Chapter 1

Epigenetics in Melanoma Development and Drug Resistance

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.70983

Abstract

Melanomas, which originate from melanocytic cells, mainly develop in the skin but can also arise at other body sites. The disease accounts for approximately 90% of deaths related to cutaneous tumors with late stage metastatic melanoma having a very poor prognosis of 6–9 month median survival for untreated patients. Research in the last decades resulted in ground-breaking discoveries of melanoma genetics and biology. High frequency mutations in genes like *BRAF*, *NRAS* and *KIT*, which lead to hyper-activation of the MAPK signaling pathway, drive melanoma progression. Targeting the MAPK signaling pathway has successfully been translated into effective therapies that significantly improve patient survival. Despite the unquestionable importance of such genetic events, the involvement of epigenetic alterations for melanoma development, and resistance to aforementioned therapies is becoming increasingly apparent. In this chapter, epigenetic alterations commonly found in melanoma are introduced, with a focus on histone and DNA modifications and their relevance for melanoma development, progression and therapy response. Detailed knowledge about this emerging aspect of melanoma research will help to understand the plastic nature of melanoma and set the foundation for novel treatment strategies that target aberrant gene regulation on genetic and epigenetic levels.

Keywords: biomarker, drug resistance, histone modifications, DNA methylation, melanoma, targeted therapy

1. Introduction

The grim prognosis for metastatic melanoma patients and the steadily increasing rates of melanoma incidents, that are projected to continuously rise within the next decades [1], represent a challenge for healthcare systems worldwide and highlight the importance of developing and optimizing prevention strategies, diagnostic approaches and treatment regimes.

After many years of research with unsatisfying treatment options and poor clinical outcomes, last decade has seen major advances in the therapy of metastatic melanoma driven by the revolutionizing discoveries of driver mutations and immune escape mechanisms that contribute to the aggressive nature of this disease. Drugs, developed to specifically exploit these mechanisms, administered either alone or in combination, have been shown to be clinically effective treatment strategies significantly increasing survival rates of patients [2–5]. Despite these recent ground-breaking advances in melanoma therapy, no currently available treatment options are curative in the majority of responding patients nor do all patients with $BRAF^{V600E}$ mutations respond to targeted therapies. Melanoma and targeted inhibition of oncogenic $BRAF^{V600E}$ became the poster child of an exciting initial therapy success followed by long-term resistance, which has also been experienced with other promising novel treatment strategies like immune checkpoint inhibitors targeting CTLA-4 and PD-1. The benefit of these new therapies is limited by the emergence of resistance, ultimately leading to tumor relapse. While the importance of genetic alterations for the development of disease and therapy resistance is unquestionable, it turns out that epigenetic remodeling is a fundamental feature of tumor development and adaption to therapy.

This chapter will briefly introduce the concept of epigenetics focusing on epigenetic alterations, especially changes in histone and DNA modifications during melanoma development and the emergence of therapy resistance. Detailed investigations into these changes will greatly contribute to our understanding of the heterogeneous and adaptive nature of melanoma. A thorough perception of how epigenetic drivers are modulating the genetic landscape will be the foundation for the development of new treatment strategies beyond pathway and immune checkpoint inhibitors.

2. Epigenetic changes

It has long been recognized that chromatin contains information beyond the primary DNA sequence. This information that is stored "on top of" the genetic information is highly dynamic and influences gene expression patterns and phenotypes without altering the nucleotide sequence while maintaining heritability to somatic daughter cells and in some cases even offspring *via* the germline. Multiple epigenetic mechanisms have been identified including ATP-dependent chromatin remodeling, the non-coding RNAs and different histone variants [6]. Here, we focus on the two most well studied aspects of epigenetic gene regulation, DNA methylation and histone modifications.

2.1. DNA methylation

The most well studied form of epigenetic information is stored by direct covalent chemical modification of the DNA itself. Cytosine residues in CpG dinucleotides are methylated at the fifth position generating 5-methyl cytosine (5-mC) (**Figure 1**) without affecting Watson-Crick base pairing and sequence information [7]. This modification is consistently found in most eukaryotic model systems [8]. Generally speaking, DNA methylation is associated with

