



Catherine Hickson. *Apples in Cinnibar Green*. Oil on Belgian linen, 120 cm × 120 cm.

Epigenetic mechanisms of gene regulation have become increasingly recognized for their central role in cancer progression and metastasis.

Epigenetics in Human Melanoma

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Background: Recent technological advances have allowed us to examine the human genome in greater detail than ever before. This has opened the door to an improved understanding of the gene expression patterns involved with cancer.

Methods: A review of the literature was performed to determine the role of epigenetic modifications in human melanoma. We focused the search on histone deacetylation, methylation of gene promoter regions, demethylation of CpG islands, and the role of microRNA. We examined the relationship between human melanoma epigenetics and their importance in tumorigenesis, tumor progression, and inhibition of metastasis. The development and clinical application of select pharmacologic agents are also discussed.

Results: We identified several articles that have extensively studied the role of epigenetics in melanoma, further elucidating the complex processes involved in gene regulation and expression. Several new agents directly affect epigenetic mechanisms in melanoma, with divergent effects on the metastatic potential of melanoma.

Conclusions: Epigenetic mechanisms have emerged as having a central role in gene regulation of human melanoma, including the identification of several putative tumor suppressor genes and oncogenes. Further research will focus on the development of novel therapeutics that will likely target and alter such epigenetic changes.

Introduction

The overall incidence of melanoma cases worldwide is increasing faster than that of any other cancer,¹ with its early diagnosis essential for improving overall survival. Once diagnosed with metastatic melanoma (AJCC stage IV), most patients will ultimately die of their disease with-

in 2 years.² The vast majority of patients with advanced melanoma have few treatment options available, and the efficacy of these agents is low. Thus, the development of more effective treatment options will require further research to elucidate the cellular and molecular mechanisms involved with this aggressive form of cancer.

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Abbreviations used in this paper: TSG = tumor suppressor gene, HDAC = histone deacetylase, VPA = valproic acid, HAT = histone acetyltransferase.

Recently, the epigenetic mechanisms involved most often in gene silencing in melanoma have come to the forefront of research, highlighting unique perspectives on the gene regulation of melanoma and how this relates to tumor suppression and metastatic potential. This review focuses on such mechanisms and how they directly and indirectly affect the growth characteristics of human melanoma. A discussion is also included on how modifications of such genes can subsequently suppress and, in some cases, enhance human melanoma growth.

Melanoma is thought to arise through a series of genetic and epigenetic events. This most likely involves numerous irreversible changes occurring within the human genome, such as chromosomal deletions, amplification, and gene mutations. Recently, however, epigenetic events have attracted more attention due to their central involvement in cancer development and potential of therapeutic intervention. Epigenetics refers to any changes in gene expression without alterations of the DNA sequence. The hallmarks of epigenetic gene regulation are DNA methylation and histone modifications. It is increasingly recognized that these “epimutations” may occur at a much higher frequency compared to gene mutation and thus may have a greater impact on the process of melanoma tumorigenesis and metastatic potential. Furthermore, since many of the changes are reversible via pharmacologic manipulation, this area of research has become a particular interest for the future therapeutic potential of such agents in the treatment of patients with advanced melanoma.

DNA Methylation

The only clearly identified epigenetic modification of DNA in mammalian cells is the methylation of C-5 of cytosines that occurs as part of a CpG dinucleotide.³ The mammalian DNA methylation machinery is made up of two components: DNA methyltransferases (DNMTs), which establish and maintain genome-wide DNA methylation patterns, and the methyl-CpG-binding proteins, which are involved in “reading” and interpreting the methylation patterns. Properly established and maintained DNA methylation patterns are essential for mammalian development and normal functioning in humans, with changes in DNA methylation patterns being important characteristics of most, if not all, human cancers. Tumors in general have reduced levels of genomic DNA methylation and contain aberrantly hypermethylated CpG islands, but the full extent and sequence context of DNA hypomethylation and hypermethylation is still relatively unknown.

Aberrant DNA Hypermethylation in Melanoma

The molecular and cellular processes associated with promoter region CpG hypermethylation act as an alternate and/or complementary mechanism to gene deletion or mutation, resulting in the inactivation of specif-

ic gene expression and function. DNA hypermethylation contributes to gene silencing by preventing the binding of activating transcription factors and by attracting repressor complexes that induce the formation of inactive chromatin structures. Numerous tumor suppressor genes (TSGs) have been identified as being regulated by DNA hypermethylation in different types of cancer. To date, more than 50 genes have been identified to be aberrantly hypermethylated during some phase of melanoma progression and metastasis, as summarized in Table 1.^{4,21}

Although the exact function of most of these aberrantly silenced genes and their contribution to melanoma progression is still unknown, most data would support the notion that they possess a wide range of alternative gene functions such as cell cycle control, apoptosis, cell signaling, tumor cell invasion and metastasis, angiogenesis, and immune recognition.²² CpG island DNA hypermethylation seems to play a central role in melanoma progression and metastasis. In our own efforts, we have examined the methylation events involved in advanced-stage melanoma by extensively screening the methylation status of the promoter regions of 30 “cancer-related” genes utilizing a sensitive and quantitative methylation-specific polymerase chain reaction (PCR)-based assay (Q-MSP), performed on 20 melanoma cell lines and 40 human melanoma samples.⁸ Our analysis of this large panel allowed us to identify four genes (DcR1, DcR2, LOX, and TPM1) that have never before been implicated as hypermethylated in human melanoma, with an overall methylation frequency of 60%, 80%, 50%, and 10%, respectively. Thus, ongoing studies are currently underway to identify the relative importance of each of these genes on demethylation and reactivation of gene expression.

Stratifin, also known as 14-3-3 σ , was first identified as an epithelial cell antigen (HME-1) exclusively expressed in human epithelia. It has been implicated in G₂/M cell cycle arrest by p53 and acts as a TSG in colorectal cancer.²³ Gene silencing of 14-3-3 σ by CpG hypermethylation has been found to occur in many human epithelial cancers, including breast cancer,²⁴ hepatocellular carcinoma,²⁵ vulval squamous neoplasia,²⁶ gastric carcinoma,²⁷ oral carcinoma,²⁸ and epithelial ovarian cancer,²⁹ as well as prostate and endometrial carcinoma.³⁰ It was previously reported that stratifin/14-3-3 σ is downregulated when comparing primary and metastatic melanoma tissue samples by gene expression microarray.³¹ However, our data demonstrate that the 14-3-3 σ gene is highly expressed in normal skin but undetectable in normal melanocytes and in most melanoma cells. We have found that the promoter CpG islands in the 14-3-3 σ gene are heavily methylated in both normal melanocytes and most melanoma cells in a cell-lineage specific manner (S.L., unpublished data, 2009). We therefore hypothesize that the observed downregulation of

