B16 cells. Decitabine-resistant cells expressed higher levels of differentiation markers than wild-type B16 at baseline (Fig. 5A), however, re-addition of decitabine did not produce a further increase in differentiation-marker in contrast to the large upregulation seen in decitabine naïve B16. In decitabine-resistant cells, DNMT1 levels were slightly decreased at baseline compared to naive B16, however, re-addition of decitabine did not produce a further decrease in DNMT1 in the resistant cells, as shown by immunostaining (Fig. 5B) and its quantitation (Fig. 5C).

THU-decitabine inhibits tumor growth *in vivo*

Little antitumor activity has been reported with single-agent decitabine in mouse B16 melanoma *in vivo* at 0.2 mg/kg i.p. for 7 consecutive days,[7] or in A375 xenograft melanoma models at 5 mg/kg i.p. every 3 hours for a total of three injections.[8] Since DNMT1 depletion can be achieved with low concentrations of decitabine, and DNMT1-depletion by decitabine is S-phase specific, we examined alternative schedules, that emphasize exposure timings (to increase treatment windows and thereby expose melanoma cells entering S-phase at different points in time), rather than peak drug levels. For this goal, CDA, by drastically abbreviating the *in vivo* half-life of decitabine,[18] could be a major factor impeding translation of *in vitro* activity into *in vivo* activity. To address this pharmacologic barrier to *in vivo* therapy, the CDA inhibitor THU[29] was added to the treatment regimen. Mice bearing B16 syngeneic melanoma were treated with a low dose of decitabine (0.2 mg/kg) administered by the s.c. route to avoid high peak levels that cause cytotoxicity, and three times per week combined with THU (administered 30–60 minutes prior to decitabine) to increase exposure time. For comparison, a group of mice was treated with THU-decitabine i.p. (i.p. administration was expected to produce higher peak drug levels but shorter exposure time). Significant antitumor activity was produced without evidence of toxicity (Fig. 6A): there was no difference in body weights of the mice, or in white blood cell, platelet or hemoglobin levels (data not shown). The s.c. route of administration was more effective than i.p. Similar antitumor activity was observed when this regimen was used to treat mice bearing A375 xenografts (Fig. 6B).

THU-decitabine promotes expression of late-differentiation factors *in vivo*

The expression of genes modified by THU-decitabine *in vitro* was examined *in vivo*. C57Bl/6 wild type mice bearing B16 and NCr nude mice bearing A375 tumors were treated with THU followed by decitabine as described above. The results *in vivo* paralleled those *in vitro*: increases in *DCT* relative to *MITF* were observed, as were increases in *CDKN1A* and *S100A4* (Fig. 6C). That regulatory factors within the tumor stroma could be altered by the THU-decitabine regimen was examined by assessing intratumoral levels of the melanocortin ligand POMC/alpha MSH, the endothelin receptor ligand endothelin-1, the wnt/frizzle ligands wnt3a and wnt5a, the c-Kit ligand Scf, and the c-Met ligand Hgf. Significant increases in POMC and Wnt5a were observed in the syngeneic B16 model and in A375 xenografts (in which the host response is mouse and the tumor is human). Intratumoral levels of cytokines implicated in the inhibition of melanocyte differentiation (TGF-β, IL-6, IL-1β and TNF-α) were also assessed. Significant decreases in IL-1β and TNF-α were observed (Fig. 6C).

DISCUSSION

A variety of melanoma cells, decitabine concentrations and treatment durations have been evaluated *in vitro*, with a focus on inducing apoptosis. [28, 30–34] In melanoma cells, however, molecular pathways that regulate apoptosis (p16/CDKN2A-p53 and p16/CDKN2A-RB) are attenuated by genetic mutation and deletion, and therefore, are not ideal targets for direct epigenetic reactivation. The present work focused on differentiation, since normal melanocyte cell cycle exit by differentiation, and decitabine-induced cell cycle exit by differentiation, does not require p53 and p16 [9–11]. Hence, concentrations of decitabine sufficient to deplete DNMT1 in melanoma cells, but not high enough to induce annexin-staining or upregulation and phosphorylation of p53, were evaluated. These decitabine concentrations induced cell cycle exit even in p16/CDKN2A and p53-null melanoma cells, with morphologic changes of differentiation, upregulation of the key melanocyte late-differentiation driver SOX9, restoration of the expected DCT/MITF ratio, and upregulation

of canonical CDKN (p27/CDKN1B and p21/CDKN1A) that mediate melanocyte cell cycle exit by differentiation.

In previous clinical trials in which inducing apoptosis or activating immune-recognition genes were the objectives of therapy, decitabine was administered for a few days, but resulting toxicity or cytopenia required subsequent suspension of therapy for many weeks.[4–6] Since decitabine is S-phase specific, concentrated exposure only treats the fraction of the tumor population that is in S-phase in the treatment window. In the present study, concentrations of decitabine that do not induce apoptosis, but nonetheless deplete DNMT1/hypomethylate DNA, were used.[22, 28, 35] These concentrations can be non-toxic to normal hematopoietic stem cells, enabling relatively frequent exposure without myelosuppression.[20–22] Accordingly, *in vivo*, it is feasible to administer drug intermittently but frequently, to increase and distribute windows of exposure that treat malignant cells entering S-phase at different points in time, without prolonging cytostatic effects that cause myelosuppression.[22] This type of approach, referred to as “metronomic” or “extended-schedule” chemotherapy, has been more effective in melanoma models than maximum tolerated dose therapy even when applied to conventional cytotoxic drugs.[36] To maintain treatment exposure that depletes DNMT1 while avoiding high decitabine levels that cause DNA damage and apoptosis, decitabine was administered s.c. instead of i.p..[22] The minimal toxicity of this approach could facilitate adjuvant, combination and immunotherapy applications. In theory, resistance should occur less frequently than with conventional cytotoxic therapy, since there should be less selective pressure for the most apoptosis-resistant sub-clones, and DNA damage which increases clonal variation is avoided or minimized.[37]

In leukemia cell lines, decitabine resistance has been pharmacologic, that is, a failure of decitabine to deplete DNMT1, as opposed to biologic, continued self renewal despite successful intra-nuclear DNMT1 depletion.[19] Similarly, we found resistance in B16 melanoma cells to be pharmacologic. Although decitabine has an *in-vitro* half-life of 5–16 hours, *in vivo*, the half-life is <20 minutes, because of rapid destruction by CDA.[18] Additionally, malignant cells themselves can upregulate CDA to destroy cytosine analogues[19], or find sanctuary in organs such as the liver, that express high levels of CDA. Since decitabine is S-phase specific, the very large decrement in half-life *in vivo* compared to *in vitro* may account for much of the difficulty in clinical translation. THU is a potent competitive inhibitor of CDA that has been applied clinically.[22, 38] We have found that administering decitabine s.c. instead of i.p. (to avoid high peak drug levels that damage DNA), and in combination with THU to improve time-above-threshold concentration for depleting DNMT1, enhanced anti-melanoma activity.