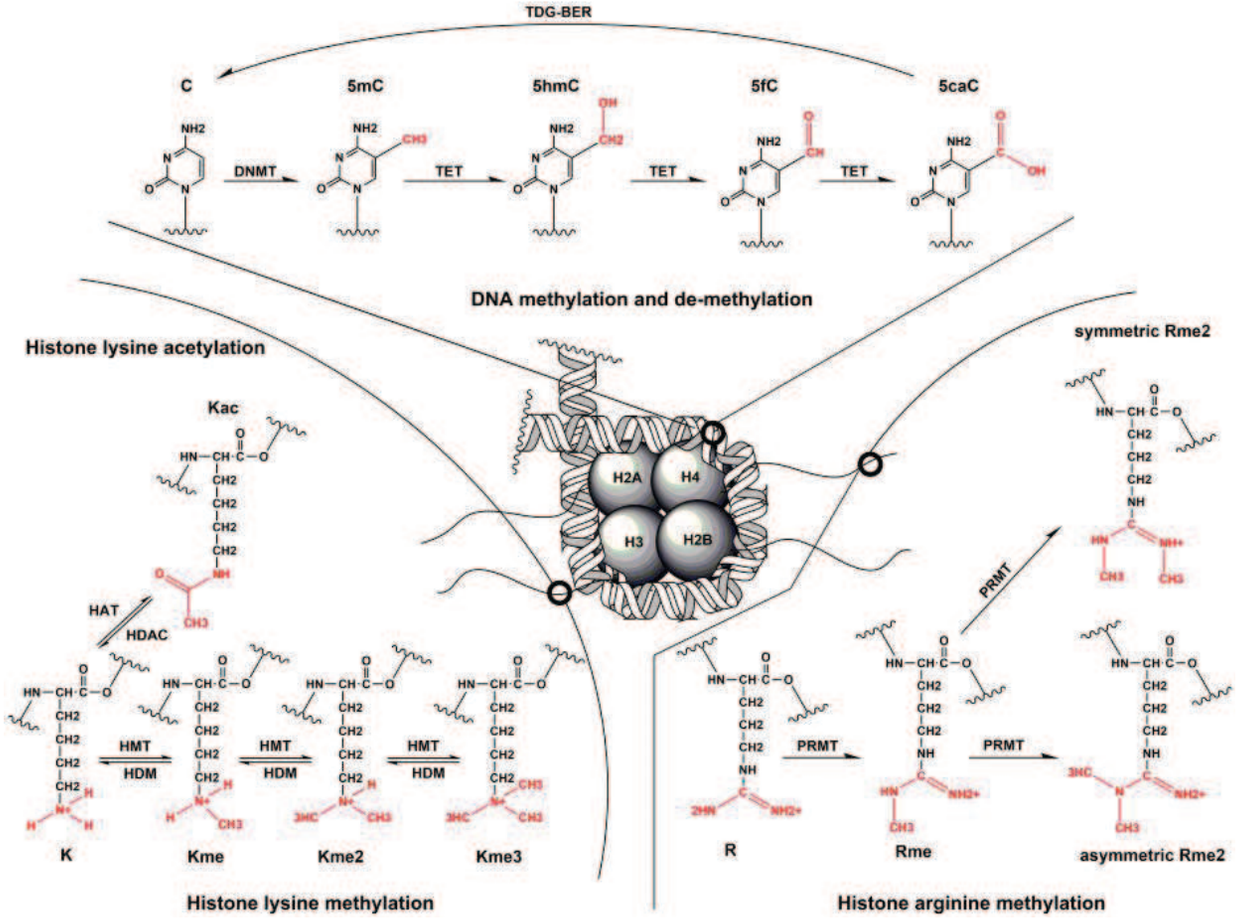

Figure 1. Schematic representation of DNA methylation and histone acetylation/methylation. The basic structural unit of eukaryotic DNA is the nucleosome. A nucleosome includes 147 bp of DNA that is wrapped around a histone octamer consisting of two copies of each core histone protein H2A, H2B, H3 and H4. The N-terminal tails of the histones protrude from the core particle and are subject to posttranslational modifications. Lysine residues (K) of several histone tails can be either acetylated (Kac) by histone acetyltransferases (HAT) or mono-, di- or tri-methylated (Kme, Kme2 or Kme3) by histone methyltransferases (HMT). These modifications can be reversed by corresponding histone deacetylases (HDAc) or histone demethylases (HDM). Additionally, arginine residues (R) can be either mono- or di-methylated (Rme or Rme2), whereby di-methylation can be presented either symmetrically or asymmetrically. Cysteine residues (C) can be directly methylated by DNA methyltransferases (DNMT) resulting in 5-methylcytosine (5mC), which can be further processed as part of active DNA demethylation to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) by ten-eleven translocation (TET) proteins. DNA demethylation is then completed by thymine DNA glycosylase (TDG)-dependent base excision repair (BER).

