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PATHOBIOLOGY IN FOCUS

Melanoma epigenetics: novel mechanisms, markers, and medicines

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The incidence and mortality rates of cutaneous melanoma continue to increase worldwide, despite the deployment of targeted therapies. Recently, there has been rapid growth and development in our understanding of epigenetic mechanisms and their role in cancer pathobiology. Epigenetics—defined as the processes resulting in heritable changes in gene expression beyond those caused by alterations in the DNA sequence—likely contain the information that encodes for such phenotypic variation between individuals with identical genotypes. By altering the structure of chromatin through covalent modification of DNA bases or histone proteins, or by regulating mRNA translation through non-coding RNAs, the epigenome ultimately determines which genes are expressed and which are kept silent. While our understanding of epigenetic mechanisms is growing at a rapid pace, the field of melanoma epigenomics still remains in its infancy. In this Pathology in Focus, we will briefly review the basics of epigenetics to contextualize and critically examine the existing literature using melanoma as a cancer paradigm. Our understanding of how dysregulated DNA methylation and DNA demethylation/hydroxymethylation, histone modification, and non-coding RNAs affect cancer pathogenesis and melanoma virulence, in particular, provides us with an ever-expanding repertoire of potential diagnostic biomarkers, therapeutic targets, and novel pathogenic mechanisms. The evidence reviewed herein indicates the critical role of epigenetic mechanisms in melanoma pathobiology and provides evidence for future targets in the development of next-generation biomarkers and therapeutics.
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EPIGENETICS: A RENAISSANCE IN CANCER PATHOBIOLOGY

Billions of dollars have been invested into our understanding of classic human genetics and its influence on phenotype and disease. Yet, variations in the DNA sequence alone do not explain the subtle phenotypic differences observed between monozygotic twins nor can they completely explain the pathobiologic differences that separate benign from malignant cellular proliferations. Epigenetics—defined as the molecular mechanisms that regulate heritable changes in gene expression without causing any changes to the DNA sequence—provides key insights into the underpinnings of such phenotypic, morphologic, and pathobiologic differences. By altering the structure of chromatin through covalent modification of DNA bases or histone proteins or by regulating mRNA translation through non-coding RNAs (ncRNA), the epigenome reserves ultimate determination over which genes are expressed and which are kept silent. This 'higher level' of gene regulation may even provide a mechanistic link between how factors such as the environment, gender, and aging influence our individual phenotype as well as our own unique susceptibilities to cancers such as melanoma, a prototype of an aggressive human malignancy.

One key difference between the genome and the epigenome is that the latter may potentially be more therapeutically reversible than mutations affecting the genetic code itself. Given that distinct subsets of malignant melanoma are driven by heterogeneous genetic mutations, this virulent form of human cancer is a prime example for examining the interplay between genetic and epigenetic events. Despite the deployment of therapies directed at specific genomic mutations in melanoma, the incidence and mortality rates from this deadly disease continue to increase worldwide—faster than that of any other potentially preventable cancer. Our understanding of how dysregulated DNA methylation and DNA demethylation/hydroxymethylation, and histone modification, as well as ncRNAs, affect cancer pathogenesis and melanoma virulence, in particular, is growing at a rapid pace and provides us with an ever-expanding repertoire of potential diagnostic biomarkers, therapeutic targets, and novel pathogenic mechanisms. We believe that this flourishing body of evidence points strongly

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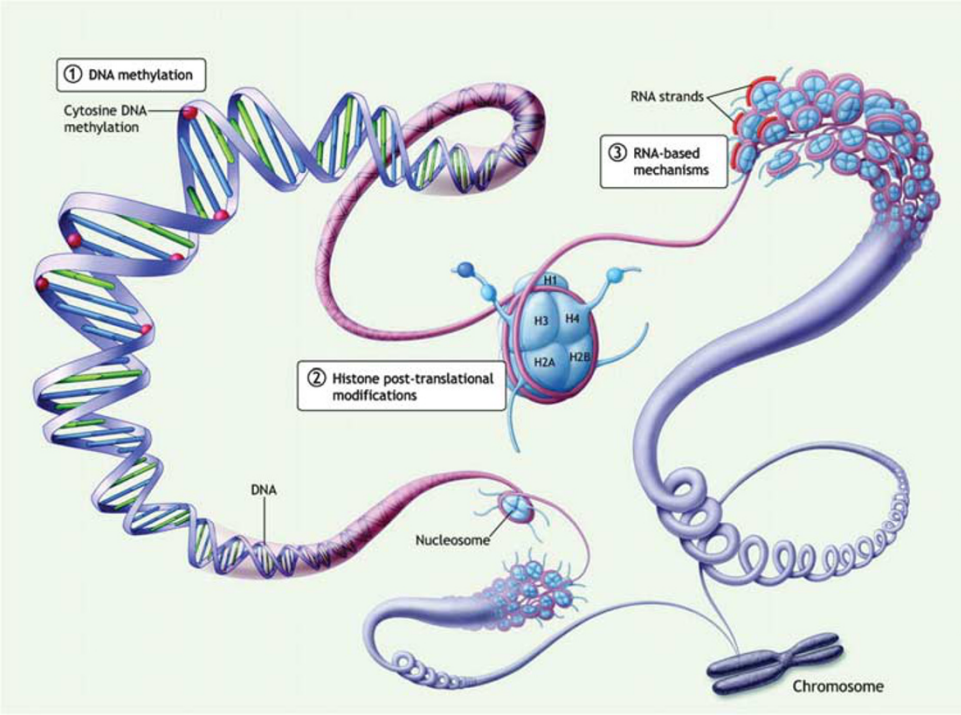

Figure 1 Summary of the three primary epigenetic mechanisms. (1) DNA methylation. (2) Histone posttranslational modifications. (3) RNA-based mechanisms, including miRNAs and large non-coding RNAs (lncRNAs). *Note*: This diagram does not illustrate its mechanisms of binding and silencing mRNAs. Reprinted with permission from Lippincott Williams & Wilkins.

towards prioritization of the cancer epigenome over a solely genome-centric viewpoint when considering the best translational approaches to virulent cancers like melanoma. In this Pathobiology in Focus, we provide a brief overview of the current understanding of epigenetic mechanisms with special attention to the cancer epigenome in melanoma, and explore the direct diagnostic and therapeutic implications and applications of these novel insights. It is critical to unravel and harness the immense power of the epigenome and direct its further clinical application in the setting of personalized medicine, particularly for cancers like melanoma, where existing diagnostic and therapeutic strategies all too often fall short.

EPIGENETICS: FOUNDATION AND PRINCIPLES

First introduced by English biologist Conrad Waddington in 1939, the term 'epigenetics' is derived from the *Greek* 'epigenesis,' connoting 'changes in gene activity during development.'[1] During a time when genetics and developmental biology were studied independently, Waddington and others stressed the critical relationship between these two emerging fields.[2] Soon it became clear that fundamental features of embryology and development demanded explanation beyond that provided by the genetic 'code.' One, for instance, was how pluripotent cells could differentiate into specialized cells, such as fibroblasts and lymphocytes, and despite sharing identical genotypes, stably maintain their distinct biologic phenotypes through generations of cell division.[1,3]

Historically, observations that were not easily explained through genetic terms but had a heritable component were considered to be 'epigenetic' phenomena. As we understand it today, however, 'epigenetics' refers more precisely to the molecular mechanisms whereby gene expression is reversibly modified in a heritable manner without changes in the DNA sequence. Such mechanisms enable the differentiation of embryonic and adult stem cells, as well as the dedifferentiation and acquisition of pluripotency by somatic cells, potentially as a consequence of environmental stimuli and cues. Moreover, epigenetic mechanisms are also likely to contribute to the development and function of self-renewing 'cancer stem cells' (CSCs). Epigenetic regulation of gene expression occurs by altering the structure and conformation of chromatin, thereby affecting the ability of transcriptional machinery to access genes and their promoters, as well as by affecting the stability of mRNA transcripts. The principal epigenetic mechanisms include DNA methylation, histone modifications, and ncRNA regulation, and we will briefly review their principles here (Figure 1). The discussion focusing on epigenetic alterations in melanoma will begin under the header 'Epigenetics and Melanoma.'