The expression of a key factor associated with melanocyte commitment, MITF, is very similar in primary melanoma cells compared to benign nevi, however, the late differentiation driver SOX9 was significantly repressed. This profile suggests lineage-commitment but impaired progressive maturation.[15, 24] SOX9 is a master regulator of neural crest differentiation that is upregulated as melanoblasts transition to a more melanocytic state[39], and cooperates with MITF to upregulate late differentiation genes such as DCT.[15] Expression of SOX9 in melanoma cells using vectors or prostaglandin D2 terminated proliferation[15] associated with activation of p21/CDKN1A directly and in cooperation with MITF.[15] Highly conserved CpG in the *SOX9* promoter were hypermethylated in MeWo melanoma cells compared to normal human melanocytes. Non-cytotoxic concentrations of decitabine substantially decreased this hypermethylation and upregulated SOX9 expression. Decitabine-induced upregulation of SOX9 was followed by upregulation of p27/CDKN1B and p21/CDKN1A, canonical CDKN that mediate cell cycle exit with differentiation.[40] Of note, in melanoma, low tumor levels of p27/CDKN1B and p21/CDKN1A are associated

with a poor prognosis.[41] Decitabine also increased expression of *S100A4*, a member of the S100 family of proteins that is implicated in neuroectodermal, mesenchymal and epithelial cell differentiation.[27] S100A4 deficient mice develop spontaneous tumors at a higher rate than wild-type controls.[42] In contrast, decitabine did not induce significant changes in S100B expression. S100B knock-out mice do not have a neoplastic phenotype.[43]

Why is *SOX9* epigenetically repressed? One possibility is that mutation or translocation of a key melanocyte early-differentiation driving gene affects its function such that corepressors instead of coactivators are recruited at late-differentiation gene targets such as *SOX9*. Since late-differentiation genes are required for progressive maturation and cell cycle exit, a proliferative advantage ensues. In this model, decitabine treatment redresses corepressor/coactivator imbalance by depleting DNMT1, and since the melanoma cells are otherwise primed to express late-differentiation genes by high expression of commitment factors (eg., MITF), late-differentiation gene expression is restored. This model of melanoma pathology and decitabine action, though consistent with the present data, remains unproven and requires further investigation.

Surrounding and/or recruited fibroblasts, endothelial, and immune cells in the tumor stroma support melanoma development through the production of several regulators of MITF, including α-MSH/POMC, HGH, SCF, and endothelin.[44] Wnt ligands are also expressed in the tumor microenvironment, including by macrophages.[45] We did observe *in vivo* decreases in Wnt5a. Wnt-Frizzled signaling displaces Groucho co-repressors from the DCT promoter and enables DCT activation by MITF. In a study of benign and malignant melanocytic tumors, Wnt5a and Frizzled, which were highly co-expressed, were significantly reduced from benign nevi to melanomas, results also consistent with a tumor suppressor role.[46] Immune cytokines in the tumor stroma have also been implicated in the inhibition of melanoma differentiation. A variety of effects on immune cytokines have been reported with epigenetic modifiers.[22, 47, 48] We observed *in vivo* decreases in IL-1β, which has been shown to inhibit MITF,[49] and TNF-α, which has been shown to inhibit tyrosinase and TRP1.[50]

Most treatment for melanoma has been oriented towards inducing apoptosis, and treatment failure may be in part because of frequent mutation or deletion of master apoptosis genes such as *p16/CDKN2A* and *TP53*. Non-cytotoxic concentrations of decitabine that deplete DNMT1 can induce melanoma cell cycle exit by p53 and p16/CDKN2A-independent differentiation. However, DNMT1 depletion by decitabine is S-phase specific, and the *in vivo* half-life of decitabine is very brief, because of CDA-mediated metabolism. Hence, non-cytotoxic doses administered frequently, possibly in combination with THU to inhibit CDA, may be more efficacious than higher doses administered less frequently. This alternative to conventional apoptosis-oriented therapy warrants further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

OA is funded by Scott Hamilton CARES foundation. YS is funded by National Institutes of Health (U54HL090513, 1R01CA138858), and Department of Defense (PR081404). PT is funded by NIH (5R01CA136776 and 5R01CA118660).

Abbreviations used

CAM	camptothecin
CDA	cytidine deaminase
DAC	decitabine
DCT	dopachrome tautomerase
DMEM	Dulbecco's Modified Essential Medium
DNMT	DNA methyl transferase
HGF	hepatocyte growth factor
i.p.	intraperitoneal
MIF	mean intensity fluorescence
MITF	microphthalmia-associated transcription factor
MSH	melanocyte stimulating hormone
NHM	normal human melanocyte
POMC	pro-opiomelanocortin
qRT-PCR	quantitative real-time polymerase chain reaction
RQ	relative number of copies of mRNA
s.c.	subcutaneous
SCF	stem cell factor
TGF	transforming growth factor
THU	tetrahydrouridine
TNF	tumor necrosis factor

References

1. Bellet RE, Catalano RB, Mastrangelo MJ, Berd D. Phase II study of subcutaneously administered 5-azacytidine (NSC-102816) in patients with metastatic malignant melanoma. Med Pediatr Oncol. 1978; 4:11–15. [PubMed: 75498]
2. Creagan ET, Schaid DJ, Hartmann LC, Loprinzi CL. A phase II study of 5,6-dihydro-5-azacytidine hydrochloride in disseminated malignant melanoma. Am J Clin Oncol. 1993; 16:243–244. [PubMed: 7687819]
3. Dahl C, Guldberg P. The genome and epigenome of malignant melanoma. APMIS. 2007; 115:1161–1176. [PubMed: 18042149]
4. Abele R, Clavel M, Dodion P, Bruntsch U, Gundersen S, Smyth J, Renard J, van Glabbeke M, Pinedo HM. The EORTC Early Clinical Trials Cooperative Group experience with 5-aza-2'-deoxycytidine (NSC 127716) in patients with colo-rectal, head and neck, renal carcinomas and malignant melanomas. Eur.J.Cancer Clin.Oncol. 1987; 23:1921–1924. [PubMed: 2449354]
5. Aparicio A, Eads CA, Leong LA, Laird PW, Newman EM, Synold TW, Baker SD, Zhao M, Weber JS. Phase I trial of continuous infusion 5-aza-2'-deoxycytidine. Cancer Chemother.Pharmacol. 2003; 51:231–239. [PubMed: 12655442]
6. Gollob JA, Sciambi CJ, Peterson BL, Richmond T, Thoreson M, Moran K, Dressman HK, Jelinek J, Issa JP. Phase I trial of sequential low-dose 5-aza-2'-deoxycytidine plus high-dose intravenous bolus interleukin-2 in patients with melanoma or renal cell carcinoma. Clin.Cancer Res. 2006; 12:4619–4627. [PubMed: 16899610]