transcriptional repression [9] and established by DNA methyltransferases namely DNMT1, DNMT3A and DNMT3B. While DNMT1 is responsible for the maintenance of DNA methylation, DNMT3A and DNMT3B catalyze the *de novo* synthesis of 5-mC [10]. Conversely, 5-mC can be removed either by replication-dependent dilution or active DNA de-methylation by ten-eleven translocation (TET) proteins. TET proteins are Fe(II)/α-ketoglutarate-dependent dioxygenases that catalyze the oxidation of 5mC to 5-hydroxymethylcytosine (5hmC) and further to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), which eventually is removed via base excision repair (BER) to restore un-methylated cytosine (**Figure 1**) [11]. Aberrant DNA methylation is a wide spread phenomenon in all cancers [12] suggesting an important role in malignant transformation.

2.2. Histone modifications

Regulatory epigenetic information is also embedded in the basic structure of chromatin and the nucleosome. The nucleosome core particle comprises 147 bp of DNA that is wrapped around an octamer of histone proteins consisting of two copies of H2A, H2B, H3 and H4 (**Figure 1**). Histones, especially the N-terminal tails, are subject to a multitude of posttranslational modifications including acetylation, methylation, phosphorylation, sumoylation, ubiquitylation or O-GlcNAcetylation with new modifications continuously identified [13]. The genome can be classified in transcriptionally active "open" euchromatin and transcriptionally inactive "closed" heterochromatin. Histone lysine acetylation affects this "open" and "closed" states by converting the charge of the affected residue at the histone tail, which decreases the histone/DNA interactions, increases DNA accessibility and therefore facilitates transcription and replication [14]. Alternatively, histone modifications can act as binding motives for transcription factors and other histone-modifying enzymes. For example, bromodomains specifically recognize acetylated lysine residues and are an important part of many chromatin-associated proteins [14]. The second very prominently studied histone modification is methylation of lysine or arginine residues. In contrast to acetylation, methylation can be present in different forms. Lysine residues can be mono-, di- or tri-methylated, while arginine residues can be mono-methylated or symmetrically or asymmetrically di-methylated, neither of which affects the charge of the amino acid side chain (**Figure 1**) [15]. Instead, methylated histone residues are recognized by a plethora of protein domains including plant homeodomain (PHD) zinc fingers, chromodomains, Tudor domains or WD40 repeats [16]. While histone acetylation is generally associated with active transcription, histone methylation has more diverse functions depending on the location of the modification. For example, H3K4me3 or H3K36me3 are usually found in active gene promoters whereas H3K9me3 or H3K27me3 are linked to transcriptional repression [13]. Histone modifications are generally reversible and dysregulation of either 'writers' (e.g. histone acetyltransferase or histone methyltransferases) or 'erasers' (e.g. histone deacetylase or histone demethylases) are attributed to the pathogenesis of human diseases [17].

3. Epigenetics in melanoma initiation and development

High-throughput DNA sequencing enabled detailed investigations into the genetic makeup of cancer and revealed hundreds of genes that are frequently mutated in melanoma [18]. Among these, a set of driver mutations has been identified that allows melanocytes to proliferate excessively, to overcome senescence and to divide indefinitely, resulting in their transformation into melanoma [19]. Despite the undeniable importance of genetic events, detailed knowledge of the molecular mechanisms of tumor initiation is still absent. This is due to the fact that such events, like epigenetic changes, are challenging to observe because models that represent individual stages of melanomagenesis are required. Nevertheless, the importance of epigenetic dysregulation in melanoma development becomes increasingly apparent, which is emphasized by the high frequency of mutations found in epigenetic regulators [20].

3.1. DNA methylation in melanoma development

One model used to investigate epigenetic alterations during melanoma development utilizes sequential cycles of anchorage blockade to transform mouse melanocytes resulting in cell lines that show different degrees of aggressiveness and *in vivo* tumor growth potential, to mimic different stages of melanomagenesis [21]. Investigating DNA and histone modifications in this model showed substantial epigenetic changes as global DNA methylation was decreased while multiple histone modifications including H4K16ac, H3K4me3, H3K27me3 and H3K9me3 were increased [21]. These findings are consistent with data from melanoma cell lines showing global hypo-methylation compared to melanocytes [22–24] with 11 out of 14 types of repetitive DNA elements being hypo-methylated [22]. Considering that repetitive DNA sequences constitute more than 45% of the human genome [25], changes of their methylation patterns affect the readout of global DNA methylation the most. Demethylation of these repetitive DNA elements has been reported to negatively influence chromatin organization, increase genetic instability or result in gene deregulation, all of which can promote tumorigenesis [26–28]. Microarray analysis of 27 common benign nevi and 22 primary invasive melanomas that covered 1505 CpG sites of regulatory regions of 807 cancer-related genes identified 26 CpG sites, associated with 22 genes that showed significant methylation differences. Of these 26 CpG sites, 19 showed significant hypomethylation with 7 hypermethylated [29].