DNA Methylation and Hydroxymethylation

In 1975, the first suggestion that DNA methylation could exert strong effects on gene expression came from two groups working independently to uncover the 'molecular switch' that turned genes on or off during development.[4,5] That 'switch'

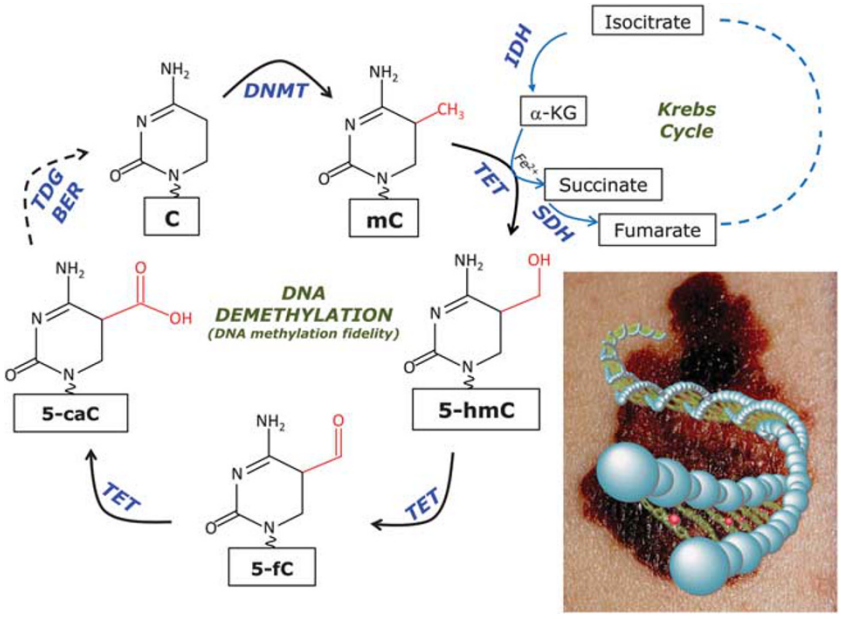

Figure 2 Active DNA demethylation pathway. The pathway involved in the Ten Eleven Translocase (TET)-dependent generation of 5-hydroxymethylcytosine (5-hmC), an epigenetic mark that is lost in melanoma (pictorially rendered to lower right). DNMT, DNA methyltransferase; TDG, thymine DNA glycosylase; BER, base excision repair; IDH, isocitrate dehydrogenase; SDH, succinate dehydrogenase.

was once thought to be DNA methylation, which occurs at the carbon-5 position of cytosine to form 5-methylcytosine (5-mC), otherwise known as the 'fifth base.'[6] Today, it is understood that this methylation does not constitute a simple 'switch' and that multiple additional tightly orchestrated epigenetic mechanisms cooperate to silence or activate genes in a context and site-specific manner. However, DNA promoter methylation is known to be critical for stabilizing the silent state of certain genes within terminally differentiated somatic cells. Here, this methylation is thought to act as a target for binding proteins that, together, prevent the reactivation of potentially deleterious germline and pluripotency genes.[7]

DNA methylation occurs on cytosine residues preceding guanine on the ipsilateral strand (thus forming a 'CpG' dinucleotide pair, wherein 'p' signifies the phosphodiester bond linking the two). CpG dinucleotide pairs are known to exist in regions enriched in CpG repeats (0.5–4 kb in length) called 'CpG islands.'[8] CpG islands are present in or near approximately 40% of mammalian gene promoters,[9] making them prominent targets for methylation, although recent studies may suggest otherwise.[7] Current understanding, however, indicates that while promoter methylation is associated with gene silencing,[10] methylation within gene bodies correlates positively with transcription.[11] Despite significant advances in our knowledge of how CpG dinucleotide and CpG island methylation influence gene expression, the precise mechanisms through which this occurs remains incomplete. However, it is known that the enzymatic methylation of cytosine is performed by DNA methyltransferases (DNMTs),[12] of which our genomes have encoded several that perform similar functions in different biological contexts.[13,14] Their function is critical during development and cellular differentiation, as well as for the faithful intergenerational propagation of specific methylation patterns, genomic imprinting, transcriptional repression of retrotransposons in both germ and somatic cells, and X-chromosome inactivation, among others.

In contrast to DNA methylation, the mechanisms underlying DNA *de*methylation are even less well understood.[15] DNA demethylation has been known to occur *passively*, specifically by the programmed failure to transmit a certain methylation pattern during a round of cell division.[15] However, *active* DNA demethylation in mammalian systems has only very recently been recognized, with accumulating evidence indicating that this occurs through the sequential, iterative oxidation of the methyl group of 5-mC and removal of the final modified group by thymine DNA glycosylase and the base excision repair pathway to yield cytosine from 5-mC (Figure 2).[15] The first and most critical step of this reaction involves oxidation of 5-mC to 5-hydroxymethylcytosine (5-hmC), which is performed by the Ten Eleven Translocase (TET) family dioxygenase enzymes.[16] Originally discovered as a human homolog of an enzyme present in *Trypanosoma cruzi*, the TET family proteins were demonstrated to be 2-oxoglutarate (or α-ketoglutarate, α-KG), iron (II)-dependent oxidases that catalyze this initial oxidation step.[17] 5-hmC is the most abundant intermediate of the active DNA demethylation pathway[18,19] and its content directly correlates with the level of differentiation in a wide

variety of human tissues.[20] In addition, both 5-hmC and TET expression/activity are tightly regulated during embryonic stem cell differentiation.[21,22]

Given its ability to initiate the removal of DNA methyl groups, TET has a putative role in maintaining DNA methylation fidelity by enabling DNA demethylation 'repair,'[23] which has earned it the epithet 'guardian of CpG islands.'[24] This would suggest that loss of TET function may have dire biologic consequences. Indeed, TET has been shown to be the most frequently mutated gene in myelodysplastic syndrome and tightly associate with reduced overall survival[25] and that its loss increases the self-renewal capacity of hematopoietic stem cells, leads to their eventual myeloproliferation.[26] Furthermore, the loss of 5-hmC has also been very recently documented in a number of solid malignancies, including breast cancer,[27] oral squamous cell carcinoma,[28] gastrointestinal stromal tumor,[29] and hepatocellular carcinoma,[30] among others. Given these observations and TET's close functional relationship to other epigenetic mechanisms,[31] one may speculate as to whether TET functions more globally as a 'guardian of the epigenome.' Understanding the precise cellular function of the TET family enzymes and the biologic significance of 5-hmC loss and dysregulated DNA demethylation is currently a high priority in cancer biology research.

In addition to TET, evidence also suggests a role for dysfunction of the Krebs cycle enzymes in DNA methylation/hydroxymethylation dysregulation. Isocitrate dehydrogenase (IDH) produces a critical co-factor, α-ketoglutarate (α-KG) for TET enzyme function and is also frequently mutated in a number of cancers.[32] Interestingly, IDH mutations not only result in loss of the necessary TET enzyme co-factor but also results in the production of oncometabolite 2-hydroxyglutarate (2-HG).[33] 2-HG competitively inhibits multiple α-KG-dependent enzymes, including the TET family 5-mC hydroxylases, as well as histone demethylases.[34] Mutations in succinate dehydrogenase, particularly in a subset of gastrointestinal stromal tumors, have also been proposed to disrupt TET function through similar mechanisms and, interestingly, are tightly associated with a number of hypermethylated genes.[29,35] Taken together, these data suggest that Krebs cycle and metabolic disarray may be involved in the malignant transformation via loss of TET function and 5-hmC and the hypermethylation of tumor suppressor genes, as will be discussed greater detail below specifically with regard to melanoma.