7. Kozar K, Kaminski R, Switaj T, Oldak T, Machaj E, Wysocki PJ, Mackiewicz A, Lasek W, Jakobisiak M, Golab J. Interleukin 12-based immunotherapy improves the antitumor effectiveness of a low-dose 5-Aza-2'-deoxycitidine treatment in L1210 leukemia and B16F10 melanoma models in mice. Clin.Cancer Res. 2003; 9:3124–3133. [PubMed: 12912964]
8. Reu FJ, Bae SI, Cherkassky L, Leaman DW, Lindner D, Beaulieu N, MacLeod AR, Borden EC. Overcoming resistance to interferon-induced apoptosis of renal carcinoma and melanoma cells by DNA demethylation. J.Clin.Oncol. 2006; 24:3771–3779. [PubMed: 16801630]
9. Inomata K, Aoto T, Binh NT, Okamoto N, Tanimura S, Wakayama T, Iseki S, Hara E, Masunaga T, Shimizu H, Nishimura EK. Genotoxic stress abrogates renewal of melanocyte stem cells by triggering their differentiation. Cell. 2009; 137:1088–1099. [PubMed: 19524511]
10. Negrotto S, Hu Z, Alcazar O, Ng KP, Triozzi P, Lindner D, Rini B, Saunthararajah Y. Noncytotoxic differentiation treatment of renal cell cancer. Cancer Res. 2011; 71:1431–1441. [PubMed: 21303982]
11. Ng KP, Ebrahem Q, Negrotto S, Mahfouz RZ, Link KA, Hu Z, Gu X, Advani A, Kalaycio M, Sobecks R, Sekeres MA, Copelan E, et al. p53 independent epigenetic-differentiation treatment in xenotransplant models of acute myeloid leukemia. Leukemia. (In Press).
12. Triozzi PL, Walker MJ, Pellegrini AE, Dayton MA. Isotretinoin and recombinant interferon alfa-2a therapy of metastatic malignant melanoma. Cancer Invest. 1996; 14:293–298. [PubMed: 8689422]
13. Steigerwald SD, Pfeifer GP. Variable DNA methylation changes during differentiation of human melanoma cells. Exp.Cell Res. 1988; 178:41–50. [PubMed: 2457505]
14. Palmieri G, Capone M, Ascierto ML, Gentilcore G, Stroncek DF, Casula M, Sini MC, Palla M, Mozzillo N, Ascierto PA. Main roads to melanoma. J.Transl.Med. 2009; 7:86. [PubMed: 19828018]
15. Passeron T, Valencia JC, Namiki T, Vieira WD, Passeron H, Miyamura Y, Hearing VJ. Upregulation of SOX9 inhibits the growth of human and mouse melanomas and restores their sensitivity to retinoic acid. J.Clin.Invest. 2009; 119:954–963. [PubMed: 19273910]
16. Sestakova B, Ondrusova L, Vachtenheim J. Cell cycle inhibitor p21/ WAF1/ CIP1 as a cofactor of MITF expression in melanoma cells. Pigment Cell Melanoma Res. 23:238–251. [PubMed: 20067556]
17. Haddad MM, Xu W, Schwahn DJ, Liao F, Medrano EE. Activation of a cAMP pathway and induction of melanogenesis correlate with association of p16(INK4) and p27(KIP1) to CDKs, loss of E2F-binding activity, and premature senescence of human melanocytes. Exp.Cell Res. 1999; 253:561–572. [PubMed: 10585280]
18. Liu Z, Marcucci G, Byrd JC, Grever M, Xiao J, Chan KK. Characterization of decomposition products and preclinical and low dose clinical pharmacokinetics of decitabine (5-aza-2'-deoxycytidine) by a new liquid chromatography/tandem mass spectrometry quantification method. Rapid Commun.Mass Spectrom. 2006; 20:1117–1126. [PubMed: 16523529]
19. Qin T, Jelinek J, Si J, Shu J, Issa JP. Mechanisms of resistance to 5-aza-2'-deoxycytidine in human cancer cell lines. Blood. 2009; 113:659–667. [PubMed: 18931345]
20. Milhem M, Mahmud N, Lavelle D, Araki H, DeSimone J, Saunthararajah Y, Hoffman R. Modification of hematopoietic stem cell fate by 5aza 2'deoxycytidine and trichostatin A. Blood. 2004; 103:4102–4110. [PubMed: 14976039]
21. Hu Z, Negrotto S, Gu X, Mahfouz R, Ng KP, Ebrahem Q, Copelan E, Singh H, Maciejewski JP, Saunthararajah Y. Decitabine maintains hematopoietic precursor self-renewal by preventing repression of stem cell genes by a differentiation-inducing stimulus. Mol.Cancer Ther. 9:1536–1543. [PubMed: 20501800]
22. Saunthararajah Y, Hillery CA, Lavelle D, Molokie R, Dorn L, Bressler L, Gavazova S, Chen YH, Hoffman R, DeSimone J. Effects of 5-aza-2'-deoxycytidine on fetal hemoglobin levels, red cell adhesion, and hematopoietic differentiation in patients with sickle cell disease. Blood. 2003; 102:3865–3870. [PubMed: 12907443]
23. Talantov D, Mazumder A, Yu JX, Briggs T, Jiang Y, Backus J, Atkins D, Wang Y. Novel genes associated with malignant melanoma but not benign melanocytic lesions. Clin.Cancer Res. 2005; 11:7234–7242. [PubMed: 16243793]