While it appears that global DNA methylation levels are decreased during melanocyte transformation, many gene-specific CpG islands are hypermethylated. Comparing 24 primary cutaneous melanomas and 5 benign nevi using the Infinium BeadChip technology covering 27,578 CpG loci in the promoter regions of 14,495 genes identified 106 hypermethylated and 44 hypomethylated CpG islands. Among the 106 hypermethylated genes, *MAPK13*, which encodes the p38 isoform, has been found to have tumor suppressive functions as retrovirus-mediated overexpression of this gene displayed cytostatic effects and reduced melanoma growth *in vitro* [30]. Another interesting target gene that has been found to be regulated by DNA hypermethylation is the master regulator of pigmentation, MITF [31]. Lauss, et al. showed that hypermethylation of CpG islands belonging to *MITF* or MITF target genes correlated with decreased expression in metastatic melanoma tumors and melanoma cell lines. Melanoma cell lines that show intrinsically low *MITF* expression displayed CpG hypermethylation while cell lines that show high endogenous *MITF* levels were characterized by hypomethylation of these CpG islands. Accordingly, treatment with the DNA methyltransferase inhibitor 5′-Aza-2′-Deoxycytidine resulted in re-expression of MITF in MITF low cell lines. However, this re-expression was not sufficient to induce expression of the MITF target gene MLANA, suggesting that DNA methylation is involved but not sufficient to regulate MITF pathway activity in melanoma [31].

Differences in DNA methylation between melanocytes and melanoma can also be attributed to mutant BRAF, the most frequently mutated gene in melanoma [32]. Knockdown of BRAF in BRAF-mutant melanoma cell lines resulted in profound alterations of the methylation landscape with changes in gene expression affecting proliferation and invasion. Furthermore knockdown of BRAF significantly decreased DNMT1 and EZH2 expression suggesting that

$BRAF^{V600E}$-mediated pathway activation has a profound influence on the epigenetic landscape [33]. Analyzing $BRAF^{V600E}$ and $BRAF^{WT}$ samples from The Cancer Genome Atlas (TCGA) revealed that $BRAF^{V600E}$ correlates with global DNA hypomethylation. Primary melanoma samples showed a significantly decreased expression of *DNMT3A*, which is mainly responsible for *de novo* DNA methylation. Interestingly, *DNMT3A* expression was not found to be decreased in BRAF mutant BRAF wild type metastatic melanoma samples, suggesting that downregulation of this DNA methylatransferase is a transient event that might be important for melanoma initiation but not for metastatic spread and maintenance of global DNA hypomethylation [34]. Furthermore, Fang, et al. showed that $BRAF^{V600E}$ drives DNA hypermethylation and gene silencing of specific target genes in a v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog G (MAFG) dependent manner in colorectal cancer and melanoma [35]. Specifically, MAFG is phosphorylated by the BRAF downstream kinase ERK1. This phosphorylation increases protein stability by reducing polyubiquitination and subsequent proteasomal degradation. MAFG then binds target gene promoters and recruits co-repressors including DNMT3B, ultimately resulting in DNA hypermethylation and gene silencing [35]. Unsupervised clustering of DNA methylation data from metastatic melanoma samples and TCGA melanoma samples identified three subgroups of melanoma tumors with differential methylation patterns [36]. These clusters did not correlate with either *BRAF*- or *NRAS*-mutation status nor primary tumor features like Breslow thickness or Clark's level, suggesting that factors beyond BRAF regulate DNA methylation in melanoma. The hypermethylated cluster (MS1) was associated with cell proliferation while the cluster with the lowest methylation levels (MS3) was associated with immunity, indicating a fundamental role of DNA methylation on melanoma and the microenvironment [36].