Table 1. — Genes Aberrantly Hypermethylated in Melanoma

Gene	Common Name	Methylation Frequency	References
APC	Adenomatous polyposis coli gene	17%	Worm et al ⁴
ASC	Apoptosis speck-like protein containing a CARD	50%	Guan et al ⁵
BST2	Bone marrow stromal cell antigen 2	50%	Muthusamy et al ⁶
CD10 (MME)	Membrane metallo-endopeptidase	13%	Shen et al ⁷
CDH1	E-cadherin	15%	Liu et al ⁸
CDH8	Cadherin 8	10%	Muthusamy et al ⁶
CDKN1B	Cyclin-dependent kinase inhibitor 1B	6.5%	Worm et al ⁹
CDKN1C (P57)	Cyclin-dependent kinase inhibitor 1C	25%	Shen et al ⁷
CDKN2A	Cyclin-dependent kinase inhibitor 2A	10%	Gonzalzo and Jones ¹⁰
CIITA- PIV	Class II transactivator, promoter IV	10%	Liu et al ⁸
COL1A2	Collagen, type 1, alpha-2	80%	Muthusamy et al ⁶
CYP1B1	Cytochrome P450, subfamily 1, polypeptide 1	100%	Muthusamy et al ⁶
DAL1	Differentially expressed in adenocarcinoma of the lung	5%	Muthusamy et al ⁶
DAPK1	Death-associated protein kinase 1	19%	Hoon et al ¹¹
DPPIV	Dipeptidyl peptidase IV	80%	McGuinness and Wesley ¹²
ER- α	Estrogen receptor alpha	42%–86%	Mori et al ¹³
GDF15	Growth/differentiation factor 15	75%	Muthusamy et al ⁶
HOXB13	Homeobox B13	20%	Muthusamy et al ⁶
HSP11	Heat shock protein H11	60%	Sharma et al ¹⁴
KR18	Zinc finger protein 160	13%	Shen et al ⁷
LOX	Lysyl oxidase	45%	Liu et al ⁸
LRRC2	Leucine-rich repeat-containing protein 2	5%	Muthusamy et al ⁶
LXN	Latexin	95%	Muthusamy et al ⁶
MDR1	Multidrug resistance 1	25%	Shen et al ⁷
Megalyn	Low-density lipoprotein-related protein 2	25%	Shen et al ⁷
MFAP2	Microfibril-associated protein 2	30%	Muthusamy et al ⁶
MGMT	O ⁶ -methylguanine-DNA methyltransferase	34%	Hoon et al ¹¹
MIB2	Skeletrophin	20%	Takeuchi et al ¹⁵
p101(PIK3R5)	Phosphoinositide-3-kinase, regulatory subunit 5	88%	Shen et al ⁷
P73	Tumor protein p73	50%	Shen et al ⁷
PCSK1	Proprotein convertase, subtilisin/kexin-type 1	60%	Muthusamy et al ⁶
PRDX2	Peroxiredoxin 2	8%	Furuta et al ¹⁶
PTEN	Phosphatase and tensin homolog	62%	Mirmohammadsadegh et al ¹⁷
PTGS2	Prostaglandin-endoperoxide synthase 2	20%	Muthusamy et al ⁶
QPCT	Glutaminyl-peptide cyclotransferase	100%	Muthusamy et al ⁶
RAR β 2	Retinoic acid receptor, beta isoform 2	70%	Hoon et al ¹¹
RASSF1A	RAS association domain family protein 1A	55%	Spugnardi et al ¹⁸
Ril (PDLIM4)	PDZ and LIM domain 4	75%	Shen et al ⁷
SOCS-1	Suppressor of cytokine signaling 1	75%	Marini et al ¹⁹
SOCS-2	Suppressor of cytokine signaling 2	75%	Liu et al ⁸
SOCS-3	Suppressor of cytokine signaling 3	60%	Tokita et al ²⁰
SYK	Protein-tyrosine kinase SYK	30%	Muthusamy et al ⁶
TFPI2	Tissue factor pathway inhibitor 2	13.5%	Nobeyama et al ²¹
THBS1	Thrombospondin 1	38%	Shen et al ⁷
THBS4	Thrombospondin 4	63%	Shen et al ⁷
TIMP3	Tissue inhibitor of metalloproteinase 3	10%	Liu et al ⁸
TM	Thrombomodulin	60%	Furuta et al ¹⁶
TNFRSF10A (DR4)	Tumor necrosis factor receptor superfamily, member 10a	10%	Liu et al ⁸
TNFRSF10C (DcR1)	Tumor necrosis factor receptor superfamily, member 10c, decoy without an intracellular domain	55%	Liu et al ⁸
TNFRSF10D (DcR2)	Tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain	85%	Liu et al ⁸
TPM1	Tropomyosin 1	10%	Liu et al ⁸
WFDC1	Wap 4-disulfide core domain 1	20%	Muthusamy et al ⁶

the 14-3-3 σ gene in the previous report is due to the existence of trace amounts of skin cell types in the primary melanoma tissue samples.

Aberrant DNA Hypomethylation in Melanoma

Epigenetic silencing of TSGs is well documented; however, comparatively little is known about the role of hypomethylation in the initiation and development of melanoma. It was proposed and recently validated that hypomethylation in cancers contributes to tumor progression by inducing genome instability via the demethylation of transposons and pericentromeric repeats and by inducing the expression of many genes, including oncogenes.³² A genome-wide hypomethylation was observed in mice that carried a hypomorphic Dnmt1 as well as in animals developing aggressive T-cell lymphomas.³³ This demonstrated the potential consequences of tumor development from spontaneously occurring or chemically induced DNA hypomethylation. The molecular basis for hypomethylation-induced tumors in this model involves chromosomal instability events accompanied by activation of endogenous retroviral elements. By analyzing and comparing the hypermethylation patterns in squamous cell carcinoma of the lung and matched normal lung tissue, it was found that extensive DNA hypomethylation in tumors occurs specifically at repetitive sequences, including short and long interspersed nuclear elements and LTR elements, segmental duplications, and subtelomeric regions.³⁴ These findings further validate the role of DNA methylation in maintaining the stability of the human genome and the suppression of transposable elements in mammalian cells.

Although global hypomethylation was found to be a relatively common event in many tumors, little information exists regarding the target genes regulated by hypomethylation. Several reports have provided clear evidence that hypomethylation in tumors causes the activation of multiple genes, including genes with suspected oncogenic activities.³² Several examples include cyclin D2, maspin,^{35,36} and R-RAS³² in gastric cancer, MN/CA9 in renal cancer,³⁷ *synuclein- γ breast cancer-specific gene 1 (SNCG/BCSG1)* in breast and ovarian cancer,³⁸ BORIS/CTCF in ovarian cancer,³⁹ heparinase in bladder cancer,⁴⁰ and hypomethylation of WNT5A, CRIP1, and S100P in prostate cancer.⁴¹

In melanoma, a group of cancer-testis antigens (CTAs) and several other genes have been identified to be aberrantly hypomethylated (Table 2).⁴²⁻⁴⁵ Expression of these genes is repressed in normal human skin melanocytes, primarily due to heavily methylated promoter regions in a cell lineage-specific

manner. Conversely, these same genes can exist in a demethylated state and are aberrantly reexpressed in subsets of melanoma cells. The biological significance of this gene reexpression continues to be poorly understood. A central question is whether their expression contributes to tumorigenesis or is only a functionally irrelevant by-product of the process of cellular transformation due to global genome hypomethylation. Current evidence would suggest that reactivation of these genes may contribute to overall tumorigenesis. It is clear that the expression of these tumor antigens can result in their recognition and possible destruction by the host immune system, with the products influencing a range of cellular processes, including cell signaling, transcription, translation, and chromosomal recombination.⁴⁶ Importantly, recent data suggest that expression of MAGE genes in cancer cells contributes directly to the malignant phenotype and response to therapy.⁴⁶

Testis-specific protein, Y-encoded (TSPY) is a repeated gene mapped to the critical region harboring the gonadoblastoma locus on the Y chromosome (GBY), the only oncogenic locus on this male-specific chromosome. It is normally expressed in the germ cells of the testis and distinct subsets of spermatogonia.⁴⁷ Elevated levels of TSPY have been observed in gonadoblastoma specimens and a variety of other tumor tissues, including testicular germ cell tumors, prostate cancer, melanoma, and liver cancer, indicating TSPY as a putative oncogene. Importantly, it was shown that ectopic expression of TSPY potentiates cell proliferation by mediating a rapid G₂/M transition of the cell cycle and promotes tumor growth in nude mice.⁴⁸ Recently, we have also shown a marked elevation in the gene expression of TSPY in melanoma, primarily a result of promoter demethylation (A.I.R., unpublished data, 2009) as the TSPY promoter CpG island is heavily methylated in normal melanocytes and significantly reduced in TSPY-expressing melanoma

Table 2. — Genes Methylated in Normal Melanocytes and Hypomethylated in Melanoma

Gene	Common Name	References
PRAME	Preferentially expressed antigen of melanoma	Sigalotti et al ⁴²
14-3-3 σ	Stratifin	unpublished data*
GAGE 1-6	G antigen 1-6	Sigalotti et al ⁴²
HMW-MAA	High molecular weight melanoma-associated antigen	Luo et al ⁴³
MAGE-A1	Melanoma antigen, family A1	Sigalotti et al ⁴²
MAGE-A2	Melanoma antigen, family A2	Sigalotti et al ⁴²
MAGE-A3	Melanoma antigen, family A3	Sigalotti et al ⁴²
MAGE-A4	Melanoma antigen, family A4	Sigalotti et al ⁴²
MAGE-A6	Melanoma antigen, family A6	Sigalotti et al ⁴²
NY-ESO-1	New York oesophageal squamous cell carcinoma 1	James et al ⁴⁴
PI5	Protease inhibitor 5/Maspin	Wada et al ⁴⁵
SSX 1-5	Sarcoma, synovial, breakpoint 1-5	Sigalotti et al ⁴²
TSPY	Testis-specific protein, Y-linked	unpublished data**

* S.L., 2009; ** A.I.R., 2009

cells. Furthermore, the use of 5-aza-2'-deoxycytidine, a demethylating agent, to treat melanoma cell lines derived from male patients who are TSPY-negative can significantly upregulate TSPY messenger RNA (mRNA) expression. These results are contradictory to those of Gallagher et al,⁴⁹ who reported TSPY promoter methylation during melanoma progression and decreased TSPY expression in metastatic melanoma. However, this study was limited by utilization of a single highly invasive metastatic melanoma cell line, a parental line of low tumorigenicity, and its two highly tumorigenic, isogenic derivative cell lines.