24. Rao P, Fuller GN, Prieto VG. Expression of Sox-9 in metastatic melanoma--a potential diagnostic pitfall. Am.J.Dermatopathol. 32:262–266. [PubMed: 20098296]
25. Latres E, Malumbres M, Sotillo R, Martin J, Ortega S, Martin-Caballero J, Flores JM, Cordon-Cardo C, Barbacid M. Limited overlapping roles of P15(INK4b) and P18(INK4c) cell cycle inhibitors in proliferation and tumorigenesis. EMBO J. 2000; 19:3496–3506. [PubMed: 10880462]
26. Cooper CD, Raible DW. Mechanisms for reaching the differentiated state: Insights from neural crest-derived melanocytes. Semin.Cell Dev.Biol. 2009; 20:105–110. [PubMed: 18935965]
27. Sherbet GV. Metastasis promoter S100A4 is a potentially valuable molecular target for cancer therapy. Cancer Lett. 2009; 280:15–30. [PubMed: 19059703]
28. Halaban R, Krauthammer M, Pelizzola M, Cheng E, Kovacs D, Sznol M, Ariyan S, Narayan D, Bacchiocchi A, Molinaro A, Kluger Y, Deng M, et al. Integrative analysis of epigenetic modulation in melanoma cell response to decitabine: clinical implications. PLoS.ONE. 2009; 4:e4563. [PubMed: 19234609]
29. Neil GL, Moxley TE, Kuentzel SL, Manak RC, Hanka LJ. Enhancement by tetrahydrouridine (NSC-112907) of the oral activity of 5-azacytidine (NSC-102816) in L1210 leukemic mice. Cancer Chemother.Rep. 1975; 59:459–465. [PubMed: 54211]
30. Gollob JA, Sciambi CJ. Decitabine up-regulates S100A2 expression and synergizes with IFN-gamma to kill uveal melanoma cells. Clin.Cancer Res. 2007; 13:5219–5225. [PubMed: 17785578]
31. Bae SI, Cheriyath V, Jacobs BS, Reu FJ, Borden EC. Reversal of methylation silencing of Apo2L/TRAIL receptor 1 (DR4) expression overcomes resistance of SK-MEL-3 and SK-MEL-28 melanoma cells to interferons (IFNs) or Apo2L/TRAIL. Oncogene. 2008; 27:490–498. [PubMed: 17653094]
32. Gallagher WM, Bergin OE, Rafferty M, Kelly ZD, Nolan IM, Fox EJ, Culhane AC, McArdle L, Fraga MF, Hughes L, Currid CA, O'Mahony F, et al. Multiple markers for melanoma progression regulated by DNA methylation: insights from transcriptomic studies. Carcinogenesis. 2005; 26:1856–1867. [PubMed: 15958521]
33. van der Velden PA, Zuidervaart W, Hurks MH, Pavey S, Ksander BR, Krijgsman E, Frants RR, Tensen CP, Willemze R, Jager MJ, Gruis NA. Expression profiling reveals that methylation of TIMP3 is involved in uveal melanoma development. Int.J.Cancer. 2003; 106:472–479. [PubMed: 12845640]
34. Muthusamy V, Duraisamy S, Bradbury CM, Hobbs C, Curley DP, Nelson B, Bosenberg M. Epigenetic silencing of novel tumor suppressors in malignant melanoma. Cancer Res. 2006; 66:11187–11193. [PubMed: 17145863]
35. Jones PA, Taylor SM. Cellular differentiation, cytidine analogs and DNA methylation. Cell. 1980; 20:85–93. [PubMed: 6156004]
36. Cruz-Munoz W, Man S, Kerbel RS. Effective treatment of advanced human melanoma metastasis in immunodeficient mice using combination metronomic chemotherapy regimens. Clin.Cancer Res. 2009; 15:4867–4874. [PubMed: 19622578]
37. Kitano H. Cancer robustness: tumour tactics. Nature. 2003; 426:125. [PubMed: 14614483]
38. Marsh JH, Kreis W, Barile B, Akerman S, Schulman P, Allen SL, DeMarco LC, Schuster MW, Budman DR. Therapy of refractory/relapsed acute myeloid leukemia and blast crisis of chronic myeloid leukemia with the combination of cytosine arabinoside, tetrahydrouridine, and carboplatin. Cancer Chemother.Pharmacol. 1993; 31:481–484. [PubMed: 8453688]
39. Cook AL, Smith AG, Smit DJ, Leonard JH, Sturm RA. Co-expression of SOX9 and SOX10 during melanocytic differentiation in vitro. Exp.Cell Res. 2005; 308:222–235. [PubMed: 15896776]
40. Chellappan SP, Giordano A, Fisher PB. Role of cyclin-dependent kinases and their inhibitors in cellular differentiation and development. Curr.Top.Microbiol.Immunol. 1998; 227:57–103. [PubMed: 9479826]
41. Florenes VA, Maelandsmo GM, Kerbel RS, Slingerland JM, Nesland JM, Holm R. Protein expression of the cell-cycle inhibitor p27Kip1 in malignant melanoma: inverse correlation with disease-free survival. Am.J.Pathol. 1998; 153:305–312. [PubMed: 9665492]
42. El Naaman C, Grum-Schwensen B, Mansouri A, Grigorian M, Santoni-Rugiu E, Hansen T, Kriajevska M, Schafer BW, Heizmann CW, Lukanidin E, Ambartsumian N. Cancer predisposition

in mice deficient for the metastasis-associated Mts1(S100A4) gene. Oncogene. 2004; 23:3670–3680. [PubMed: 15116098]