3.2. Histone modifications during melanoma development

Remarkable insights into the importance of histone modifications for melanoma development have been revealed using a zebrafish model in which the human $BRAF^{V600E}$ gene is under the control of the *mitfa*-promoter crossed onto a p53 loss-of-function background. This model develops nevi that eventually progress into melanoma [37]. Like in most genetically engineered animal models, only a small fraction of genetically identical melanocytes transform into melanoma, highlighting the importance of molecular events beyond genetic alterations to drive melanoma development. To address this problem and investigate melanoma initiation in more detail, Kaufman, et al. developed a triple transgenic zebrafish model (p53/BRAF/crestin:EGFP) in which a crestin/enhanced green fluorescent protein (crestin:EGFP) allows the visualization of neural crest stem/progenitor cells, the precursors of melanocytes [38]. Melanomas, which developed in these animals reestablish crestin:EGFP expression indicating that these cells reverse into a neural crest progenitor state. Knockout of sox10, a master regulator of neural crest identified and regulated by acetylation of histone 3 lysine 27 (H3K27Ac), significantly delayed melanoma onset. H3K27Ac in super enhancers at the *SOX10* locus was also found to be enriched in human melanoma cell lines indicating that epigenetic regulation of SOX10 expression is an important step of melanoma initiation [38].

Several histone-modifying enzymes have been shown to function aberrantly and contribute to melanoma progression. The H3K9me3-specific histone methyltransferase SET domain bifurcated 1

(SETDB1) is recurrently amplified within a region of chromosome 1 and shows a high expression in melanoma compared to nevi or normal skin [39]. Using the same zebrafish model as described above (*mitfa*-promoter crossed into a p53 loss-of-function background), Ceol, et al. identified that *SETDB1* amplification accelerates melanoma onset and increased invasivness. This was found to be independent of SETDB1 enzyme activity. Instead SETDB1 is part of a multimeric H3K9 methyltransferase complex including the H3K9me3 methyltransferase SUV39H1. Overexpression of SUV39H1 in the same zebrafish model also resulted in accelerated melanoma onset suggesting analogue functions of the entire H3K9 methyltransferase complex influencing melanoma development, at least partially by abrogating oncogene-induced senescence [39].

Another deregulated histone-modifying enzyme during melanoma development is the H3K27me3-specific histone methyltransferase enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2). EZH2 and H3K27me3 have been found to be elevated in aggressive melanoma cell lines and metastatic tumor samples. The expression of tumor suppressors RUNX3 and E-cadherin was found to be suppressed by EZH2 dependent H3K27me3 [40]. Accordingly, EZH2 is a major factor for melanoma initiation and progression. Knockout of EZH2 in a genetically engineered $NRAS^{Q61K}$ melanoma mouse model reduced the number of melanomas and prevented metastasis formation [41]. Mechanistically, this was mediated by EZH2 target genes including deoxycytidine kinase (*DCK*), adenosylmethionine decarboxylase 1 (*AMD1*) and WD repeat domain 19 (*WDR19*) which are suppressed in H3K27me3-dependent manner [41]. A possible explanation for EZH2 facilitating melanoma development could be by enabling senescence evasion. Knockdown of EZH2 in melanoma cells reestablished a senescence phenotype partially by reactivating p21/CDKN1A transcription, which was found to be independent of H3K27me3. Instead, at the transcriptional start site of p21/CDKN1A, H3K14ac was increased as a result of decreased recruitment of histone deacetylase 1 (HDAC1), which correlated with transcriptional activation [42]. This is in line with reports that EZH2, as part of the Polycomb repressive complex 2 (PRC2), is able to recruit histone deacetylases, which shows functional synergy with H3K27me3 in mediating target gene silencing [43]. Later, it was found that the non-canonical NF-kB pathway regulates EZH2 expression by direct binding of NF-kB2 to the EZH2 promoter. Inhibition of NF-kB2 induced a senescence-like phenotype, which was reversible upon EZH2 re-expression [44]. Not suprisingly, pharmacological inhibition of EZH2 has been shown to impair melanoma growth *in vitro* and *in vivo* [41, 44, 45] and has emerged as a interesting target in multiple cancer types [46].

A more specific example how histone modifications promote melanoma progression is found in the case of the telomerase reverse transcriptase (TERT). Recently, activating *TERT* promoter mutations result in new transcription factor binding sites within the promoter, have been identified in up to 71% of all melanomas, which equals or even exceeds the frequency of *BRAF* and *NRAS* mutations, suggesting a key role for this genetic alteration in melanoma development [47]. Interestingly, mutations in the *TERT* promoter frequently co-occur with BRAF mutations [48–50]. In fact it was found that MAPK pathway inhibition decreased H3K4me3 and H3K9ac in the mutant *TERT* promoter region. This resulted in loss of RNA polymerase II (Pol II) recruitment and decreased *TERT* transcription. Mechanistically, ERK2 directly binds mutant *TERT* promoters and inhibits HDAC1 repressor complex recruitment, which results in active *TERT* transcription [51].