Maspin, a mammary serine protease inhibitor, was originally reported to be a TSG in breast cancer.⁵⁰ The expression pattern of the maspin gene differs among cancer types and normal tissue, with its implication as a TSG still questioned. Wada et al⁴⁵ reported that immunoreactivity for maspin was negative in normal skin melanocytes and 40 melanocytic nevi, but 5 of 40 (12.5%) melanomas were found to be positive, with methylation inversely correlated with maspin protein expression, both in vitro and in vivo.⁴⁵ The expression of maspin in a subset of melanoma cells requires further investigation to determine the significance of aberrant maspin expression.

Clinical Applications

Methylated Genes as Therapeutic Targets

Since hypermethylation can be reversed by specific drugs, the use of DNA demethylating agents has been exploited to investigate their therapeutic ability to “reactivate” such genes upon demethylation with subsequent gene expression contributing to the regression of established tumors. Drugs that inhibit DNA methyltransferases were shown to have the potential to reactivate silenced genes and induce differentiation or apoptosis of malignant cells. The most intensively studied class of such agents is DNA methyltransferase inhibitors, including 5-azacytidine (5-aza, azacytidine) and 5-aza-2'-deoxycytidine (decitabine), currently approved for human use in the United States for the treatment of patients with myelodysplastic syndrome.

It appears that epigenetic alterations in cancer cells affect virtually every cellular pathway involved in cell cycle progression, apoptosis, cell survival, angiogenesis, and immunogenicity. Therefore, it is not surprising that “epigenetic drugs” display pleiotropic activities. Combinations of demethylation drugs with chemotherapy, interferon, and tumor vaccines have been proposed to increase their clinical efficacy, since genes involved in chemotherapy and/or interferon resistance and cancer-testis antigens are all modulated by promoter methylation. In murine experiments, the combination therapies have shown clear benefits in eliminating cancer.^{51,52} Some clinical trials also have shown limited but promising results.⁵³ These clinical

trials demonstrated that demethylation agents (decitabine) can be combined safely with carboplatin and cause epigenetic changes in patients with solid tumors. Decitabine can also be safely administered with high-dose interleukin-2 (IL-2) and may enhance the activity of IL-2 in melanoma. When 5-aza-2'-deoxycytidine is combined with IL-2, objective responses occurred in 31% of melanoma patients.⁵⁴

In our own efforts, we found that subcutaneous injection of 5-azacytidine as a single agent in melanoma xenograft models resulted in a significant delay in tumor growth in several nude mice models. While the exact mechanism of this observation is not clear, we suggest that this significant tumor inhibition effect is due to inhibition of several known genes involved in tumor angiogenesis in combination with the possible reactivation of several known TSGs. Although reactivation of TSGs and inhibition of tumor growth by demethylation agents have been well documented, there are always concerns in regard to the lack of specificity of current demethylation agents that may result in the global hypomethylation of the genome. Specifically, it may be possible to not only reexpress previously silenced TSGs, but also activate “tumor-promoting genes.” Further research is needed to determine the appropriate patient selection and dosing schedules. As we identify new genes and improve our current understanding of the epigenetic mechanisms involved in melanoma, we foresee the development of an array-based DNA methylation assay able to identify a panel of methylated genes within a freshly procured melanoma sample. In doing so, we can then modulate these suspected genes for treatment, possibly treating patients with a demethylating agent.

DNA Methylation as Biomarkers in Human Cancer

Aberrantly hypermethylated or hypomethylated genes can serve as biomarkers in clinical use for early detection of disease, tumor classification, and response to treatment with classical chemotherapy agents, target compounds, and epigenetic drugs. Indeed, as more genes are being identified, several studies have investigated the potential utility of Q-MSP for gene methylation analysis of clinical samples. Identification and validation of methylation markers for human cancer are active areas of research, with other investigators finding such markers in prostate cancer, such as the GSTP1 gene.⁵⁵ Several studies have demonstrated the utility of detecting circulating methylated tumor-related genes in the peripheral blood of cancer patients as a potential marker for disease progression, metastatic potential, and therapeutic response to treatment.⁵⁶ For instance, it was reported that circulating methylated RASSF1A was significantly less frequent for biochemotherapy responders compared with nonresponders, with methylation of RASSF1A significantly correlated with

overall survival and biochemotherapy response.⁵⁷ Mori et al¹³ reported that estrogen receptor A (ER-A) methylation can predict melanoma progression, with serum methylated ER-A an unfavorable prognostic factor. Thus, detection of the hypermethylated DNA of TSGs may contribute to a more sensitive classification system for melanoma, with the further identification of prognostic markers as predictors of outcome to treatment.

DNA methylation mapping has revealed that many human cancers of different tumor histologies may possess unique profiles of hypermethylated CpG islands. We recently examined the methylation status of 30 genes in clinical melanoma samples using Q-MSP.⁸ We found that SOCS1, SOCS2, RAR β 2, DcR1, and DcR2 are the most frequently methylated genes in melanoma, with methylation frequencies of 90%, 80%, 60%, 60%, and 85%, respectively. Another five genes, RECK, IRF7, PAWR, DR5, and Rb, which were found to be highly methylated in other forms of malignancies, were not methylated in melanoma at all. This result further validates that different tumor types possess different panels of methylated genes. Identifying the tumor type-specific methylation events may provide biomarkers for the differential diagnosis of malignant diseases. This may have a direct impact on the development of targeted therapeutics, such as utilizing demethylating agents to reactivate latently suppressed gene expression of TSGs.

Histone Deacetylation

The successful transcription of DNA within the nucleus of a human cell requires that the segment to be transcribed must be accessible to numerous transcription factors and cofactors. Gene promoter methylation serves to block the binding of transcription factors and subsequent downstream gene expression by acting as a direct steric hindrance, recruiting MB methyl-CpG-binding proteins and/or interfering with RNA polymerase activity.⁵⁸ DNA-binding histones are directly modified to affect the strength with which they bind and subsequently segregate DNA from transcriptional machinery. Acetylation of lysine residues on histones is associated with transcriptionally active DNA where deacetylation is associated with tightly bound, inactive DNA. Additionally, promoter methylation can affect histone activity through the stepwise recruitment of other transcriptional modulators to the methylation site, as histone acetylation similarly affects promoter methylation levels. Each of these processes provides a means of controlling gene expression for the maintenance of cell homeostasis and has been shown deregulated in many cancer types. Histone deacetylases (HDACs), implicated in the development of tumors of various histologies through the alteration of normal gene expression patterns and inhibition of DNA repair,⁵⁹ have thus become major therapeutic targets in cancer.

Eukaryotic DNA is found in the nucleus packaged into dense chromatin, a tightly twisted compaction composed of DNA, histones (H1, H2A, H2B, H3, H4) and nonhistone proteins. The functional unit of chromatin is the nucleosome, and it consists of a histone octamer of two H2A-H2B dimers and an H3-H4 tetramer around which ~147 bp of core DNA is wound 1.7 times. Histone H1 acts to link nucleosomes together for a higher order compaction of chromatin. Between each nucleosome lies a stretch of ~50 bp, which gives the overall appearance of “beads on a string” in actively transcribed euchromatin. The NH₂-terminal histone domain extends from the core as a charged “histone tail.” Amino acid residues such as lysine and arginine contribute to the overall basic properties of the histone tail and to the binding of the negatively charged DNA phosphate backbone. Posttranslational modification of these and other residues on the nucleosomal tails primes the sites for unique interaction with chromatin remodeling complexes and transcription factors, thus allowing for a variety of ways to affect chromatin assembly and gene expression. Specifically, reversible acetylation of the ϵ -amino group on N-terminal tail lysine residues reduces the positive charge of the histone and alleviates the charge attraction between it and DNA, relaxing chromatin and making DNA more available to transcription factors. Lysine acetylation can also recruit transcriptional activators such as the SWI/SNF adenosine triphosphatase (ATP)-dependent chromatin remodeling complex^{60,61} and regulate gene expression through the modification of higher-order chromatin folding.⁶² The enzymes responsible for acetylating and deacetylating lysine are histone acetyltransferases (HATs) and HDACs, respectively. In this manner, chromatin can switch between being open (euchromatin) or closed (heterochromatin) to transcriptional activity.