43. Nishiyama H, Knopfel T, Endo S, Itohara S. Glial protein S100B modulates long-term neuronal synaptic plasticity. Proc.Natl.Acad.Sci.U.S.A. 2002; 99:4037–4042. [PubMed: 11891290]
44. Hsu MY, Meier F, Herlyn M. Melanoma development and progression: a conspiracy between tumor and host. Differentiation. 2002; 70:522–536. [PubMed: 12492494]
45. Nitzki F, Zibat A, Konig S, Wijgerde M, Rosenberger A, Brembeck FH, Carstens PO, Frommhold A, Uhmann A, Klingler S, Reifenberger J, Pukrop T, et al. Tumor stroma-derived Wnt5a induces differentiation of basal cell carcinoma of Ptch-mutant mice via CaMKII. Cancer Res. 70:2739–2748. [PubMed: 20233865]
46. Bachmann IM, Straume O, Puntervoll HE, Kalvenes MB, Akslen LA. Importance of P-cadherin, beta-catenin, and Wnt5a/frizzled for progression of melanocytic tumors and prognosis in cutaneous melanoma. Clin.Cancer Res. 2005; 11:8606–8614. [PubMed: 16361544]
47. Leoni F, Zaliani A, Bertolini G, Porro G, Pagani P, Pozzi P, Dona G, Fossati G, Sozzani S, Azam T, Bufler P, Fantuzzi G, et al. The antitumor histone deacetylase inhibitor suberoylanilide hydroxamic acid exhibits antiinflammatory properties via suppression of cytokines. Proc.Natl.Acad.Sci.U.S.A. 2002; 99:2995–3000. [PubMed: 11867742]
48. Mishra N, Brown DR, Olorenshaw IM, Kammer GM. Trichostatin A reverses skewed expression of CD154, interleukin-10, and interferon-gamma gene and protein expression in lupus T cells. Proc.Natl.Acad.Sci.U.S.A. 2001; 98:2628–2633. [PubMed: 11226290]
49. Kholmanskikh O, van Baren N, Brasseur F, Ottaviani S, Vanacker J, Arts N, van der BP, Coulie P, De Plaen E. Interleukins 1alpha and 1beta secreted by some melanoma cell lines strongly reduce expression of MITF-M and melanocyte differentiation antigens. Int.J.Cancer.
50. Martinez-Esparza M, Jimenez-Cervantes C, Solano F, Lozano JA, Garcia-Borron JC. Mechanisms of melanogenesis inhibition by tumor necrosis factor-alpha in B16/F10 mouse melanoma cells. Eur.J.Biochem. 1998; 255:139–146. [PubMed: 9692912]

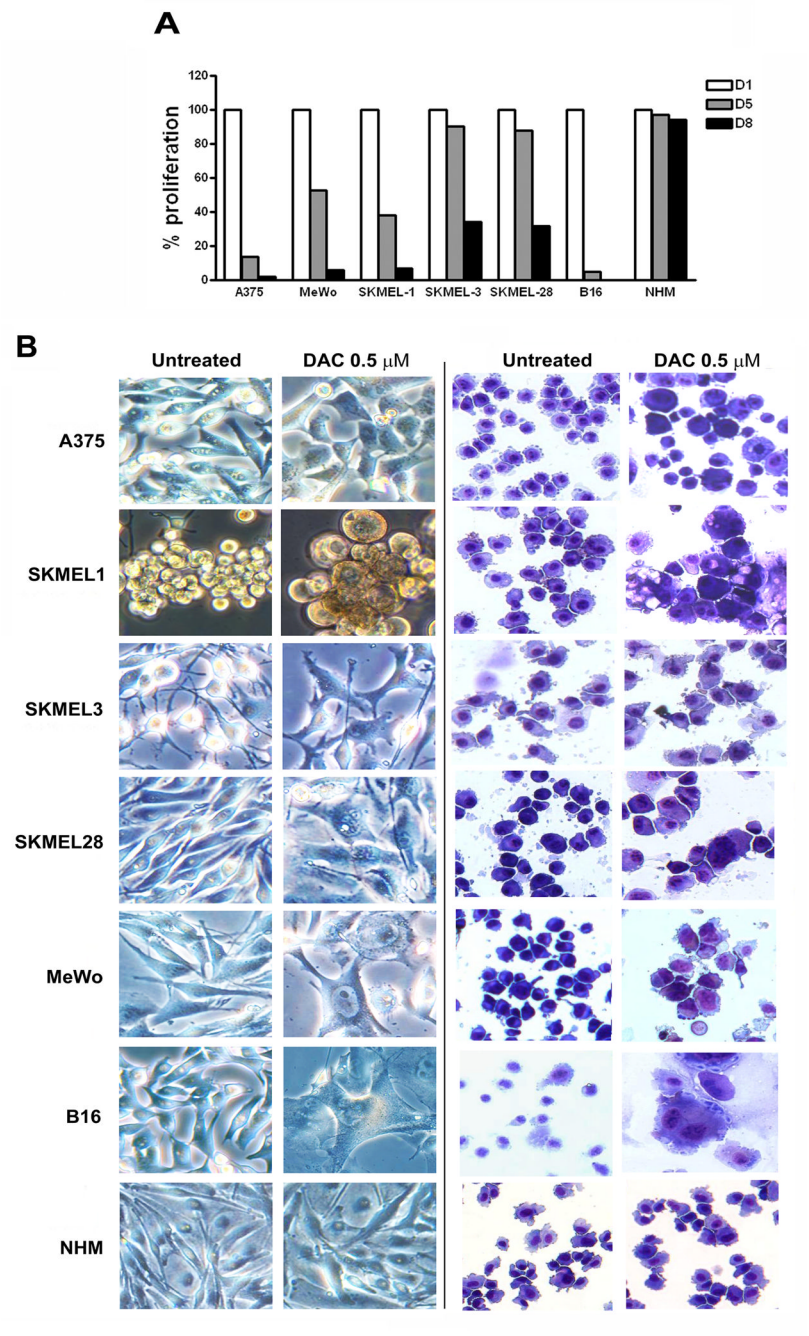

Figure 1. Effects on proliferation and morphology

(A) Decitabine 0.5 μM decreased melanoma cell-line but not normal melanocyte proliferation. Human and mouse melanoma cell lines normal human melanocytes (NHM) were cultured with decitabine 0.5 μM added on days 1 and 4. Cell counts by automated counter. Proliferation is expressed relative to vehicle treated control. **(B) Decitabine 0.5 μM induced morphologic changes of differentiation**. Human and mouse melanoma cell lines and normal human melanocytes (NHM) were cultured *in vitro* with decitabine 0.5 μM added on days 1 and 4. Images were obtained on day 8 by phase- contrast (left columns of the

panel) or Giemsa staining of cytospins of equal number of cells (right columns of the panel). All images identical 100× magnification.