A systematic overview of the epigenomic landscape of two phenotypically distinct melanocyte cell models that are characterized by low or high tumorigenicity showed distinct chromatin states associated with melanomagenesis. Specifically, chromatin state transitions characterized by loss of histone acetylation marks like H3K27Ac, H2BK5Ac and H4K5Ac and di-/trimethylation of H3K4 in regulatory domains associated with signaling pathways important for melanoma including phosphatidylinositol 3-kinase (PI3K), interferon (IFN) γ-, LKB1-, TRAIL- and platelet-derived growth factor (PDGF)-mediated signaling was observed, again emphasizing the link between epigenetic changes and melanoma development and progression [52].

Especially, loss of H3K4 methylation seems to be a key factor for melanoma growth and the highly problematic intratumor heterogeneity frequently observed in melanoma [53]. The histone 3 K4 demethylases jumonji/ARID1 (JARID1/KDM5B/PLU-1/RBP2-H1) defines a subpopulation of slow cycling melanoma cells, which is important for continuous growth of melanoma tumors. Interestingly, this subpopulation was found to be highly dynamic, as isolated KDM5B-positive and negative melanoma cells give rise to a heterogenous population consisting of both subpopulations [54] which highlights the variable nature of the epigenetic landscape in melanoma.

3.3. Epigenetic modifications as biomarkers and prognostic factors in melanoma

Because of the profound differences in DNA methylation patterns between melanocytic nevi and melanoma, several studies have investigated the suitability of DNA methylation as a predictive biomarker in melanoma. Unsupervised hierarchical clustering of 27 common benign nevi and 22 primary invasive melanomas resulted in separation of the two sample cohorts. Specifically, 22 genes were identified that significantly distinguished melanomas from nevi whereas 14 of these genes were validated in a separate set of 25 melanomas and 29 nevi [29] suggesting that analysis of differential DNA methylation patterns could be used as melanoma biomarkers. Later on Gao, et al. investigated the methylation differences of common nevi, dysplastic nevi, primary melanomas and metastatic melanomas and established a diagnostic algorithm based on promoter methylation patterns of *CLDN11, CDH11, PPP1R3C* which was able to distinguish dysplastic nevi from melanomas with a specificity of 89% and sensitivity of 67% [55]. DNA methylation changes, however, are not limited to melanoma development (nevi *versus* primary melanoma) but are also apparent in melanoma progression (primary melanoma *versus* metastatic melanoma). DNA methylation profiling using Illumina Infinium Human Methylation 450 K Beadchips of 14 normal nevi, 33 primary melanomas and 28 melanoma metastases identified gene promoters that were hypermethylated during melanoma development or melanoma progression [56]. Promoter methylation of several identified genes including *HOXA9, MEOX2, RBP1, TFAP2B, TWIST1* and *AKT3* were shown to be suitable biomarkers to distinguish between nevi, primary and metastatic melanoma. AKT3 and TFAP2B protein expression was also confirmed as biomarkers suitable for staining by immunohistochemistry. Furthermore, Wouters, et al. were able to correlate hypomethylation of *MEOX2, OLIG3* and *PON3* promoter hypomethylation with increased overall free survival [56]. Another major player in melanoma development that has been shown to be regulated by DNA methylation is Phosphatase and Tensin Homolog (*PTEN*). *PTEN* inactivating mutations or deletions have been found in 12% of the

TCGA melanoma cohort [57]. However, loss of PTEN expression is a more frequent event as reduced expression has been observed in approximately 50% of stage IIIB/C melanomas with a complete loss in 20–25% of all samples which correlated with decreased overall survival [58]. Accordingly, *PTEN* promoter methylation was found in 60.7% in the TCGA melanoma cohort and was an independent predictor for impaired patient survival [59]. Besides gene-specific DNA methylation, hypomethylation of repetitive DNA elements has also been associated with patient survival. Two studies report contradicting findings. Sigalotti, et al. analyzed cell lines isolated from 42 stage IIIC patients and reported that hypomethylation of 2 out of 3 CpG sites within Long Interspersed Nucleotide Element-1 (LINE-1) sequences correlated with improved prognosis and 5 year overall survival [59]. In contrast, Ecsedi, et al. extracted genomic DNA from primary melanoma and found that hypomethylation of 6 CpG sites associated within LINE-1 sequences in 46 primary melanomas correlated with decreased relapse-free survival of the corresponding patients and was also found to be associated with increased metastatic capacity [60]. A possible explanation for these contradictory results could be fundamental differences in the way how samples were analyzed. Sigalotti, et al. isolated and cultured melanoma cells from primary tumor tissue which might have affected the DNA methylome leading to the observed differences.