Chromatin Structure and Histone Modification

While pathologists routinely speak of heterochromatin and hyperchromatic nuclei, it is useful to review the concept of chromatin as a functional DNA scaffold that may respond to external cues and instruct the activity and function of the DNA that it envelops. For over 50 years, we have known that histones may be post-translationally modified. Chromosomal DNA is packaged into nucleosomes with DNA wrapped around highly alkaline histone protein octamers, which consist of subunits (H2A, H2B, H3, and H4) and other variants. Histone modifications may either activate or silence transcription depending on the nature and location of the modification by controlling the accessibility of DNA to the transcriptional machinery and by recruiting or excluding additional protein complexes.[36] At least 130 posttranslational modifications of histone proteins have been identified in human cells, the best studied of which include methylation and acetylation.[37] Histone methylation was thought to be an irreversible process for many years until the first histone demethylase (KDM1A) was discovered in 2004.[38]

The physical association of histone modifications with anatomical segments of the genome is, in part, determined by the specific modifier. For example, acetylation events may be found in active promoters and enhancers (as with H3K27ac: acetylated histone H3 on lysine 27), in transcribed gene bodies (as with H3K36me3: trimethylated histone H3 on lysine 36), or in association with heterochromatic or repressed regions (as with H3K9me3 and H3K27me3).[39] Interestingly, studies have shown that DNMTs physically associate with histone deacetylases, which reverse histone acetylation, as well as with histone methyltransferases,[40] and, together, favor closed chromatin conformations near gene regulatory regions thereby perpetuating 'silent' epigenetic states through multiple generations of cell division.[41] In addition, certain modifications, such as the methylation of H3K9 (histone 3, lysine 9), tightly associates with aberrant heterochromatin formation and silencing of tumor suppressors, such as cyclin-dependent kinase inhibitor 2a (CDKN2a), in cancer cells.[42] Moreover, perturbation of the overall histone profile, or 'histone code,' has been shown to have prognostic relevance in various cancers.[43]

Polycomb group (PcG) proteins are epigenetic repressors that associate with specific posttranslational histone modifications and are essential for the transcriptional regulation of cell differentiation and development.[44,45] PcG-mediated repression is associated with and is thought to involve both biochemical and physical modulation of chromatin structure. Given the recent emergence of complex data that has identified novel components of the PcG proteins and their putative roles in cancer, it is of interest that such histone modifications were actually the last of the three main epigenetic mechanisms to be associated with malignancy.[41]

Regulation of Gene Expression by ncRNAs

While $<2\%$ of the total genomic sequence encodes for proteins, at least 90% of the genome is actively transcribed into ncRNA, otherwise known as the 'dark matter' of the genome.[46] The first eukaryotic ncRNA to be discovered was a large RNA named H19, originally described as having a putative tumor suppressor function in Wilms' tumor[47] and later found to be involved in the process of genomic imprinting.[48] As we understand them today, ncRNAs are a heterogeneous group of RNAs that are generally classified into two groups based on their lengths, ranging anywhere

from 18–25 to 10,000 nucleotides in length.[49] Small ncRNAs are <200 nucleotides in length and within this category, the microRNAs (miRNAs) are the most well studied.[49] miRNAs bind to miRNA response elements contained within their target mRNA transcripts and subsequently recruit the RNA-induced silencing complex, which antagonizes target mRNA stability and/or translation.[50] In this manner, ncRNAs regulate a wide variety of complex cellular processes, including gene silencing, gene transcription, DNA imprinting, DNA demethylation, chromatin structure dynamics, and RNA interference.[51] A number of oncogenic and tumor suppressive ncRNAs, particularly miRNAs, have also been recently described in melanoma and will be discussed in greater detail below.

The second class of ncRNAs that have recently been described are the long ncRNAs (lncRNAs), which range anywhere from 200 nucleotides to ~100 kb.[46] Unlike the miRNAs, lncRNAs bind other protein complexes and also form a secondary structure, although the primary sequence and molecular factors that influence these dynamics remain unknown.[52] However, like the miRNAs, lncRNAs have been implicated in a variety of gene regulatory roles, including chromosome dosage compensation, genomic imprinting, epigenetic regulation, cell cycle control, nuclear and cytoplasmic trafficking, transcription, translation, splicing, and cell differentiation, among others.[52] It has become clear that dysregulation of ncRNAs, including the miRNAs and lncRNAs in particular, are a critical factor in the pathobiology of cancer.

This overview, although limited and rudimentary in the context of a rapidly emerging database, bears testimony to the diversity and pleiotropism inherent to epigenetic mechanisms of gene regulation. We will now apply these concepts to a form of human cancer that serves as a paradigm for clinical virulence, mechanistic complexity, and therapeutic challenge: malignant melanoma.

EPIGENETICS AND MELANOMA

Aberrant DNA Methylation

In 1983, Feinberg and Vogelstein[53] first reported that 'substantial hypomethylation' of CpG dinucleotide was present in human cancer cells. Since this discovery, alterations to DNA methylation throughout the genome have been well documented in cancer. Global genome-wide methylation is now known to be reduced very early in the neoplastic progression of carcinogenesis.[54–56] Teleologically, given the additional cellular and environmental functions required for neoplastic cells to proliferate and eventually metastasize, one may hypothesize that hypomethylation allows previously benign cells to express and experiment with novel gene products to exert a survival advantage. In addition, DNA hypomethylation, specifically in or around centromeric repeats and other repetitive sequences, has been shown to contribute to chromosomal instability.[57] In addition to global hypomethylation, cancer cells concurrently and paradoxically display localized hypermethylation of CpG islands of multiple tumor suppressor genes early in carcinogenesis,[58–60] which has been deemed 'one of the most precocious hits in tumorigenesis.'[60] In general, the tendency toward hypermethylation has been described in multiple human cancers, including melanoma, and has also been termed the 'CpG island methylator phenotype.'[61,62]

The DNA methylation status of cutaneous melanoma has been extensively studied and has been demonstrated to have prognostic and therapeutic significance. Hypermethylation of specific tumor suppressor genes, as well as those involved in cell-cycle regulation, DNA repair, cell signaling, transcription, and apoptosis, have been reproducibly described in cutaneous melanoma.[63] The CDKN2A promoter has been shown to be hypermethylated in a substantial fraction of primary cutaneous melanoma samples and is associated with both increased Ki-67 index and reduced patient survival.[64] Of interest, CDKN2A, which encodes negative regulators of cell cycle progression p16 and p14 and is inactivated in the majority of sporadic cutaneous melanomas, is also the most frequently mutated gene inherited in familial cutaneous melanoma.[65,66] In a study of 86 metastatic melanoma specimens, four tumor suppressor genes were found to be frequently hypermethylated.[67] Retinoic acid receptor-β2 was the most commonly methylated gene in this series (70% in primary and metastatic melanoma specimens),[67] and has also been described to be silenced in multiple other human cancers.[68] RAS association domain family protein 1A (RASSF1A), which is critical for mitochondrial apoptosis and cell cycle arrest, was found to be methylated in 57% of melanoma specimens, O6-methylguanine DNA methyltransferase (MGMT, discussed in greater detail below) in 34%, and apoptosis mediator death-associated protein kinase in 19%.[67] Indeed, the number of tumor suppressor genes that are hypermethylated in melanoma is accumulating.[69] By contrast, specifically hypomethylated genes have been less documented in melanoma.

Besides the regional aberrant methylation status of gene promoters, melanoma also exhibits *global* hypomethylation within the bulk genome, but the degree is not sufficient to distinguish the benign nevus from melanoma.[70] However, we have preliminarily noted that, unlike 5-mC, the loss of 5-hmC by immunohistochemistry can distinguish melanomas from physiologic melanocytes and benign melanocytic proliferations, wherein 5-hmC nuclear immunoreactivity remains high.[71] We have also found that a strong correlation exists between the loss of 5-hmC and with the parameters of poor prognosis in melanoma, including Breslow depth, mitotic rate, and ulceration, as well as with lower overall survival, suggesting a potential predictive value of loss of 5-hmC.[71] Others have since reproduced these findings.[72,73]