While this concept is simplified in lysine acetylation and gene transcription, there remains uncertainty as to which of the many cyclically interactive regulatory mechanisms actually steers the overall epigenetic procession of tumor formation and progression, such as which process precedes and which predominates. Fuks et al⁶³ show that the methyl-CpG-binding protein, MeCP2, binds methylated CpG (met-CpG) sites and recruits not only HDAC activity, but also histone H3 lysine-9 (K9)-specific histone methyltransferase (HMT) activity. HDACs and HMTs work together, specifically at a met-CpG site, by removal of the acetyl group from H3 K9 to allow methyl addition, an initiation factor for the formation of transcriptionally inactive heterochromatin.^{63,64} Tamaru and Selker⁶⁵ and Jackson et al⁶⁶ describe the complementary event of methylated H3 K9 leading to the methylation of DNA in *Neurospora crassa* and *Arabidopsis thaliana*, respectively. In this manner, DNA methylation and various histone modifications act on each other to repress chromatin. The

myriad ways histone tail residues can be modified (eg, acetylation, methylation, ubiquitination), the interdependent relationship of covalent modifications on different tails with their effects, and the propagation of those effects among nucleosomes collectively provide many different routes toward gene repression or activation. Specific combinations of histone modifications confer the overall expression status of a region of chromatin, the proposed “histone code.”⁶⁷⁻⁷⁰

Histone Acetyltransferases and Deacetylases

HAT activity is generally associated with transcriptional activation, while the opposite is true for HDACs. The names imply specificity for histones; however, it is now known that HATs and HDACs affect numerous other proteins involved in many different cellular processes.⁷¹ Thus, the more general terms of “lysine acetyltransferase” (KAT) and “lysine deacetylase” (KDAC) are used to emphasize activity rather than target. Moreover, lysine acetylation has recently been connected to the regulation of protein stability through either directly or indirectly affecting protein interaction with cell ubiquitination machinery.⁷² HATs and HDACs are commonly found in multi-subunit complexes where their activities and substrate specificities may be altered by cofactors within the complex, and vice versa.⁶⁸ Given the wide functional variety of subunits present in different complexes, acetylation becomes a multifaceted influence over many cellular events. Furthermore, HAT mutation, translocation, deletion, and overexpression, as well as HDAC overexpression and underexpression, have been implicated in the development of various tumors.⁷³

Histone Acetyltransferases

HATs transfer an acetyl group from acetyl-CoA to the ε-amino group of lysine residues. In addition to acting on N-terminal histone tails, the HAT-catalyzed acetylation of core histones has been described with the proposed effect of recruiting ATP-dependent chromatin remodeling complexes.⁷³⁻⁷⁶ Three groups of HATs are recognized based on conserved sequence and generally function as coactivators for transcriptional activation machinery: GNAT [general control non-depressible 5 (Gcn5)-related *N*-acetyltransferase], MYST [named for family members MOZ, Ybf2-Sas3, Sas2, and Tip60 and also includes HBO1, MOF and MORF (MOZ-related factor)], and p300/CBP (adenoviral E1A-associated protein, 300 kDa; CREB-binding protein). Members of the GNAT family acetylate histones H3 and H4 as well as nonhistone transcriptional activators and include human GCN5, HAT1, and PCAF (p300/CREB-binding associated factor). MYST HATs have a characteristic highly conserved 370 bp MYST domain with acetyl-CoA binding site and function in numerous nuclear processes including transcriptional activation and DNA

repair.⁷⁷ Finally, p300 and CBP are involved in cellular proliferation, differentiation, and apoptosis. They are known as global transcription coactivators that can acetylate each of the core histones as well as nonhistone proteins such as p53, Rb, and E2F.^{78,79}

Notably, MYST members Tip60 and HBO1 present in a complex with the putative melanoma tumor suppressors ING3 and ING4, respectively.⁸⁰⁻⁸³ Additionally, both Tip60 and HBO1 functionally link NF-κB,⁸⁴⁻⁸⁶ while acetylation by Tip60, GCN5, and PCAF can stabilize the transcription factor c-Myc,^{87,88} each of which are putative melanoma oncoproteins.⁸⁹⁻⁹⁴ Furthermore, both CBP and p300 have been shown to associate with microphthalmia-associated transcription factor (MITF), a melanocyte lineage survival oncogene⁹⁵⁻⁹⁷ that transcriptionally regulates melanoma invasiveness, proliferation, and apoptosis,⁹⁸ is mostly upregulated in metastatic melanomas,⁹⁹ and is associated with decreased survival in metastatic melanoma patients.¹⁰⁰

Histone Deacetylases

Currently, 18 known human HDACs have been described.^{101,102} They are separated into four classes based on structural homology to yeast HDACs, mechanism of enzyme activity, and cellular localization. Classes I, II, and IV comprise the “classic HDAC family.” Each of the 11 members has Zn²⁺ cofactor-dependent catalytic activity and can thus be inhibited by HDAC inhibitors that target the enzyme’s Zn²⁺ ion pocket. Class I (HDAC-1, -2, -3, and -8) is thought to be expressed ubiquitously in all cell types and shares homology with yeast Rpd3. The presence of class II (class IIa = HDAC-4, -5, -7, -9; class IIb = HDAC-6, -10) is more restricted to smooth muscle, heart, pancreas, and brain, among a few other tissues, and is homologous with HDA1. Additionally, class I is mostly localized to the nucleus, whereas class II may be found shuttling between the nucleus and cytosol. Class II is further divided into IIa and IIb as HDAC-6 and HDAC-10 have two catalytic domains instead of the sole domain found in all other classified HDACs. HDAC-11 is separated into class IV as it has structural homology with both Rpd3 and HDA1 while having similar tissue localization to class II.¹⁰³ Class III is composed of the sirtuins (SIRT 1-7), so named for their homology to yeast transcriptional repressor silent information regulator 2 (Sir2). This group is unique in that it is NAD⁺ cofactor-dependent for the catalytic formation of nicotinamide (currently studied for sirtuin inhibitor activity) and *O*-acetyl-ADP-ribose. Sirtuins have been implicated in aging, with overexpression in yeast leading to increased lifespan and knockdown decreasing lifespan by 50%.¹⁰⁴ More recently, sirtuins have been implicated in neurodegenerative disorders, HIV, and cancer.

In addition to acting in transcriptionally repressive complexes, HDACs may influence growth arrest, differentiation, and apoptosis.^{105,106} With the myriad down-

stream effects of HDAC activity on tumor development, HDACs have become a major epigenetic target for tumor reversion and prevention. However, while much is known about HDACs mechanistically, their class and isoform-specific roles in cancer have only begun to be elucidated. The development of HDAC inhibitors that are both broad-spectrum and selective has helped to identify individual deacetylase function in normal and cancerous cells. Moreover, with the development of sir-tuin inhibitors, which are functionally different from classic HDAC inhibitors, a new field of targeted epigenetic cancer therapy has evolved.

HDAC Inhibition in Melanoma

Regulation of histone modifications is a promising approach for epigenetic control over and reversal of tumor development. Decreasing upregulated oncogene expression and increasing downregulated TSG and/or DNA repair gene expression are each proposed avenues. In the complex malignant transformation process of melanoma, gene expression profiles reveal a loss of expression of genes that act to counter tumor cell formation, including cell cycle regulators and proapoptotic genes.¹⁰⁷ Many of these TSGs are shown to be downregulated jointly through promoter hypermethylation and the reversible deacetylation of lysine residues by HDACs of local histones.¹⁰⁸ Furthermore, HDACs are known to act on proteins that regulate cellular differentiation, proliferation, gene expression, and death.^{105,109} Therefore, HDAC inhibitors are currently being studied as treatment against the development of malignant melanoma.

With the recent surge of interest surrounding epigenetic therapies for patients with cancer, many structurally diverse HDAC inhibitor compounds are being developed. Most of the described HDAC inhibitors can be structurally separated into four main groups: short-chain fatty acids (ie, butyrate and valproic acid [VPA, valproate]), hydroxamic acids (ie, trichostatin A [TSA] and suberoylanilide hydroxamic acid [SAHA, Zolanza™, vorinostat]), benzamides (ie, MS-275 and CI-994), and cyclic tetrapeptides with or without a Zn²⁺-binding L-2-amino-8-oxo-9,10-epoxydecanoic acid (AOE) moiety (ie, depsipeptide [FK228, FR901228, romidepsin] and trapoxin [TPX]). Members of these groups inhibit class I and class II HDACs by targeting the Zn²⁺ pocket and exhibit a wide range of potency and specificity for individual HDACs or HDAC classes. The HDAC-I-selective inhibitor Bis-pyridinium diene, however, does not have a Zn²⁺ binding group and in this way is unrelated to other HDAC inhibitors.¹¹⁰ Class III inhibitors (SIRTis) do not act through a similar mechanism due to the lack of Zn²⁺ as a SIRT cofactor. By regulating levels of NAD⁺ and competitive inhibitors nicotinamide and NADH, sir-tuin activity can be modulated. Moreover, they exhibit equally variable potency and selectivity to that of classes

I and II, although only for SIRTis. The SIRTis suramin, including such derivatives as NF675 and some indoles, are within the nanomolar IC₅₀ range, which is more potent than that of the hydroxamates.¹¹¹ Chemical variations on core, active components have allowed for such diversity in drug development. Given the dramatic response of cutaneous and peripheral T-cell lymphoma patients to SAHA and depsipeptide, as well as general HDAC inhibitor effectiveness in culture, researchers and clinicians have focused their efforts over the last decade to synthesize and uncover novel HDAC inhibitors with increased selectivity and antitumor activity for both the study of enzyme biology and clinical application. Promising results for the treatment of human melanoma with HDAC inhibitors in addition to further mechanistic knowledge regarding HDACs, HDAC inhibitors, and melanoma have recently been described.