Besides changes in DNA methylation (5-mC), genome wide loss of the DNA demethylation intermediate 5-hydroxymethylcytosine (5-hmC) has recently been found to be a hallmark of melanoma [61]. Specifically, it has been shown that 5-hmC levels are progressively lost in melanoma compared to benign nevi, which was accompanied by decreased expression of TET family members and IDH2. Re-establishing 5-hmC by overexpression of TET2 reduced tumor growth and invasion suggesting an important function for 5-hmC in melanoma pathology. Accordingly, high levels of 5-hmC were found to negatively correlate with Breslow depth and predict better survival [61]. These findings were confirmed later on and suggest that 5-hmC analysis by immunohistochemistry could be a promising candidate as a prognostic biomarker in melanoma [62].

Presumed correlations between histone modifications and melanoma progression with prognosis have not been investigated compared to DNA methylation. This is in part because of technical challenges eminent by direct assessment of histone modifications [63]. Martinez, et al. performed immunohistochemical analyses of 10 benign nevi, 25 primary cutaneous melanomas without metastases, 19 primary cutaneous melanomas with metastases and 33 metastatic melanomas using an antibody specifically detecting H3K79 trimethylation and H3T80 phosphorylation (H3K79me3T80ph). They found a significant increase of H3K79me3T80ph in melanoma compared to nevi seemingly identifying a subset of primary melanomas with metastatic potential [64]. Another strategy to utilize histone modifications as biomarkers and prognostic factors that avoids the technical difficulties of direct assessment of histone modifications is to investigate the expression levels of histone-modifying enzymes. Along this line, it has been reported that the expression of the H3K27-specific histone methyltransferase EZH2 is increased during melanoma progression. However, only metastatic melanomas showed a significant increase compared to nevi [65]. Accordingly, analyses of EZH2 expression of TCGA melanoma samples showed a significantly shorter survival of patients with high EZH2 expression. Additionally, EZH2 high patients developed distant metastases faster, suggesting

a role for EZH2 in metastasis formation [41]. In contrast to EZH2, KDM5B has been found to be significantly downregulated during melanoma development. About 70% of the investigated nevi samples showed a KDM5B expression compared to 10 and 30% in primary and metastatic melanoma samples, respectively [66].

To our knowledge and despite the wealth of epigenetic changes that differentiate melanocytes and melanoma, no epigenetic biomarkers are used in the clinic to date.

4. Impact of epigenetic modifications on melanoma therapy

4.1. Acquired drug resistance, an obvious problem in melanoma therapy

Despite tremendous advances in developing innovative cancer therapies within the last few years, mechanisms for treatment failure are still not fully understood. Targeted inhibition of oncogenic $BRAF^{V600E}$ melanomas became the poster child of exciting initial therapeutic responses unfortunately followed by long-term resistance. Development of therapy resistance is the major obstacle for the successful use of targeted therapies, where almost all patients, who respond initially, are relapsing, irrespectively of single or combined inhibition of the MAPK pathway [67]. Furthermore, 15–20% of mutant *BRAF* tumors do not respond to targeted therapy in the clinical setting [68], suggesting the presence of pre-existing resistance mechanisms. Resistance to MAPK pathway inhibition has been shown to involve emergence of genetic mutations in *RAS* or *MEK*, amplification of mutant *BRAF* or alternative BRAF splicing [69, 70]. However, such genetic resistance mechanisms are absent in approximately 40% of patient samples, indicating the involvement of other mechanisms contributing to therapy failure [67]. Among these mechanisms, the upregulation of CRAF [71] or the SOX10-mediated activation of TGF-β that results in increased EGFR and PDGFRβ expression [72] that have been reported to mediate non-genetic resistance. Elevated EGFR and PDGFRβ levels have been shown to be reversible after discontinuing BRAF and MEK inhibitor treatment, while expression of EGFR or treatment with TGF-β resulted in a slow cycling drug-resistant phenotype [72]. This observation reflects findings by our group [73] and others [74, 75] of reversible multidrug-tolerant slow-cycling state following stressors like drug treatment. Beside failure of BRAF inhibition, a recent study found that dynamic and recurrent non-genomic alterations following chronic BRAF inhibitor treatment also affect tumor immunity possibly resulting in cross resistance to anti PD-1 therapy [76].