In addition, certain subtypes of the nevi, as well as of malignant melanoma, also recapitulate this inverse relationship. The retention of 5-hmC nuclear staining was

very recently documented in certain benign nevic subtypes, including the Spitz nevus, whereas the loss of 5-hmC was found to be a feature in multiple melanoma subtypes, including both acral and mucosal melanoma.[73] Moreover, the loss of 5-hmC may be common to malignant melanoma of varying etiologies; melanomas arising in chronically sun-damaged skin as well as those arising in sun-protected areas, too, demonstrate this epigenetic phenomenon.[73] Further work is clearly indicated, however, to expand on these findings to determine whether retention and loss of 5-hmC are truly universal in benign and malignant melanocytic lesions, irrespective of clinicopathologic variants. Moreover, determination of how other epigenetic alterations involving histone modifications and miRNA expression may relate to tumor subtypes awaits further study. Interestingly, it was recently reported that increasing morphologic atypia in dysplatic melanocytic nevi corresponds to progressive loss of 5-hmC nuclear staining, a finding that also tightly associates with increasing nuclear diameter (Figure 3).[74] This provides pathologic evidence that loss of TET function, as evidenced by reduced levels of 5-hmC and resulting epigenomic instability, may be critical to the pathogenesis of melanoma. In addition to melanoma, the loss of 5-hmC has been documented uniformly and universally in a number of human cancers, independent from, but reminiscent of, the global reductions in 5-mC discussed above.[20] For example, 5-hmC loss has been reported in cancers of the brain, breast, lung, liver, stomach, pancreas, colon kidney, prostate, ovary, uterus using a variety of methods, including liquid chromatography-mass spectrometry, anti-5hmC antibody-based radio-immune dot blots, and immunohistochemistry.[20,75]

Despite these promising observations, the mechanism underlying 5-hmC loss in cancer, in general, and in melanoma, more specifically, remains elusive. Interestingly, whereas IDH1 expression is similar between nevi and melanoma, we find that IDH2 is significantly downregulated in melanoma as are all three TET genes, with the most marked decrease in TET2.[71] These data, in part, may shed light on previous findings indicating that mutations in IDH1 or 2 are present in up to 10% of melanomas[76] and that 5-hmC levels in IDH1-mutant gliomas compared with wild-type IDH1 do not differ substantially.[75] Moreover, overexpression of wild-type IDH2 in a zebrafish melanoma model increases 5-hmC levels and prolongs tumor-free survival compared with mutant IDH2.[71] Importantly, overexpression of TET2 reverses the genome-wide 5-hmC distribution from global loss, as is seen in melanoma, toward one resembling a benign nevus-like pattern. In keeping with this, TET2-overexpressing melanoma cells give rise to smaller tumors compared with mutated TET2 melanoma cells,[71] and TET2 expression has very recently been shown to be significantly higher in nevi than in superficial spreading melanoma and cutaneous metastatic disease.[72] Taken together, TET family enzyme dysfunction and the concomitant loss of 5-hmC and resulting epigenomic instability provide a plausible pathogenic mechanism to explain the inappropriate methylation of tumor suppressor genes, which has been widely observed in various human cancers. Notably, whereas the replicative fidelity of DNA polymerases are well known,[77] our understanding of DNMT fidelity is only very recently beginning to emerge.[23,78–80] Further investigation into this important area of cancer epigenetics promises to shed insights into this critical aspect of the dysregulated cancer epigenome. In light of our preliminary insights into epigenetic fidelity regulation in melanoma, the loss of 5-hmC may be a direct reflection of loss of the TET family 'guardian' or fidelity function, which may prove to be central to the epigenetic dysregulation and resultant pathobiology of melanoma and other cancers.

Melanoma Cell Longevity through Histone Modifications

Of the dysregulated epigenetic mechanisms involved in the pathogenesis of melanoma, aberrant histone modifications are among the least documented. While this may be, in part, due to the more challenging laboratory techniques required to delineate histone modifications,[81] closer examination of this aspect of the epigenome will likely provide missing links between modifications to DNA bases and their overall influence on chromatin structure and transcriptional regulation. Therapeutic inhibition of histone deacetylase in melanoma cell lines has been shown to improve apoptotic efficiency through upregulated CDK inhibitor p21 expression, suggesting that aberrant histone deacetylation may have pathogenic role in melanoma through the downregulation of apoptotic mechanisms.[82] Indeed, histone hypoacetylation has been demonstrated to downregulate other proapoptotic proteins, including the Bcl-2 family proapoptotic proteins (Bim, Bax, and Bak),[83] as well as tumor suppressor genes, such as phosphatidylinositol 4, 5-bisphosphate 5-phosphatase A, a negatively regulator of the PI3K/Akt signaling pathway.[84]

In addition to histone hypoacetylation, aberrant histone methylation also appears to have a pathogenic role in melanoma. Increased expression of EZH2 is tightly associated with highly proliferative and aggressive subtypes of melanoma, as well as in cancers of the endometrium, prostate, and breast.[85] It is also tightly associated with loss of tumor-suppressive cell cycle inhibitor p16 in melanoma and endometrial carcinoma.[85] Interestingly, EZH2 expression in patient melanoma specimens, as demonstrated by immunohistochemistry, has been shown to increase incrementally from benign nevi to melanoma, and is also significantly higher in invasive melanoma than it is in *in situ* melanoma or in benign melanocytic lesions.[86] EZH2 is the subunit of polycomb repressor complex 2 (PRC2) that is responsible for catalyzing the transcriptionally repressive methylation of H3K27, and it appears that EZH2 upregulation in this context represses the expression of tumor suppressor genes.[85]

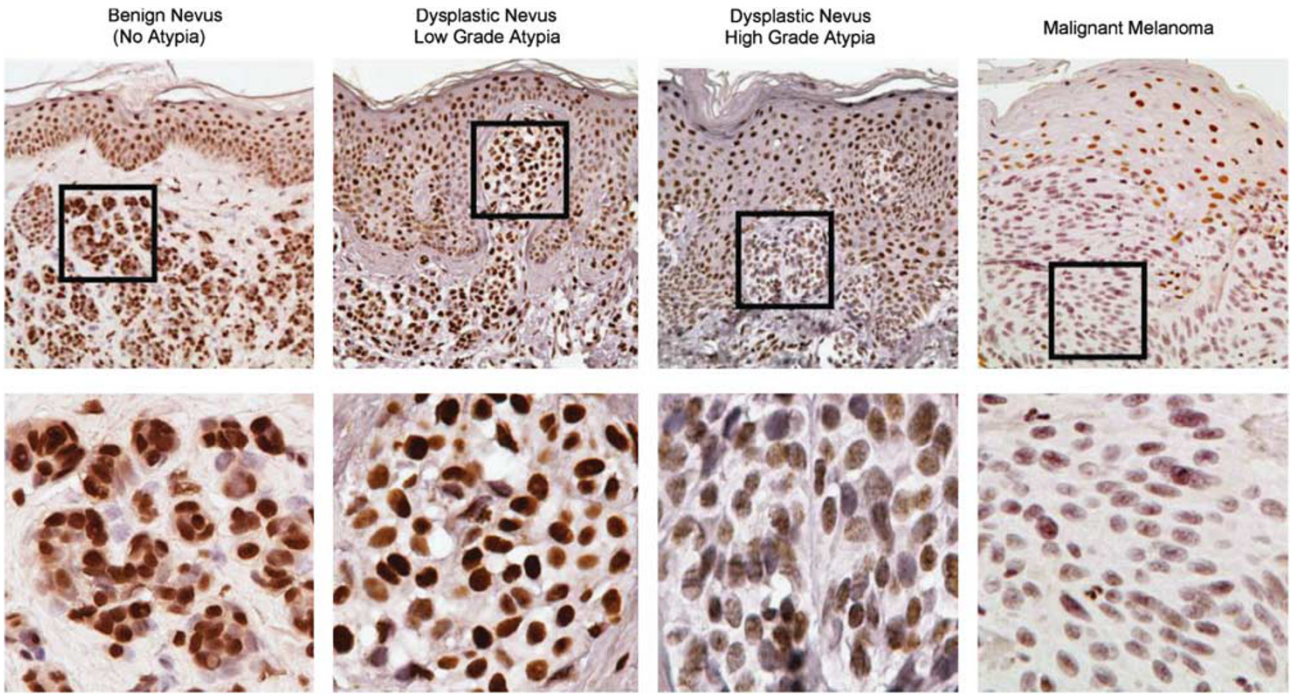

Figure 3 Increasingly 'dysplastic' melanocytic lesions show progressive loss of immunohistochemical staining for 5-hydroxymethylcytosine (5-hmC). Immunohistochemical staining with 5-hmC demonstrates progressive loss of 5-hmC with progression from benign nevus (no dysplasia) to low- and high-grade dysplastic nevi, and finally to melanoma. Sections at $\times 200$ are shown above with selected areas that are further magnified in the panels directly below. From Larson *et al.*,[74] reprinted with permission from Nature Publishing Group.