HDAC Inhibitor Function

It has been established that hyperacetylation of N-terminal tail lysines of histones H3 and H4 is associated with transcriptional activation, whereas hypoacetylation is associated with transcriptional repression.¹⁰⁶ The cell type-specific antitumor effects of HDAC inhibitors are generally thought to result from the re-expression of TSGs and increased acetylation of non-histone proteins active in multiple cellular processes. Notably, protein acetylation may either stabilize the protein or promote its degradation, ie, HDAC2 and p300 are rapidly degraded upon treatment with VPA or butyrate.^{112,113} Multiple HDAC inhibitors have been shown to inhibit angiogenesis through attenuation of vascular endothelial growth factor (VEGF)¹¹⁴ and also to induce generation of reactive oxygen species (ROS),¹¹⁵ premature chromatid separation,¹¹⁶ autophagic cell death,¹¹⁷ and senescence¹¹⁸ in transformed cells. More general effects of treatment with HDAC inhibitors are the induction of intrinsic (mitochondrial) and extrinsic (death receptor-mediated) apoptotic pathways, growth arrest, and differentiation in vitro and in vivo,^{73,119} albeit only about 2% to 20% of genes show a change in expression after HDAC inhibitor treatment in tumor cells, most of those genes being involved in cell growth and survival.^{71,105} Recently, Boyle et al¹²⁰ described an equal number of genes repressed or reactivated in melanoma cell lines treated with either butyrate or suberic bishydroxamate (SBHA), consistent with findings in other tumor cell types.

Apoptosis and the Cell Cycle

Human melanoma cells are known to be highly resistant to proapoptotic stimuli. However, they express high levels of wild-type p53, the master regulator of DNA repair, and primary melanomas do not often show mutation of p53, inconsistent with most other tumor types. Treatment with HDAC inhibitors induces apoptosis in most

melanoma cell lines via mitochondrial and caspase-dependent pathways, sparing normal melanocytes.^{120,121} Poly-ADP ribose protein (PARP) cleavage by caspases is an apparent hallmark of HDAC inhibitor-induced apoptosis in melanoma.¹²² Hydroxamates TSA, SAHA, and SBHA upregulate proapoptotic proteins such as Bax, Bim, Bak, Bid, caspases 3 and 8, and Apaf-1 while deregulating antiapoptotic proteins such as Bcl2, Bcl-xL, XIAP, and Mcl-1 in multiple tumors.¹²¹⁻¹²⁴ Tables 3 and 4^{73,121-149} list genes involved in numerous cellular processes that are shown to be up- or downregulated in various tumor histologies upon treatment with one or more HDAC inhibitors. Facchetti et al¹⁴⁶ also describe melanoma cell

death using the short-chain fatty acid VPA as being heavily modulated by these and the survivin protein.

While p53-independent HDAC inhibitor-induced apoptosis is common for most drug classes, it was recently reported that sodium butyrate-induced apoptosis of metastatic UCD-Mel-N, A375, and primary cutaneous SB2 human melanoma cells was mediated via a p53-dependent induction of Bax through HDAC1 repression.¹²³ HDAC1 overexpression was found to make the cells more resistant to butyrate where HDAC1 underexpression conferred butyrate sensitivity and was correlated with increased p53 acetylation, preceding Bax induction. Small molecule IKK1 inhibitor parthe-

Table 3. — Genes Upregulated in Various Tumor Histologies With HDAC Inhibitors

Gene	HDAC Inhibitor	References	Mechanistic Information
APAF1	TSA, SAHA, depsipeptide	Soengas et al, ¹²⁴ Peart et al ¹²⁵	
ARF4	Depsipeptide	Kobayashi et al ¹²⁶	
BAK1	SAHA, SBHA, depsipeptide	Zhang et al, ¹²¹ Peart et al ¹²⁵	
Bam	TSA, butyrate	Bandyopadhyay et al ¹²³	p53-dependent; butyrate-induced apoptosis of melanoma cells associated with HDAC1-dependent induction of proapoptotic proteins, Bax, Bam, PUMA, NOXA, and acetylation of p53; HDAC1 confers resistance to butyrate-induced apoptosis
Bax	TSA*, SAHA, SBHA, butyrate*	Bandyopadhyay et al, ^{123*} Zhang et al, ¹²¹ Munshi et al ¹²⁷	p53-dependent apoptosis*
Bid	SBHA	Zhang et al ¹²¹	
Bim	SAHA, SBHA, depsipeptide	Zhang et al, ¹²¹ Peart et al ¹²⁵	
Bim EL	SBHA	Flørenes et al ¹²²	
CAECAM5	TSA, MS-275	Lee et al, ¹²⁸ Hess-Stump ¹²⁹	
CASP3,8	SBHA	Zhang et al ¹²¹	
CD24	VPA	Milutinovic et al ¹³⁰	
CD40,80,86	TSA, VPA	Khan et al ¹³¹	
CDH1	VPA	Milutinovic et al ¹³⁰	
CDKN3	SAHA, depsipeptide	Peart et al ¹²⁵	
CRBP1	SIRTi	Pruitt et al ¹³²	
DAPK3	SAHA, depsipeptide	Peart et al ¹²⁵	
E-CADHERIN	TSA, SIRTi	Pruitt et al, ¹³² Ou et al ¹³³	
FLJ23028	Depsipeptide	Kobayashi et al ¹²⁶	
GATA4	Depsipeptide	Wu et al ¹³⁴	Depsipeptide decreased both CpG and H3K9 methylation of the promoter
GATA4,5	SIRTi	Pruitt et al ¹³²	
gp100/pMel17	Depsipeptide	Kobayashi et al ¹²⁶	
GSN	MS-275	Gore et al ¹³⁵	
H-2D	TSA, VPA	Khan et al ¹³¹	
HDAC3,5	SAHA, depsipeptide	Peart et al ¹²⁵	
HLA-C,DPA1,F	VPA	Milutinovic et al ¹³⁰	
HSPB1	VPA	Milutinovic et al ¹³⁰	
L1-CAM	Butyrate	Kuwajima et al ¹³⁶	Melanoma metastasis: member of the Ig superfamily of cell adhesion molecules
LMP2,7	TSA, VPA	Khan et al ¹³¹	
MAD	SAHA, depsipeptide	Peart et al ¹²⁵	
MAGEB2	VPA	Milutinovic et al ¹³⁰	
MCL1	SAHA, depsipeptide	Peart et al ¹²⁵	
Mel-CAM	Butyrate	Kuwajima et al ¹³⁶	Melanoma metastasis: member of the Ig superfamily of cell adhesion molecules
MLH1	SIRTi	Pruitt et al ¹³²	

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nolide can induce the ubiquitination and proteasomal degradation of HDAC1 in tumor cells and upregulation of suppressed genes such as p21WAF1/CIP1, thereby leading to the p53- and Bax-mediated apoptosis now understood in melanoma.¹⁵⁰ Parthenolide is not an HDAC inhibitor; it appears to act through the activation of the PIKK ATM and currently undefined downstream effects leading to HDAC1 ubiquitination.