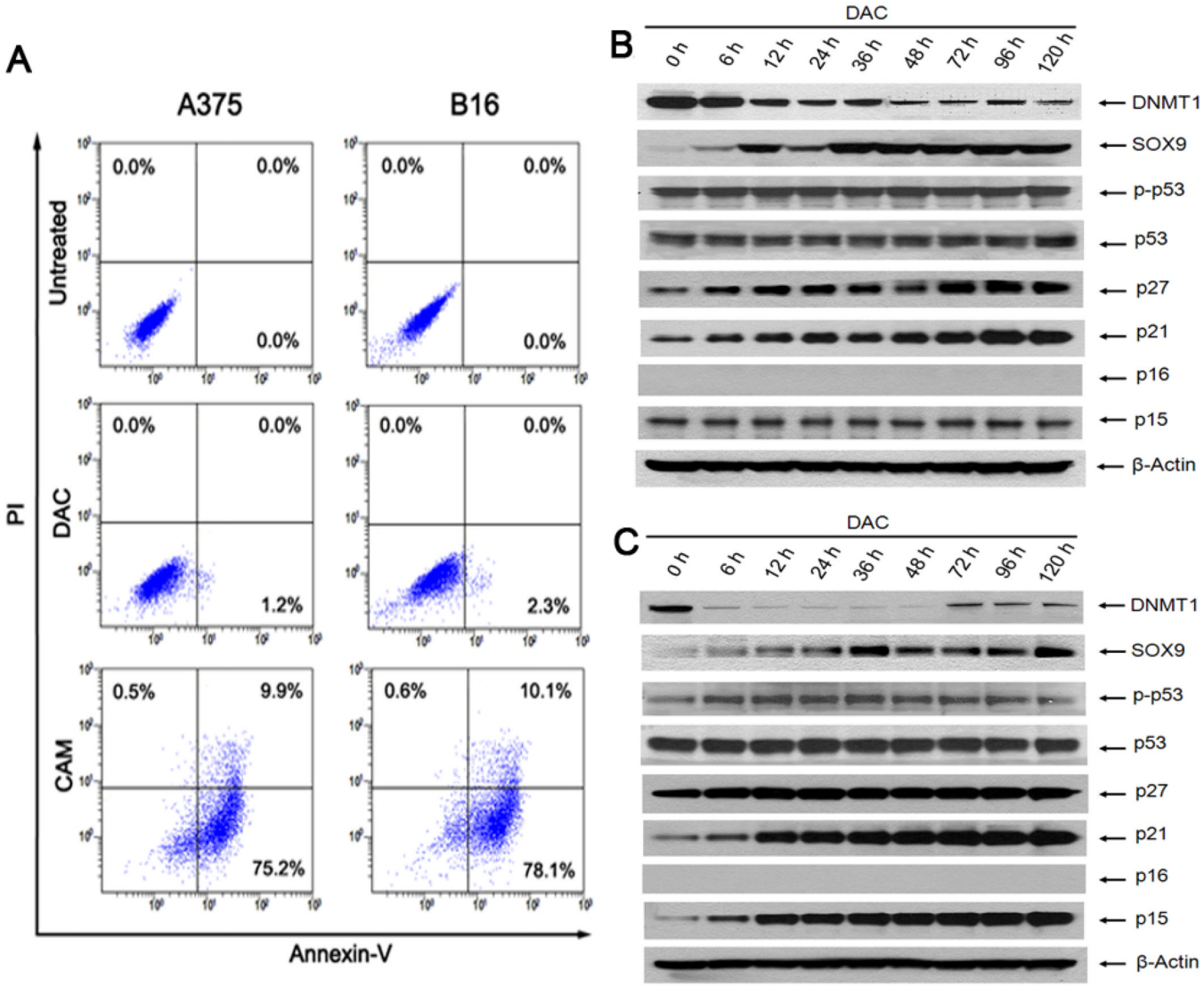

Figure 2. Effects on apoptosis and differentiation pathways

(A) The concentration of decitabine (DAC) used did not induce apoptosis as measured by Annexin/PI-staining 24 hours after addition of drug. Camptothecin (CAM) was used as a positive control for apoptosis. **(B) Response of A375 human melanoma cells**. A375 cells were cultured with decitabine 0.5 μM added at 0h. Protein levels measured by Western blot. **(C) Response of B16 murine melanoma cells**. B16 cells were cultured with decitabine 0.5 μM added at 0h. Protein levels measured by Western blot.

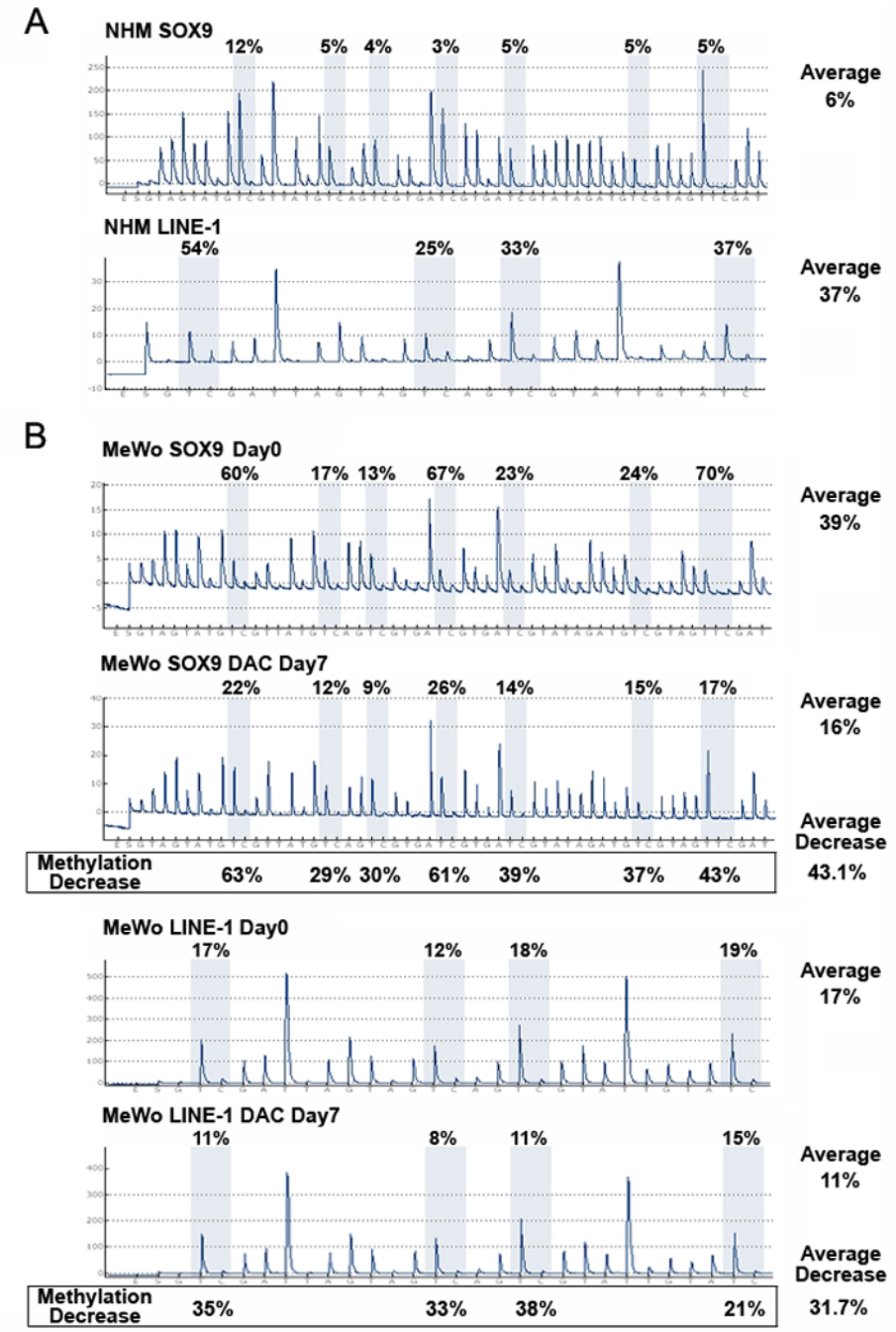

Figure 3. Decitabine effects on *SOX9* proximal promoter and LINE-1 transposon CpG methylation