Additional histone-modifying enzymes have also demonstrated oncogenic potential in melanoma. The histone methyltransferase SETDB1 (SET Domain, Bifurcated 1) is recurrently amplified in melanoma and accelerates tumor development in zebrafish melanoma models harboring the common BRAF(V600E) mutation.[87] SETDB1 catalyzes the trimethylation of histone H3K9 and thereby promotes the repression of target genes.[87] SETDB1 overexpression has been shown to cause significant downregulation of a group of genes enriched for the development-regulating homeobox (HOX) genes.[87] Dysregulated HOX genes are known to be associated with a number of hematologic malignancies and support the immortalization of leukemic cells.[88] Interestingly, whereas the BRAF(V600E) mutation is present both in many melanomas and in benign melanocytic nevi,[89] elevated SETDB1 protein is present in human melanomas, but not in nevi or in normal melanocytes.[87] Moreover, emerging evidence is further suggestive of a *bona fide* oncogenic role for SETDB1 in both non-small-cell and small-cell lung cancers.[90] Additionally, its overexpression in this context may also correlate with chemosensitivity to clinically approved mithramycin, an antitumoral antibiotic that binds to the minor groove of the DNA double helix, thereby displacing transcriptional activators, and shown to suppress basal SETDB1 promoter activity.[90] Taken in aggregate, these data strongly support that dysregulation of the histone modification system contributes to the loss of tumor suppressors or enhanced longevity/proliferative capacity in melanoma and other cancers.

A Role for MiRNAs and Other NcRNAs in Melanoma

The prognostic and pathobiologic importance of ncRNAs in melanoma have been well established and represent an active area of investigation (Table 1).[91] Indeed, an array of miRNAs and other ncRNAs have been shown to exhibit either tumor-suppressive capabilities or pro-oncogenic and/or prometastatic potential involving multiple molecular pathways. The tumor-suppressive function of miRNAs, in part, may be mediated through interactions with PcG proteins. miR-200c was shown to be significantly more downregulated in both primary and metastatic melanomas compared with benign melanocytic nevi, and its overexpression in melanoma cell lines appears to result in significantly reduced cell proliferation, migratory capacity, and expression of key transporters involved in melanoma drug resistance.[92] Bmi-1 (B lymphoma Mo-MLV insertion region 1 homolog) is a PcG protein component of the PRC1, which, as described earlier, comprises an important class of transcriptional repressors that orchestrate changes in chromatin structure and thereby regulate gene activity.[93] Bmi-1, specifically, is an important transcriptional repressor of the Ink4a/Arf locus, which encodes two distinct gene products, including tumor suppressors $p16^{ink4a}$ (p16) and $p19^{Arf}$ (p19).[94] p16 inhibits CDK activity and thereby blocks entry into the cell cycle,

Table 1 Oncogenic/prometastatic and tumor-suppressive miRNAs (mi-Rs) reported in melanoma

miRNAs in melanoma						
miRNA	Target/function	Expression in malignant melanoma	Sample/source	Clinical utility	Publication year	Reference number(s)
Oncogenic or prometastatic						
miR-let-7 family	NRAS	↓	Cell lines	Detection, progression	2008	179
miR-195	Wee1 kinase	↑	Cell lines	Prognosis	2013	180,181
miR-221, -222	p27, c-KIT (miR-221)	↑	Cell lines, serum	Detection	2009, 2011	154,182
miR-193b	Mcl-1 (Bcl-2 family)	↓	Cell lines	Chemosensitization	2011	183
miR-15b	BIM1	↓	Serum	Prognosis	2012	155
miR-199a-5p	SWI/SNF	↑	Serum	Prognosis	2012	155
miR-424	HIF-1α/HIF-2α	↑	Serum	Prognosis	2012	155
miR-432-5p	Unknown	↑	Serum	Prognosis	2012	155
miR-1908, 199a-5-, 199a-3p	ApoE, DNAJA4; loss promotes angiogenesis	↑	Tissue	Prognosis	2012	100
miR-214	Integrin-β3	↑	Tissue	Prognosis	2011	184
Tumor suppressive						
miR-205	E2F	↓	Tissue	Therapeutic	2011	185
miR-137	c-Met, YB1, MITF, EZH2	↓	Tissue-derived cell lines	Prognosis	2013	153
miR-26a	SODD	↓	Cell lines	Progression, therapeutic	2013	186
miR-33a	Pim-1, CDK6, CCDN1	↓	Serum	Prognosis	2012	155
miR-34a	PNUTS (PPP1R10)	↓	Cell lines	Detection, therapeutic	2008	187
miR-125b	c-Jun	↓	Cell lines	Therapeutic	2013	188
miR-9	NF-ĶB1	↓	Tissue	Prognosis	2012	144
miR-18b	p53	↓	Cell lines	Therapeutic	2013	189
miR-573	MCAM	↓	Cell lines	Therapeutic	2013	190

whereas p19 promotes p53 stability, and in doing so, arrests cell cycle progression and promotes apoptosis.[94] Interestingly, overexpression of miR-200c in melanoma cell lines results in significant downregulation of Bmi-1 and shows a similar phenotype to Bmi-1 knockdown melanoma cell lines.[92] Moreover, miR-200c overexpression significantly inhibits melanoma xenograft growth and metastasis *in vivo*, which correlates with diminished expression of Bmi-1 as well as reduced levels of epithelial cadherin (E-cadherin).[92] Several other miRNAs, including miR-612, have been demonstrated to suppress the epithelial–mesenchymal transition and metastasis in other human cancers.[95] Both miR-200 and E-cadherin are expressed at lower levels at the deep invasive tumor margin and associate clinically with increased melanoma thickness and disease progression.[96] Taken collectively, these data suggest that miR-200c exhibits tumor-suppressive function by targeting Bmi-1 and upregulating tumor suppressor and cell adhesion molecules; thus, its downregulation, as observed in primary and metastatic melanoma samples, appears to contribute to the molecular pathogenesis of melanoma.

Several miRNAs have been found to exhibit oncogenic or prometastatic capabilities. Elevated levels of wild-type p53 directly upregulates miR-149 expression, which is also increased in fresh human metastatic melanoma isolates.[97] miR-149, in fact, targets and reduces glycogen synthase kinase-3α levels, which increases the expression of antiapoptotic Bcl-2 family protein Mcl-1 known to produce apoptotic resistance in melanoma cell lines.[97] Similarly, miRNA-21 is significantly increased in primary melanoma tissues compared with benign nevi and is tightly associated with increased proliferation and decreased apoptosis.[98] In addition, a cluster of 14 miRNAs on the X chromosome (miR-506-514 cluster) was found to be consistently and significantly overexpressed in nearly all patient biopsy samples of metastatic melanoma, regardless of mutation status in NRAS or BRAF.[99] Notably, inhibition of the expression of this cluster in melanoma cell lines, or one of its subclusters, led to significant abrogation of cell growth,

induction of apoptosis, reduced invasiveness, and decreased colony formation *in vitro*.[99] Indeed, a number of miRNAs exhibit oncogenic potential in melanoma through inhibition of apoptosis. In addition, a cooperative network of miRNAs (miRNA-1908, miR-199a-5p, and miR-199a-3p) that endogenously promotes metastatic invasion, angiogenesis, and colonization in melanoma has also been recently identified.[100] These miRNAs appear to target apolipoprotein E, which normally suppresses invasion and metastasis.[100] Moreover, patients whose primary melanomas express higher levels of miR-199a-3p, miR-199a-5p, or miR-1908 have been shown to have significantly shorter metastasis-free survival times than patients whose primary melanomas express lower levels of each of these miRNAs.[100] Interestingly, highly metastatic melanoma cell lines that are pretreated with a cocktail of locked nucleic acids targeting these miRNAs for downregulation show reduced ability to metastasize to multiple distant organs upon their injection into mice.[100]

Preliminary evidence also implicates several long ncRNAs in the pathobiology of melanoma. HOTAIR, one such lncRNA that has been associated with metastatic behavior, was found to be significantly overexpressed in lymph nodes containing metastatic melanoma compared with matched primary melanoma specimens.[101] Moreover, its knock down in cell lines suppressed melanoma cell motility, invasiveness, and extracellular matrix degradation.[101] Interestingly, recent evidence suggests that HOTAIR, through direct scaffolding interactions with histone-modifying enzymes, may facilitate changes to chromatin structure.[101] Similar roles for other ncRNAs have gained significant attention in the basic science literature, such as Xist, recently shown to silence the X chromosome by exploiting and inducing three-dimensional chromosome structural alterations.[102] Additional putative oncogenic lncRNAs have been reported in melanoma, including SPRY4-IT1[103] and Llme23,[104] and reports of additional lncRNA with either oncogenic or tumor-suppressive roles in melanoma pathobiology will likely follow. Altogether, there is substantial preliminary evidence to suggest that lncRNAs, in addition to miRNAs, are progressively dysregulated and may promote melanomagenesis through the loss of either tumor-suppressive function or promotion of oncogenic or prometastatic molecular pathways. While many facts remain elusive, including the precise mechanisms or drivers underlying their dysfunction, as well as their basic regulatory mechanisms, ncRNAs provide a most bountiful area of further investigation in melanoma and cancer pathogenesis and therapy.