Treatment of human melanoma cell lines A2058 and HMV1 with combination LAQ824 (hydroxamate) and 13-cis-retinoic acid (CRA) showed synergistic inhi-

bition of growth leading to G₂ arrest (A2058) and apoptosis (HMV-1).¹⁴⁴ Synergy was associated with increased RARb2 expression, sensitizing the cells to the retinoid CRA. Retinoids are natural and synthetic vitamin A analogs with roles in epithelial cell growth and differentiation. A phase II clinical trial of retinol in patients with advanced cancer, 15 of 65 having melanoma, showed limited activity overall.¹⁵¹ Low retinoid sensitivity in a number of tumors, including melanoma, is thought to be due to RARb2 promoter methylation and decreased expression. LAQ824/CRA-

Table 3. — Genes Upregulated in Various Tumor Histologies With HDAC Inhibitors (continued)

Gene	HDAC Inhibitor	References	Mechanistic Information
<i>MMP2</i>	VPA	Milutinovic et al ¹³⁰	
NOXA	TSA, butyrate	Bandyopadhyay et al ¹²³	p53-dependent apoptosis
OSMRβ	TSA	Lacrouette et al ¹³⁷	Expression of OSMRbeta allows it to activate the signal transducer and activator of transcription 3 (STAT3) and inhibit proliferation
<i>p16</i>	VPA, depsipeptide	Valentini et al, ¹³⁸ Wu et al ¹³⁴	Depsipeptide decreased both CpG and H3K9 methylation of the promoter
<i>p19</i>	TSA, SAHA	Chinnaiyan et al, ¹³⁹ Moore et al ¹⁴⁰	
<i>p21WAF1/CIP1</i>	TSA, SAHA, depsipeptide, MS-275	Glaser et al, ¹⁴¹ Moore et al, ¹⁴⁰ Lee et al, ¹²⁸ Sasakawa et al ¹⁴²	Upregulation through activation of Sp1 sites of p21 promoter following HDAC inhibitor treatment even in those not constitutively expressing p21; lack of p21 in about 30% of all melanoma biopsies ¹⁴³ ; p21 upregulation independent of p53
<i>p57</i>	TSA	Moore et al ¹⁴⁰	
<i>PRDX1</i>	SAHA, depsipeptide	Peart et al ¹²⁵	
PUMA	TSA, butyrate	Bandyopadhyay et al ¹²³	p53-dependent apoptosis
<i>QPCT</i>	VPA	Milutinovic et al ¹³⁰	
RAP1A	SAHA, depsipeptide	Peart et al, ¹²⁵ Kobayashi et al ¹²⁶	
RARβ2	LAQ824+CRA,TSA	Kato et al, ¹⁴⁴ Ou et al ¹³³	
RB1CC1	Depsipeptide	Kobayashi et al ¹²⁶	
S1P-1,-3	TSA	Koh et al ¹⁴⁵	S1P (S1P-1,-2 and -3) inhibits cell migration; upon treatment with TSA, S1P stimulates cell migration of A2058 cells
<i>SALL3</i>	Depsipeptide	Wu et al ¹³⁴	Depsipeptide decreased both CpG and H3K9 methylation of the promoter
<i>SFRP1,2</i>	SIRT1	Pruitt et al ¹³²	
<i>SPINK2</i>	VPA	Milutinovic et al ¹³⁰	
Survivin	SAHA, VPA	Facchetti et al ¹⁴⁶	When treated with VPA, survivin is upregulated in VPA-resistant cells and downregulated in VPA-sensitive melanoma cells
TAP1,2	TSA, VPA	Khan et al ¹³¹	
Tapasin	TSA, VPA	Khan et al ¹³¹	
TFIIB	Depsipeptide	Kobayashi et al ¹²⁶	
<i>TNF</i>	SAHA, depsipeptide	Peart et al ¹²⁵	
TNFAIP6	Depsipeptide	Kobayashi et al ¹²⁶	
<i>TNFRSF9</i>	TSA, depsipeptide	Moore et al, ¹⁴⁰ Sasakawa et al ¹⁴²	
<i>TNFRSF12A</i>	SAHA, depsipeptide	Peart et al ¹²⁵	
<i>TNFSF9</i>	TSA, SAHA, depsipeptide	Moore et al, ¹⁴⁰ Peart et al ¹²⁵	
<i>TNFSF10B</i>	TSA	Moore et al ¹⁴⁰	
TRAIL-R1,R2	SAHA, VPA	Facchetti et al ¹⁴⁶	
<i>TUBA</i>	TSA, SAHA, MS-275	Glaser et al ¹⁴¹	
<i>WIF1</i>	VPA	Milutinovic et al ¹³⁰	

Genes in italics are described only for tumor histologies other than melanoma.

* Specifies related HDAC inhibitor, reference, and mechanistic information.

induced apoptosis was associated with generation of reactive oxygen species and induction of SM22 gene expression, while p21 was increased in both cell lines. Similarly, HMV-1 xenograft responded better to in vivo treatment than did A2058. The results indicate variability with the combination therapy; however, each cell line exhibited between 60% and 80% growth inhibition. Therefore, HDAC inhibitors such as the hydroxamate LAQ824 may serve to sensitize cancerous cells to retinoid treatment while enhancing retinoid effects.

Clinical Applications

MS-275 is an HDAC1/3 inhibitor that has been shown to be competitive with cytotoxic agents like 5-fluorouracil in regard to diminishing tumor growth in nude mice. In a recent phase I clinical trial testing oral MS-275 in patients with refractory solid tumors or lymphomas, 2 patients showed partial remissions and 6 showed prolonged disease stabilization.¹³⁵ One metastatic melanoma patient on the lowest dose regimen had disappearance of all but one small nodal metastasis. Overall, the drug was

Table 4. — Genes Downregulated in Various Tumor Histologies With HDAC Inhibitors

Gene	HDAC Inhibitor	References	Mechanistic Information
BCL2	TSA, butyrate	Bandyopadhyay et al, ¹²³ Flørenes et al ¹²²	
Bcl-XL	TSA, SAHA, SBHA, depsipeptide	Moore et al, ¹⁴⁰ Peart et al, ¹²⁵ Flørenes et al ¹²² Facchetti et al, ¹⁴⁶ Zhang et al ¹²¹	Antiapoptotic
Bcl-Xs	SAHA	Facchetti et al ¹⁴⁶	Proapoptotic
<i>bFGF</i>	SAHA, VPA, MS-275, suramin	Hess-Stumpp et al, ¹²⁹ Dua et al ¹⁴⁷	
<i>BST2</i>	SAHA, depsipeptide	Peart et al ¹²⁵	
<i>CD3E,G</i>	SAHA, depsipeptide	Peart et al ¹²⁵	
<i>CDK2AP1</i>	SAHA, depsipeptide	Peart et al ¹²⁵	
<i>Cyclin A2</i>	TSA, depsipeptide	Moore et al, ¹⁴⁰ Sasakawa et al ¹⁴²	
<i>Cyclin B2</i>	TSA, SAHA, depsipeptide	Lee et al, ¹²⁸ Peart et al ¹²⁵	
Cyclin-E	VPA	Valentini et al ¹³⁸	
E-CADHERIN	TSA, butyrate	Ou et al, ¹³³ Kuwajima et al ¹³⁶	Butyrate increased cell-cell aggregation with downregulation of cadherin and upregulation of CAM
<i>HDAC4</i>	SAHA, depsipeptide	Peart et al ¹²⁵	
<i>HIF1</i>	SAHA, VPA, MS-275	Bolden et al, ¹⁴⁸ Chinnaiyan et al, ¹³⁹ Hess-Stumpp et al ¹²⁹	
<i>IGF</i>	Suramin	Dua et al ¹⁴⁷	
<i>IL8</i>	SAHA, depsipeptide	Peart et al ¹²⁵	
Ku70,80	SAHA	Munshi et al ¹²⁷	
LEF1	TSA, SAHA, butyrate, LBH589	Yokoyama et al ¹⁴⁹	
MCL1	SBHA	Zhang et al ¹²¹	
M-MITF (melanocyte-specific MITF)	TSA, SAHA, butyrate, LBH589	Yokoyama et al ¹⁴⁹	
<i>MYC</i>	SAHA, depsipeptide	Peart et al ¹²⁵	
<i>PDGF</i>	Suramin	Dua et al ¹⁴⁷	
<i>RAD21,51L3</i>	SAHA, depsipeptide	Peart et al ¹²⁵	
RAD50	SAHA	Munshi et al ¹²⁷	
S1P-2	TSA	Koh et al ¹⁴⁵	S1P (S1P-1, -2, and -3) inhibits cell migration; upon treatment with TSA, S1P stimulates cell migration of A2058 melanoma cells
SOX10	TSA, SAHA, butyrate, LBH589	Yokoyama et al ¹⁴⁹	
Survivin	SAHA, VPA	Facchetti et al ¹⁴⁶	When treated with VPA, survivin is upregulated in VPA-resistant cells and downregulated in VPA-sensitive melanoma cells
<i>TIE2</i>	SAHA, VPA, MS-275	Hess-Stumpp et al ¹²⁹	
<i>TNFRSF8,14</i>	SAHA, depsipeptide	Peart et al ¹²⁵	
<i>TNFSF13</i>	TSA, SAHA, MS-275	Glaser et al ¹⁴¹	
<i>TP53</i>	SAHA, depsipeptide	Peart et al ¹²⁵	
<i>VEGF</i>	SAHA, VPA, depsipeptide, MS-275, suramin	Sasakawa et al, ¹⁴² Dua et al, ¹⁴⁷ Lafon-Hughes et al ⁷³	
XIAP	SBHA	Zhang et al ¹²¹	

Genes in italics are described only for tumor histologies other than melanoma.