(A) *SOX9* promoter and LINE-1 repetitive element CpG methylation in normal human melanocytes (NHM). Methylation measured by pyrosequencing. *SOX9* promoter CpG are in a conserved region of the proximal promoter (figure S3). **(B) *SOX9* promoter and LINE-1 CpG methylation in p53 and p16-null MeWo melanoma cells before day 0 and after day 7 decitabine.** Decitabine 0.5 μM added on day 1.

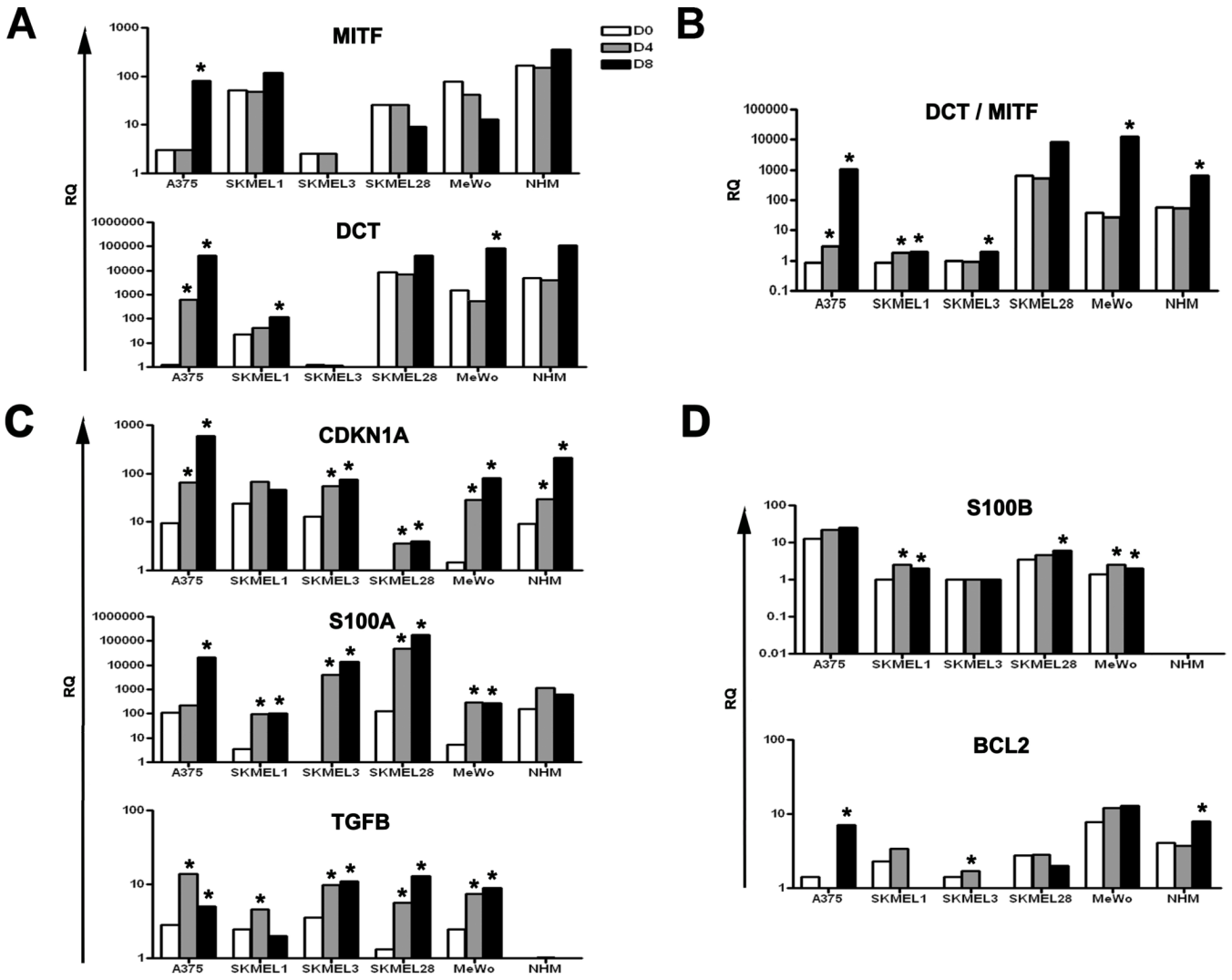

Figure 4. Effects on gene expression

Gene expression measured by QRT-PCR on day 0, 4 and 8. Human melanoma cells lines and normal human melanocytes (NHM) were cultured *in vitro* with decitabine at 0.5 μM on day 1, 4, and 7. **(A) *MITF* and *DCT* expression. (B) The ratio of *DCT*/*MITF* expression. (C) *p21/CDKN1A, S100A, TGFB* – genes previously reported to be upregulated by decitabine treatment of melanoma cells. (D) *S100B, BCL2* – genes previously reported as not being upregulated by decitabine treatment of melanoma cells**.