CANCER CELL 'STEMNESS' AND THE EPIGENOME

In 2006, the American Association for Cancer Research determined that a CSC is 'a cell within a tumor that possesses the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor.'[105] First discovered in hematopoietic malignancies in the 1960s and 1970s,[106,107] CSCs have been identified in a variety of solid tumors, including cancers of the breast,[108] brain,[109] colon,[110] and melanoma.[111] Although their existence has previously been a matter of debate,[112] CSCs, also referred to as cancer-initiating cells,[113] are thought to potentially represent oncogenic derivatives of normal-tissue stem or progenitor cells,[114,115] may develop in certain forms of cancer as a consequence of the EMT, and/or evolve spontaneously during tumor progression.[116,117] We have observed that melanoma cells acquire CSC markers with evolution from benign nevi to primary melanoma to metastatic melanoma,[74] and, interestingly, a similar progression is observed with respect to loss of 5-hmC, as we have delineated above. This strongly suggests that the acquisition of melanoma 'stemness' with tumor progression may be in some way related to the loss of DNA hydroxymethylation (Figure 4). This hypothesis is, in part, grounded in the regulatory role played by DNA methylation in the maintenance and function of embryonic stem cells, which CSCs may, in part, recapitulate.[31]

EMT is a complex molecular and cellular process by which epithelial cells lose their differentiated characteristics, including cell–cell adhesion, and acquire mesenchymal features, such as motility, invasiveness, and a heightened resistance to apoptosis.[118] This 'transition' has been proposed to be instrumental to the acquisition of 'stemness' by both non-transformed and tumor cells.[119,120] EMT and acquisition of the 'stem-like' phenotype has also been implicated in the development of chemoresistance in many human cancers.[118] The loss of E-cadherin, a 'calcium-dependent transmembrane adhesion' molecule critical for epithelial cell–cell adhesion, is a hallmark of the EMT.[118] Its loss, in part, further stabilizes the mesenchymal state through β-catenin-mediated upregulation of EMT-inducing transcription factors.[118] Mechanistically, E-cadherin loss is thought to be either genetic or epigenetic, and epigenetic mechanisms are steadily entering the limelight.[121] As melanocytes do not belong to the epithelial lineage, 'EMT' cannot be strictly used to describe melanoma pathobiology. Moreover, melanoma stem cells need not coincide with all cells exhibiting an EMT-like phenotype, in that self-renewal resulting in localized tumorigenic rather than purely invasive growth is emblematic of CSC behavior. Indeed, it has been reported that differentiated melanocytes express E-cadherin, which allows them to maintain homophylic adhesion with keratinocytes in the basal layer of the epidermis.[116] Of note, the loss of E-cadherin, the quintessential hallmark of the EMT in epithelial tumors, is also present in late-stage, metastatic melanoma to lymph nodes[122,123] and very recently described (2013) to be present in desmoplastic melanoma,[117] a tumor that rarely metastasizes. More importantly, the loss of E-cadherin as well as the aberrant expression of neural cadherin (N-cadherin) marks the critical transition from the radial-growth phase to the vertical-growth phase in melanoma,[124,125] an event that is associated with acquisition of potential for metastasis, has also been reported. Nonetheless, the ability of melanoma to show stem cell-driven

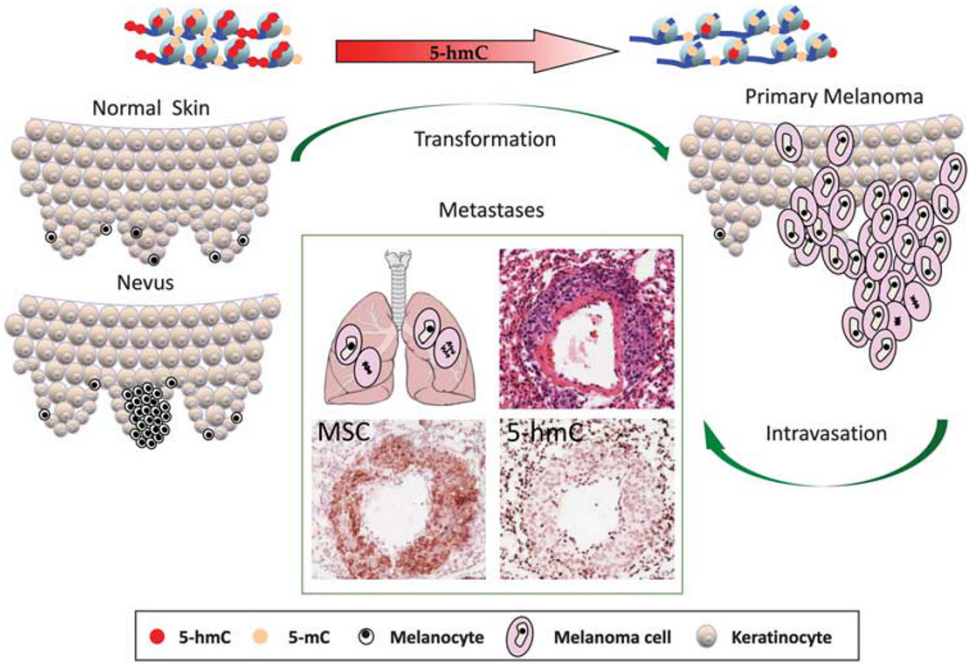

Figure 4 Schematic representation of potential interplay between epigenetics (loss of 5-hydroxymethylcytosine (5-hmC)) and melanoma stem cell (MSC) expression during melanoma progression. Melanomagenesis may originate in 'normal skin,' or in a pre-existing benign or dysplastic nevus as a result of transformation of melanocytes/nevus cells, a process that appears to be associated with progressive loss of 5-hmC and concomitant heightened activity of self-renewing, stem-like cells. Intravasation of primary melanoma as a consequence of dermal invasion transports cells epigenetically programmed for malignant behavior to lymph nodes and vital organs. Experimental models of metastases reveal cells expressing MSC markers to be relatively devoid of 5-hmC, consistent with epigenetic interplay with the stem cell component of the tumor at the metastatic site. (Lung diagram adapted from courtesy of Patrick J Lynch, medical illustrator; C Carl Jaffe, MD, cardiologist. http://creativecommons.org/licenses/by/2.5/.)

tumorigenesis at primary and metastatic sites, as well as to toggle between tumorigenesis and EMT-like phenotypes, implicates the likelihood of robust genomic–epigenomic regulatory interactions. In this regard, reprogramming of EMT-inducing transcription factors collaborate with BRAF activation towards the dedifferentiation, loss of E-cadherin, gain of invasive properties, and malignant transformation of melanoma.[116]