well tolerated and did not exhibit any significant long-term toxicity. Combination treatment of the SIRT1/2 selective inhibitor suramin with the xanthine derivative pentoxifylline has been shown to synergistically inhibit antitumor and antimetastatic activity in B16F10 melanoma cells.¹⁴⁷ Coordinated suppression of matrix-metalloprotease 9 (MMP-9) was also observed. Pentoxifylline is a tumor necrosis factor-alpha (TNF- α) inhibitor used clinically to improve patient blood flow. Suramin is a potent inhibitor of proangiogenic proteins such as PDGF and VEGF, and its antiproliferative effectiveness in culture has led to clinical trials for various malignant cancers. Due to its broad range toxicity at effective doses in single-agent clinical trials, suramin has been utilized in combination therapy as a chemosensitizing agent.^{111,147}

VPA has rapidly become an important HDAC inhibitor in the clinical arena. It exhibits antitumor activ-

ity in combination with epirubicin, an anthracycline chemotherapeutic agent, in patients with solid tumors despite anthracycline resistance.¹⁵² A phase I trial using this combination in patients with advanced solid tumors has recently been completed (Table 5). VPA has induced dose-dependent G₀/G₁ phase arrest, apoptosis, and sensitization to cisplatin and etoposide in M14 human melanoma cells.¹³⁸ In addition, the cell cycle regulating tumor suppressor p16INK4A, either mutated or with homozygous deletion in 75% melanoma cell lines and associated with late-stage melanoma, was shown to be increased following VPA treatment, correlating with increased senescence of M14 cells. VPA plus the ribonucleotide reductase inhibitor hydroxyurea have been shown to synergistically induce apoptosis in human melanoma cell line SK-Mel-37 via a p21, p27, and caspase-3 influenced mechanism.¹⁵³

Table 5. — Current HDAC Inhibitor Clinical Trials for Patients With Melanoma or Other Solid Tumors

HDAC Inhibitor	Combination	Title	Phase	Malignancy	Sponsors	Stage	Identifier
VPA	Azacytidine (DNMTi)	Azacytidine and Valproic Acid in Patients With Advanced Cancers	I	Metastatic or nonresectable refractory tumors	M. D. Anderson Cancer Center, Celgene Corporation	Ongoing	NCT00496444
VPA	Hydralazine (Aprisoline)	Phase II Study of Epigenetic Therapy to Overcome Chemotherapy Resistance in Refractory Solid Tumors	II	Refractory solid tumors	National Institute of Cancerologia, Psicofarma S.A.DE C.V. Conacyt	Completed	NCT00404508
VPA	Epirubicin (anthracycline) 5-fluorouracil Cyclophosphamide	Phase I Trial of Valproic Acid and Epirubicin in Solid Tumor Malignancies	I	Advanced solid tumors	H. Lee Moffitt Cancer Center & Research Institute, National Cancer Institute (NCI), Pfizer	Completed	NCT00246103
VPA	Dosing: sunitinib, sorafenib, dasatinib, erlotinib, lapatinib, and lenalidomide	Valproic Acid-Based 2-Agent Oral Regimens for Patients With Advanced Solid Tumor	I	Advanced solid tumors	M. D. Anderson Cancer Center	Recruiting	NCT00495872
VPA	Bevacizumab (VEGF-mAb)	Valproic Acid and Bevacizumab in Patients With Advanced Cancer	I	Metastatic or nonresectable solid tumors	M. D. Anderson Cancer Center	Recruiting	NCT00530907
MS-275	–	Safety and Efficacy Study of a New Chemotherapy Agent to Treat Metastatic Melanoma	II	Nonresectable metastatic melanoma (stage IV)	Bayer Schering Pharma AG, Germany	Completed	NCT00185302
MS-275	–	MS-275 in Treating Patients With Advanced Solid Tumors or Lymphoma	I	Nonresectable or metastatic solid tumors; lymphoma	National Cancer Institute (NCI)	Completed	NCT00020579
MS-275	Isotretinoin (Retinoid)	MS-275 and Isotretinoin in Treating Patients With Metastatic or Advanced Solid Tumors or Lymphomas	I	Metastatic, progressive, refractory, or nonresectable solid tumors; lymphoma	Sidney Kimmel Comprehensive Cancer Center, National Cancer Institute (NCI)	Completed	NCT00098891

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Given VPA's pan-selectivity and low potency, a new VPA-derived class of HDAC inhibitors has been described with micromolar antiproliferative activity, as opposed to VPA millimolar potency, and has been based on current knowledge of organosulfur moieties that increase HDAC inhibition.¹⁵⁴ Largazole, a natural depsipeptide with a "masked" thiol group, is a novel prodrug for its "unmasked largazole thiol" form. The largazole thiol has potency for HDAC-1, -2, and -3 in the picomolar range, as exhibited in a panel of malignant melanoma cell lines.¹⁵⁵ It appears that largazole is a potent agent, with only its close relative, FK228, being near this therapeutic range. The development of such potent and selective drugs could lead to decreased patient dosing and diminished side effects. Dehmel et al¹⁵⁶ are currently pursuing novel trithiocarbonates as competitive, potent HDAC6-selective inhibitors.

FK228 has shown significant tumor growth inhibition *in vitro* and *in vivo* in human melanoma cell lines

through the Rap1-mediated suppression of the Ras-Raf-MEKK1/2-ERK1/2 pathway.¹²⁶ It is currently being examined in several clinical trials, including a phase II trial for patients with nonresectable stage III or IV melanoma (Table 5). More recently, Murakami et al¹⁵⁷ showed a marked upregulation of the cell surface antigen gp100/pm117 and caspases 3 and 7 in human melanoma cell lines following FK228 treatment, correlating with an increase in p21WIF1/CIP1 expression. Furthermore, Fas death receptor expression was induced in murine B16/F10 melanoma cells while major histocompatibility class I (H-2D) antigen increased in B16/F10 as well as human melanoma cell lines, sensitizing the B16/F10 cells to CTL-mediated killing. Campoli and Ferrone¹⁵⁸ and Khan et al^{131,159} have similarly described how HDAC inhibitors can induce the expression of HLA class I and II cell-surface antigens and costimulatory molecules in tumor cells, including melanoma.

Table 5. — Current HDAC Inhibitor Clinical Trials for Patients With Melanoma or Other Solid Tumors (continued)

HDAC Inhibitor	Combination	Title	Phase	Malignancy	Sponsors	Stage	Identifier
SB939	—	SB939 in Treating Patients With Locally Advanced or Metastatic Solid Tumors	I	Locally advanced or metastatic solid tumors	National Cancer Institute of Canada	Recruiting	NCT00504296
SB939	—	Phase I Dose Escalation Study of Oral SB939	I	Locally advanced or metastatic solid tumors, advanced hematologic malignancies	S*Bio	Recruiting	NCT00741234
MGCD0103	—	Phase I/II Study of MGCD0103 (MG-0103) in Combination With Gemcitabine	I	Advanced refractory solid tumors	Celgene Corporation, MethylGene Inc.	Completed	NCT00372437
CUDC-101	—	A Phase I Study of the Safety, Pharmacokinetics, and Anti-Tumor Activity of CUDC-101 in Patients With Advanced Solid Tumors	I	Advanced refractory solid tumors	Curis, Inc.	Recruiting	NCT00728793
LBH589	—	Dose-Escalating Study of LBH589 in Adult Patients With Advanced Solid Tumors	I	Advanced solid tumors	Novartis	Recruiting	NCT00739414
CHR-3996	—	Safety Study of the Histone Deacetylase Inhibitor, CHR-3996, in Patients With Advanced Solid Tumours	I	Malignant solid refractory tumors	Chroma Therapeutics	Recruiting	NCT00697879
FR901228 (FK228)	—	FR901228 in Treating Patients With Unresectable Stage III or Stage IV Malignant Melanoma	II	Nonresectable intraocular melanoma or melanoma of the skin (stage III or IV)	Eastern Cooperative Oncology Group, National Cancer Institute (NCI)	Ongoing	NCT00104884

This list was compiled from www.clinicaltrials.gov with specific "identifiers."

On the other hand, the human melanoma oncogene M-MITF (melanocyte-specific MITF) was down-regulated via TSA, sodium butyrate, SAHA, and LBH589 treatment of melanoma cells.¹⁴⁹ Systemic LBH589 treatment of mouse UACC62 melanoma xenograft models decreased tumor growth and M-MITF mRNA levels while the models showed drug tolerance. Interestingly, Dashwood and Ho¹⁶⁰ describe the presence of a potent, natural isothiocyanate HDAC inhibitor, sulforaphane, in cruciferous vegetables such as broccoli. They provide evidence that a moderate intake of broccoli sprouts can induce histone hyperacetylation, observable in circulating blood, above what one would receive from such classic HDAC inhibitors as SAHA. Bruserud et al¹⁶¹ have noted that the long-term toxic effects for many HDAC inhibitors are not widely known, with light to severe side effects ranging from gastrointestinal and neurologic (nausea, vomiting, and anorexia) effects to hematologic (thrombocytopenia, neutropenia), metabolic, and cardiovascular problems. Despite the presence of such side effects, many patients have seen reversion of tumor growth and increased lifespan. This is an exciting time for epigenetic research and therapy. The level of understanding of the molecular mechanisms behind HDAC inhibition and selectivity continues to grow, and the horizon is becoming brighter with the imposing dawn of epigenetic therapy for cancer.