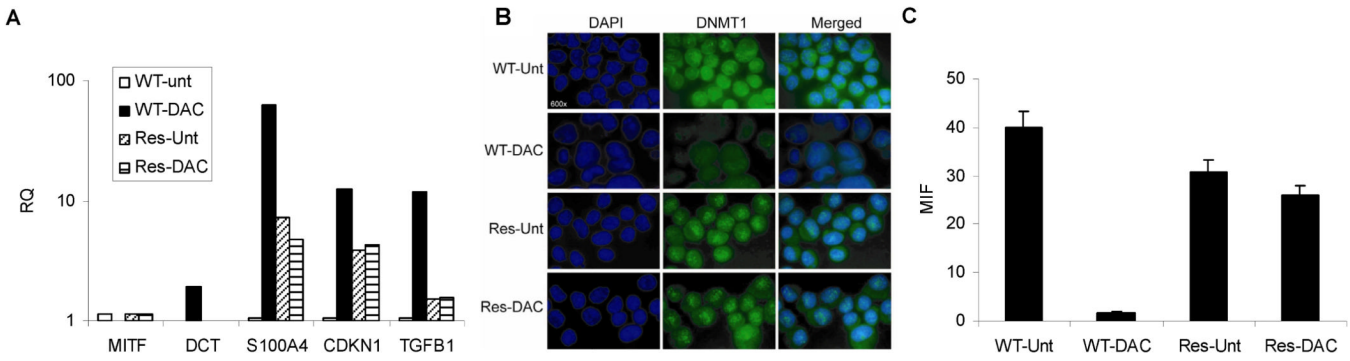

Figure 5. Gene and DNMT1 expression of resistant cells

B16 melanoma cells were maintained in culture with decitabine for 90 days. Decitabine-resistant and wild-type control B16 melanoma cells were analyzed for **(A) gene expression by QRT-PCR with and without further exposure to decitabine at 0.5 μM** and **(B and C) DNMT levels using immunofluorescence with Q-Dots**. WT-Unt = wild-type B16 untreated control, WT-DAC = decitabine treated, Res-Unt = decitabine resistant B16 untreated, Res-DAC = decitabine treated.

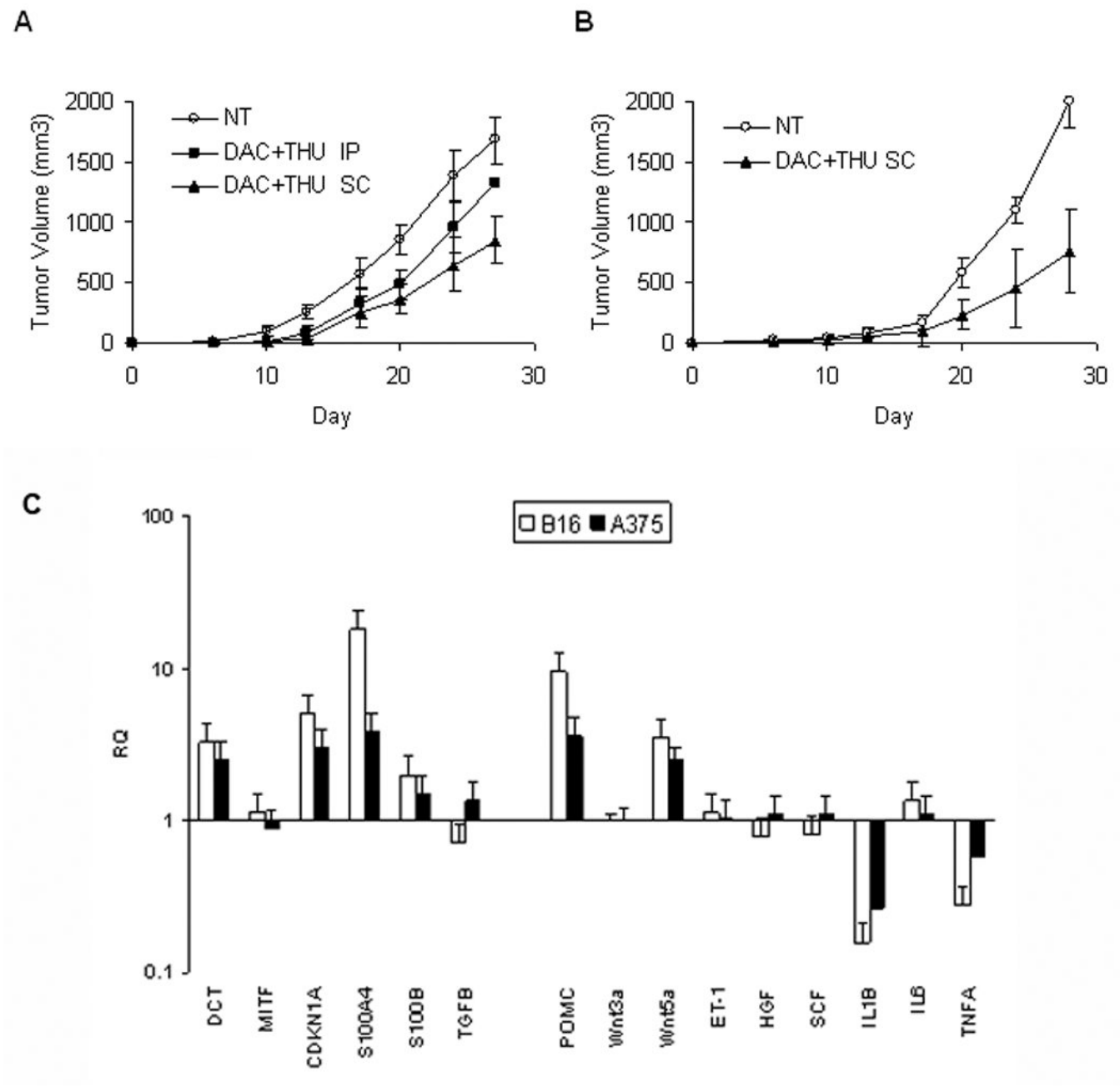

Figure 6. Effects of decitabine (DAC) and THU (THU) on tumor growth and gene expression *in vivo*

(A) B16 melanoma cells were implanted s.c. on day 1. Beginning on day 3 groups of mice were treated with THU 30–60 minutes prior to DAC either i.p. or s.c. A group was not treated (NT). Data represent mean tumor volume ± SEM, n = 7 mice per group. **(B) A375 cells were implanted s.c. on day 1**. Beginning on day 3, mice were treated with THU-DAC s.c. as above. Data represent mean tumor volume ± SEM, n = 7 mice per group. **(C) Effects of THU-DAC on intratumoral gene expression *in vivo***. B16 and A375 cells were implanted s.c. on day 1. Beginning on day 10, mice were treated with decitabine at 0.2 mg/kg 3×/week and THU at 4 mg/kg 2×/week. A group of mice was not treated (NT). Tumor was harvested on day 24. Regulators of MITF and melanocyte differentiation markers were

assessed by QRT-PCR (murine and human specific primers were used for B16 and A375 respectively). Values are presented as the ratio of gene expression in treated versus non-treated groups (mean ± SD, n = 4 mice per group).