It must be noted that EMT-like behavior, although often reflected by loss of E-cadherin, also requires the upregulation of cell surface molecules necessary for invasion and metastasis, as well as matrix metalloproteases (MMPs), which degrade the extracellular matrix and facilitate invasion of cells with mesenchymal characteristics.[126] The expression of $\alpha V\beta 3$ integrin, which, in addition to E-cadherin loss and N-cadherin expression, also tightly associates with the transition from the radial- to vertical-growth phase in melanoma.[127] Furthermore, this integrin induces the expression of MMP-2, an enzyme that degrades the collagen within the basement membrane.[128] While the epigenetic regulation of this integrin and MMP expression has yet to be described in melanoma or other cancers, it is very likely that dysregulated epigenetics are involved in their upregulation in this context. Furthermore, evidence is emerging to support that melanoma stem cells may be oncogenic derivatives of normal-tissue stem or progenitor cells. Latexin, a negative regulator of hematopoietic stem cell populations,[129] was recently shown to reduce the risk of old stem cells transforming into CSCs[130] and is known to be down-regulated in approximately 50% of melanomas.[131] Notably, the CpG island promoter of the latexin gene has been shown to be universally hypermethylated in melanoma cell lines and other cancers.[131] These findings further demonstrate that epigenetic mechanisms may be critical to the development of melanoma or CSCs. Studies also show that histone modifications have important roles in melanoma stem cells. For instance, a distinct subpopulation of slow-cycling melanoma cells positive for jumonji/ARID1 (JARID1B) was found to be required for continuous tumor growth.[132] JARID1B (KDM5B/PLU-1/RBP2-H1) is a member of the highly conserved family of JARID1 H3K4 demethylases, which are known to be involved in development, cancer, and stem cell biology.[133] JARID1B is highly expressed in benign nevi, whereas in aggressive primary melanomas and melanoma metastases, single cells comprising 5–10% of the total population have high JARID1B expression.[134] JARID1B levels are elevated in highly regenerative tissues as well as in cancer, wherein it regulates the transcription of oncogenes, such as BRCA1 in breast cancer, through direct interaction with promoter sites.[135] Indeed, demethylation of H3K4 has been shown to support the transformation of hematopoietic precursors to leukemia stem cells via regulation of the

developmental HOX gene family.[136] JARID1B has been associated with either positive or negative cell cycle control, depending on the type of cancer (ie, positive cell cycle control in melanoma, negative in breast cancer).[137,138] Interestingly, JARID1B has been shown to have a stabilizing effect on hypophosphorylated pRB,[137] which is normally regarded as an immediate-acting antiproliferative mechanism. It has been proposed that JARID1B's antagonistic effect on proliferation may ultimately permit the maintenance of a slow-cycling tumor sub-population.[132] Of interest, recent evidence suggests that this slow-cycling sub-population may be distinct from melanoma subpopulations with 'stemness' or EMT-like associated features.[132]

Finally, evidence points to the involvement of miRNAs both in cancer pathobiology and in the regulation of cancer 'stemness.' As discussed above, the miRNAs are a subset of ncRNAs that negatively regulate gene expression by targeting and degrading the mRNA transcript or inhibiting its translation.[139] With more than 200 miRNAs described in humans, many have been implicated as putative oncogenes or tumor suppressor genes that are involved in the regulation of 'stemness' and metastasis in various human cancers.[140] The miR-200 family members (miR-200s, to include miR-200a, miR-200b, miR-200c, miR-141, miR-429), in particular, are key tumor-suppressive regulators of the EMT.[141] They control 'stemness' by directly targeting transcription factors such as transcriptional repressor of E-cadherin Zeb1/2 (zinc-finger E-box-binding HOX 1/2).[142] Interestingly, the miR-200s are downregulated in various cancer types but, more specifically, in cancer cells undergoing the EMT or with other stem-like features, as will be further explored below.[143] In addition, a recent study reported that miR-9 is downregulated in metastatic melanoma compared with primary melanomas and that its knockdown in melanoma cell lines enhances cell proliferation and migration capacity.[144] Furthermore, overexpression of miR-9 in metastatic melanoma cell lines induces significant downregulation of the NF-κB1-Snail1 pathway and a concomitant increase in E-cadherin expression. Taken together, these data support that epigenetic mechanisms, miRNAs in particular, have a key role in regulating EMT-like changes in melanoma.

More recent evidence has identified miR-22 as a potent proto-oncogenic miRNA that deranges the epigenetic landscape of the cell.[145] miR-22 has been shown to enhance the repopulating capacity and stem cell function of hematopoietic stem/progenitor cells.[146] *In vivo* models demonstrate that miR-22 triggers myelodysplastic-like syndromes and hematological malignancies and that its expression correlates directly with poor survival rates.[146] Interestingly, miR-22 has also been shown to enhance the EMT by repressing miR-200, leading to the upregulation of Zeb1 and Zeb2, and subsequent repression of E-cadherin expression.[31] These results shed light on the possible mechanisms underlying the change from the epithelioid to spindle cell morphology during the first wave of 5-mC loss in mouse cutaneous carcinogenesis observed in a landmark report by Fraga *et al.*[60] miR-22 overexpression has also been shown to instigate higher rates of tumor invasiveness and metastasis, as well as a progressive decrease, in disease-free survival rate in breast cancer mouse models.[31] Further analysis has revealed that miR-22 directly targets and reduces the expression of the critical DNA-demethylating enzyme and 5-mC oxidase TET2, resulting in a marked reduction in 5-hmC levels and a concomitant increase in 5-mC levels in the genome of mouse hematopoietic stem/progenitor cells.[146] The resulting loss of demethylase function has been shown to lead to genomic hypermethylation and silencing of the miR-200 promoter.[146] Indeed, derangement of this miR-22-TET2 pathway has been deemed to be one of the most frequent events in hematologic malignancies.[146] Overall, miR-22 appears to have consistent, principal proto-oncogenic potential through the dysregulation of the DNA demethylation apparatus, enhancement of the EMT, and enabling of cancer cell stemness.

EPIGENOMIC BIOMARKER APPLICATIONS IN MELANOMA

Many of the epigenetic markers discussed above have direct diagnostic utility. For example, studies indicate that, in addition to the oncogenic implications of hypermethylated genes, methylation status of certain genes may provide direct prognostic implications in patients with melanoma. Global levels of long-interspersed element-1 (LINE-1) methylation in short-term tumor cell cultures grown from patients with nodal metastatic melanoma have been shown to significantly predict overall survival in patients with stage IIIC cutaneous melanoma.[147] Moreover, identification of these epigenetic hallmarks circulating as free DNA in the serum of patients with melanoma using methylation-specific PCR is also an area of active investigation.[148] In addition, the loss of 5-hmC, as demonstrated through immunohistochemistry, may aid in distinguishing malignant melanocytic lesions from dysplastic or borderline melanocytic lesions wherein 5-hmC staining is relatively more intense.[71–73] The diagnostic utility and prognostic significance of loss of 5-hmC by immunohistochemistry, as has been demonstrated in melanoma,[71] also has been recapitulated in other human tumors, including oral squamous cell carcinoma,[28] gastrointestinal stromal tumor,[29] and hepatocellular carcinoma.[30]

miRNAs may also have powerful prognostication potential in melanoma. Patient melanoma specimens expressing lower levels of miRNA-205 by immunohistochemistry have been shown to associate tightly with significantly shorter melanoma-specific survival, independent of melanoma stage, age, gender, or Breslow depth.[149] Interestingly, miRNA-205 *over*expression in patient melanoma samples has been shown to result in lower levels of Zeb2 expression and increased expression of E-cadherin, suggesting that this particular miRNA may also be involved in suppressing the

EMT.[150] Indeed, *in vitro* and *in vivo* models have demonstrated that miR-205 overexpression impedes melanoma cell migration and invasion.[150] Furthermore, miR-205 expression progressively decreases from benign to dysplastic nevi, as well as in melanomas, in both clinical specimens and cell lines.[150] Another miRNA, miR-29c, was demonstrated to be significantly downregulated in AJCC stage IV melanoma specimens compared to primary tumors, with elevated expression significantly predicting disease-free and overall survival.[151] Several other miRNAs, including miRNA-31[152] and miRNA-137,[153] also exhibit tumor-suppressive function in melanoma by interfering with a number of oncogenic pathways. Interestingly, both of these miRNAs appear to downregulate EZH2, the histone methyltransferase component of PRC2 discussed above,[93] the expression of which progressively increases from benign nevi to dysplastic nevi to localized and metastatic melanoma, where its expression is associated with a poor 5-year prognosis.[153] These findings emphasize the relevance of dysregulated epigenetic 'cross-talk' mechanisms in the pathobiology of melanoma and demonstrate their tumor-suppressive functions. Moreover, this epigenetic insight offers the potential application of prognostic biomarkers in melanoma and other melanocytic lesions.