Epigenetics of MicroRNA in Melanoma

MicroRNAs (miRNAs) are endogenous ≈22-nt noncoding RNAs. They play a key role in regulating gene expression by interacting with mRNA — either by inhibiting mRNA translation¹⁶²⁻¹⁶⁵ or by causing mRNA degradation.¹⁶⁶⁻¹⁶⁸ There are currently hundreds of confirmed miRNAs in humans, and computational predictions suggest that the total count might be more than 1,000.¹⁶⁹ The regulatory nature of miRNAs, combined with the large number of presumptive target genes, suggests that they are essential regulators of a wide range of cellular processes. Recent evidence is emerging that particular miRNAs may play an important role in human cancer epigenetic pathogenesis.

It is important to understand the basic molecular mechanisms involved in miRNA-mediated gene silencing. Briefly, miRNA is originally transcribed by RNA polymerase II as a long primary miRNA (pri-miRNA).¹⁷⁰ It is then processed into a 60–70 nucleotide miRNA precursor (pre-miRNA) by Drosha, a member of nuclear RNase III family. The pre-miRNA is transported from the nucleus to the cytoplasm by RanGTP/exportin5, where it is subsequently cleaved by DICER to generate 20- to 22-nucleotide duplexes. Generally, only one strand of the duplex serves as mature miRNA.^{171,172} Single-strand miRNA is incorporated into a ribonucleoprotein effector complex known as the RNA-induced silencing complex

(RISC). This complex identifies target messages based on complementarities between the “guide” miRNA and the mRNA and results in either endonucleolytic cleavage of targeted mRNA or translational repression.¹⁷³⁻¹⁷⁵

Most recently, researchers have shown that miRNAs is not only an inactivating factor for translation. It plays a more diverse role in the regulation of gene expression.¹⁷⁶ Li et al¹⁷⁷ identified several double-stranded RNAs (dsRNAs) that activate E-cadherin, p21(WAF1/CIP1) (p21), and VEGF gene expression by targeting noncoding regulatory regions in gene promoters. They reported synthesized 21-nt dsRNAs targeting selected promoter regions of human genes that, when transfected into human cell lines, resulted in long-lasting and sequence-specific induction of the target genes. Ørom et al¹⁷⁸ also found that miRNA miR-10a interacts with the 5′ untranslated region of ribosomal protein mRNAs and enhances their translation.

Since miRNA-mediated regulation can affect the expression of hundreds of genes on several chromosomes, unique patterns of altered miRNA expression provide complex fingerprints that may serve as diagnostic markers for tumorigenesis.^{176,177} A few studies have examined the miRNA profiles within melanoma cell lines¹⁷⁹ or tumor samples.^{180,181} Zhang et al¹⁸¹ examined 45 primary cultured melanoma cell lines by array comparative genomic hybridization (aCGH) and observed that many genomic loci are frequently affected (85.9%) by copy number abnormalities in melanoma cells that contain miRNA-coding sequences. Among the NCI-60 cell lines examined by Gaur et al,¹⁷⁹ the melanoma cell lines clustered into an independent terminal branch based on miRNA expression.

Increasing evidence shows that expression of miRNA genes is deregulated in human cancer, thus epigenetically adjusting their target gene mRNA expression accordingly.¹⁸²⁻¹⁹⁰ Specific overexpression or underexpression has been shown to correlate with particular tumor histologies, with miRNA overexpression resulting in downregulation of TSGs, whereas their underexpression could lead to oncogene upregulation.¹⁸²⁻¹⁸⁵ For example, let-7, downregulated in lung cancer, suppresses Ras,¹⁸⁶ while mir-15 and mir-16, deleted or downregulated in leukemia, suppress BCL2,¹⁸⁷ and mir-17-5p and mir-20a control the balance of cell death and proliferation driven by the proto-oncogene c-Myc.¹⁸⁸ Clear evidence indicates that miRNA polycistron mir-17-92 serves as an oncogene in lymphoma¹⁸⁹ and lung cancer¹⁹⁰; mir-372 and mir-373 are novel oncogenes in testicular germ cell tumors and act by neutralizing p53-mediated cyclin-dependent kinase (CDK) inhibition, possibly through direct inhibition of the expression of the tumor-suppressor LATS2.¹⁹¹

Several miRNAs have been identified as playing a key role in human melanoma epigenetic pathogenesis. Bemis et al⁵⁶ reported the expression of mature miR-

137 in melanoma cell lines capable of downregulating MITF expression in melanoma, with MITF previously shown to be a master regulator of melanocyte development, survival, and function.^{98,192,193} They further identified a 15-bp variable nucleotide tandem repeat sequence, which alters the processing and function of miR-137 in melanoma cell lines.⁵⁶ Integrin $\beta(3)$ is known to play an important role in melanoma progression and invasion. Müller et al¹⁹³ determined miRNA let-7a to be an important regulator of integrin $\beta(3)$ expression. The repressed expression of integrin $\beta(3)$ accompanies reduced invasive potential of melanoma cells transfected with synthetic let-7a molecules, as observed in Boyden chamber assays.

On the other hand, the induction of integrin $\beta(3)$ expression was achieved in melanocytes by transfection with let-7a anti-miRs, resulting in invasive behavior of transfected melanocytes.¹⁹³ Therefore, the loss of let-7a expression is involved in the development and progression of melanoma. Schultz et al¹⁹⁴ found that members of the let-7 family of miRNAs were significantly downregulated in primary melanomas compared with benign nevi. Overexpression of let-7b in melanoma cells in vitro downregulated the expression of cyclins D1, D3, and A, as well as cyclin-dependent kinase (Cdk) 4, all of which have been described to play a role in melanoma development. The effect of let-7b on protein expression is due to targeting of 3'-untranslated regions (3'-UTRs) of individual mRNAs. In line with its downmodulating effects on cell cycle regulators, let-7b inhibited cell cycle progression and anchorage-independent growth of melanoma cells. One of these, miR-370, is embedded in a CpG island. Although 5-aza-CdR increased miR-370 expression by 2.1-fold in malignant cells, the expression in nonmalignant cells was unchanged.

The transcriptional regulation of miRNA expression has recently been examined in tumors, with one mechanism identified as the epigenetic modification of DNA via methylation and/or HDAC. Saito et al¹⁹⁵ demonstrated that the induction of a subset of miRNAs was followed by the inhibition of DNA methylation and HDAC. One of these miRNAs — miR-127, located within a CpG island — is generally downregulated in most cancer cells compared with corresponding normal cells. MiR-127 was found to be upregulated following treatment with chromatin-modifying drugs, while the target gene CL6 was translationally repressed.¹⁹⁵ Meng et al¹⁹⁶ reported that another CpG island-embedded miRNA, miR-370, showed IL-6-driven methylation regulation in cholangiocarcinoma cells. The authors demonstrated that IL-6 can enhance the growth of cholangiocarcinoma cells by repressing the expression of miR-370 epigenetically. The demethylation agent 5-aza-2'-deoxycytidine had an opposite effect on the expression of miR-370 but only in malignant cells. Among its predicted targets, the oncogene MAP3K8 was identified, which may explain the

altered growth of tumor cells in this context. These data illustrate the complex network involving an inflammation-associated cytokine, DNA methylation, expression of miRNA, and its target protein-coding gene.¹⁹⁶

In lung adenocarcinoma, Brueckner et al¹⁹⁷ reported that the let-7a-3 locus is generally hypomethylated and also that its expression can be epigenetically modulated. We were unable to find any reports describing the regulation of miRNA expression and DNA demethylation/HDAC inhibitor drugs in melanoma, although these two agents are undergoing studies for potential therapeutic efficacy in a variety of solid tumors, including melanoma. However, a group has identified the promyelocytic leukemia zinc finger (PLZF) transcription factor as a repressor of miR-221 and miR-222 by direct binding to their putative regulatory region in melanoma. Specifically, PLZF silencing in melanomas unblocks miR-221 and miR-222, which in turn controls the progression of the neoplasia through downmodulation of p27Kip1/CDKN1B and c-KIT receptor, leading to enhanced proliferation and differentiation blockade of the melanoma cells, respectively. In vitro and in vivo functional studies confirmed the key role of miR-221/-222 in regulating the progression of human melanoma, thus suggesting that targeted therapies suppressing miR-221/-222 may prove beneficial in advanced melanoma.¹⁹⁸

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