In addition, miRNAs may serve as prognostic biomarkers when detected in the circulation. Serum levels of miR-221 has been shown to distinguish between patients with melanoma *in situ* from those with stage I–IV melanoma.[154] Furthermore, several miRNAs detected in the serum of patients at the time of primary melanoma diagnosis have been shown to reflect overall tumor burden and to accurately and significantly predict risk of recurrence.[155] Because there exists conflicting data regarding their utility and practical reproducibility of various assays,[81] more research and translational development is required before such approaches are brought to the bedside. Nevertheless, miRNAs represent a very appealing epigenomic marker of prognosis and certainly deserve much further exploration.

EPIGENOMIC THERAPEUTIC APPLICATIONS IN MELANOMA

Unlike genomic mutations, epigenetic alterations in cancer are, in principle, therapeutically reversible, and a number of epigenetic therapies have already received FDA approval (Table 2). Sole use of DNMT inhibitors for the treatment of melanoma has yielded mixed results, with early studies suggesting enhanced capacity for experimental metastasis in xenograft models.[156] In contrast, very recent preliminary data suggest that HDAC inhibitors in nanomolar concentrations may have some therapeutic benefit.[157] While the sole use of these epigenetic therapies in melanoma continues to be an active area of clinical investigation, recent studies have shown great promise for their adjunctive use with various treatment regimens, for example, immuno-, chemo- and radiotherapeutic strategies. For example, DNMT and HDAC inhibitors upregulate the expression of a number of critical melanoma cell surface molecules, including major histocompatibility complex and costimulatory molecules, as well as the melanoma antigen encoding gene (MAGE-1) tumor antigen.[158–160] Animal models demonstrate modest benefits using combined HDAC inhibitors with or without[157] adoptively transferred, gp100 melanoma antigen-specific T cells.[161] The adjunctive use of these epigenetic therapies to upregulate the expression of such critical cell surface target antigens with existing immunotherapies, including interferon-α, ipilimumab, and melanoma peptide vaccines,[162,163] is a promising area of active investigation.[164]

Table 2 Current FDA-approved epigenetic agents

Current FDA-approved epigenetic agents			
Class	Agent name	Condition	Year
DNMT-I	Azacitidine (Vidaza™)	Myelodysplastic syndromes	2004
	Decitabine (Dacogen®)	Myelodysplastic syndromes	2006
HDAC-I	Vorinostat (Zolinza™)	Cutaneous T-cell lymphoma	2006
	Romidepsin (Istodax®)	Cutaneous T-cell lymphoma	2009

In addition to their combinatorial use with immunotherapies, epigenetic agents may also support and enhance the effectiveness of standard chemotherapeutic or radiotherapeutic regimens. Alkylating agents are thought to exert their antitumor activity by inducing either DNA double-strand breaks or interstrand crosslinking.[165] However, a DNA repair protein called MGMT can remove alkyl lesions induced by these agents, inhibiting their cytotoxic effects.[165] Accordingly, elevated expression of MGMT has been shown to contribute to chemoresistance to alkylating agents in multiple human malignancies, including melanoma,[165] and has been attributed to aberrant methylation patterns.[166] This has provided the rationale for the combined use of DNMT inhibitors alongside alkylating agents, an approach recently shown to have promising results in phase I/II studies in patients with metastatic melanoma.[167] Other epigenetically regulated mediators of chemosensitivity to alkylating agents have also been identified that may be therapeutically upregulated with DNMT inhibitors.[168] Furthermore, DNMT and HDAC inhibitors also have the ability to restore apoptotic capacity by upregulating epigenetically silenced effectors such as Apaf-1,[169] caspase-8,[170] and p16,[171] and thereby enhancing chemosensitivity to the DNA-intercalating agent doxorubicin,[169] DNA crosslinking agent cisplatin, and topisomerase inhibitor etoposide.[171] This combination also has shown promising results in phase I/II clinical trials.[172] Given their demonstrated ability to restore the apoptosome in melanoma, HDAC inhibitors also may radiosensitize human melanoma cells.[173,174] Taken together, the potential adjunctive role of DNMT and HDAC inhibitors used in

Table 3 On-going clinical trials of epigenetic agents for the treatment of melanoma

Ongoing clinical trials of epigenetics agents for the treatment of melanoma				
Class	Epigenetic agent	Investigation	Phase	ClinicalTrials.gov Identifier
DNMT inhibitor	Azacitidine (Vidaza™)	Azacitidine and recombinant interferon alfa-2b in patients with stage III or IV melanoma that cannot be removed by surgery	I	NCT00217542
		Decitabine and pegylated-interferon in melanoma	I/II	NCT00791271
	Decitabine (Dacogen®)	Decitabine with temozolomide and panobinostat (HDAC inhibitor) for resistant, metastatic melanoma	I/II	NCT00925132
		Decitabine and temozolomide for patients with metastatic melanoma	I/II	NCT00715793
		Decitabine with vemurafenib for melanoma	I/II	NCT01876641
HDAC inhibitor	Vorinostat (Zolinza™)	Vorinostat and proteasome inhibitor NPI-0052 for melanoma	I	NCT00667082
		Vorinostat for metastatic for unresectable melanoma	II	NCT00121225
		Vorinostat for metastatic/recurrent ocular melanoma	II	NCT01587352
	Romidepsin (Istodax®)	Romidepsin for melanoma	I	NCT01638533
	Panobinostat	Panobinostat with ipilimumab for unresectable, stage III/IV melanoma	I	NCT02032810

conjunction with conventional chemo-, immuno-, and radiotherapeutic strategies is an active and exciting area of investigation (Table 3).[69] Of note, several miRNAs have also demonstrated efficacy in animal models and this class of novel therapeutics is being actively investigated.[29,35] Further characterization of the epigenetic regulation of cell surface molecule expression, apoptotic mediators, and other related pathways is likely to further illuminate this promising area of cancer research.

MELANOMA AND ITS EPIGENOME: LOOKING FORWARD

With the incidence of melanoma increasing worldwide and the consistently poor prognosis associated with advanced cases,[175] strategies for earlier detection, risk stratification, and enhanced therapeutic efficacy are desperately needed. Moving beyond a concept focused primarily on accumulated mutations to the DNA sequence as the central driver of carcinogenesis or melanomagenesis, the evidence reviewed herein points to a paradigm shift to consider gene expression also in the context of the epigenome. Such an approach provides an opportunity to explore, identify, and deploy new diagnostic and therapeutic strategies. This, in part, will depend on furthering our understanding of how the discrete categories of epigenetic changes interact with and regulate one another, as well as the mechanisms that disrupt these systems. For example, a recent study demonstrated that BRD4 is significantly upregulated in primary and metastatic melanoma tissues compared with melanocytes and benign nevi.[176] BRD4 is a bromodomain and extraterminal domain (BET) family protein that exerts key roles at the interface between chromatin remodeling and transcriptional regulation by binding to acetylated histones and recruiting specific coactivating or corepressing chromatin-modifying enzymes to target promoters.[177,178] Newly developed, cell-permeable small-molecule inhibitors of BET proteins have shown very promising anti-melanoma activity *in vivo*, regardless of BRAF or NRAS mutational status. This final example illustrates the critical nature of advancing our understanding of epigenetic 'cross-talk' mechanisms in melanoma and other cancers. Further investigation into epigenetic fidelity maintenance mechanisms and their dysregulation in melanoma and other cancers will also be critical to our understanding the therapeutic manipulation of the cancer epigenome.

As we have discussed, epigenetic mechanisms such as DNA methylation and hydroxymethylation, histone modifications, and ncRNAs are critical to the regulation of gene expression, phenotypic plasticity, cell cycle regulation, apoptosis, and other critical biologic functions in both normal and cancer cells. Furthermore, distinct epigenetic hallmarks show promise for assisting in distinguishing between benign and malignant lesions under the microscope and in the blood, and may also provide critical prognostic information. We have also illustrated the ways in which the epigenome can be harnessed to unlock the expression of molecules critical to the success of chemo-, immuno-, and radiotherapeutic strategies. In summary, there is justification for great optimism that future advancements in our understanding

of the melanoma and cancer epigenome will translate into direct diagnostic and therapeutic benefits for patients who are afflicted by this virulent form of human malignancy.

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