Even though immunotherapies like IL-2, adoptive T-cell transfer or antibodies that block CTLA-4 or PD-1 have shown long-term responses in some patients [77–80], many patients eventually relapse as melanoma cells escape immune surveillance. Genetic mechanisms like loss or mutation of specific antigens or parts of the major histocompatibility complexes that are involved in antigen presentation, have been attributed to immune evasion [81]. More recently, loss of function mutations in interferon-receptor signaling and in antigen presentation have been linked to resistance to PD-1 inhibition in three of four investigated patients [82]. Beside these genetic alterations that cause immunotherapy resistance, the expression of several melanoma antigens is linked to the dynamically regulated expression of NGFR

[83] or can be reversibly lost in response to inflammation [84]. Another study found a correlation between a mesenchymal transcription signature, including WNT5A and ROR2, with resistance to anti-PD-1 therapy in metastatic melanoma [85] suggesting the involvement of epithelial-mesenchymal transition in immunotherapy failure.

In the following paragraphs, the current knowledge about epigenetic mechanisms contributing to drug resistance in melanoma is summarized.

4.2. Epigenetic alterations and targeted therapy

One of the most clinically relevant observations that point towards non-genetically regulated drug resistance is the concept of drug holidays, which describes the phenomenon of intermittent treatment schedules or treatment interruption. This delays the emergence of resistance. One of the first reports describing the benefit of treatment interruption was a case study of a patient diagnosed with an adenocarcinoma of the lungs. After initial chemotherapy, the patient enrolled in a phase I study of the orally active epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor gefitinib. After 18 month of drug response, the disease eventually progressed and was treated with a different combination of chemotherapy. One year after discontinuation of the initial treatment, gefitinib re-treatment resulted in a significant response [86]. Similar observations were further reported for patients treated with BRAF or BRAF/MEK inhibitors in which re-treatment with BRAF inhibitors resulted in a significant response after disease progression during an earlier treatment with BRAF or BRAF/MEK inhibitors [87]. A multi-institutional retrospective study later on found that 43% of patients that received re-treatment with BRAF inhibitors after disease progression and treatment interruption showed a clinically significant response [88]. Studies using vemurafenib-naive, primary human-patient-derived melanoma xenograft mouse models showed that vemurafenib resistance could be delayed by intermittent dosing schedules compared to continuous treatment [89].

The reversibility of drug resistance observed in clinical settings matches well with findings of slow cycling subpopulations that have been found to allow for reversible drug tolerance *in vitro.* One of the first reports of such a drug-tolerant subpopulation showed that a very small fraction of cancer cells including melanoma survives treatment with drug concentrations 100-fold higher than the IC50 [74]. These surviving cells were found to be mainly quiescent and in G1 arrest, they eventually continued growth in the presence of the drug. Importantly, drug withdrawal re-sensitized these drug-tolerant cells and re-established the same cellular heterogeneity as found in the initial sensitive population. Mechanistically, the surviving drug-tolerant cells exerted an altered chromatin state with increased expression levels of the histone demethylase KDM5A (JARID1A/RBP2) and concomitantly reduced levels of H3K4me2/3. RNAi-mediated knockdown of KDM5A confirmed that this histone demethylase is important for the establishment of the reversible drug-tolerant state [74]. This observation of an epigenetically regulated mainly G1 arrested state surviving exposure to high drug concentrations is similar to the previously mentioned slow cycling KDM5B^{high} subpopulation that is important for continuous melanoma growth [54]. KDM5B^{high} cells have been found to be enriched upon drug treatment and resemble a slow cycling drug-tolerant state in melanoma

as shRNA-mediated knockdown of KDM5B increased sensitivity to different drugs [90]. In accordance with the dynamic nature of KDM5A and KDM5B positive subpopulations, we have observed that chronic exposure to external stressors, rather than specific drug treatment, initiates an innate cellular response whereupon cells adopt a slow cycling, multidrug-tolerant phenotype [91]. Continuous exposure of melanoma cells to sub lethal BRAF inhibitor concentrations for 12 days initiated a cellular transformation and not the selection of a pre-existing subpopulation, which resulted in a slow cycling, mainly G1 arrested phenotype. These so called induced drug-tolerant cells (IDTCs) were unresponsive to 20-fold higher BRAF inhibitor concentrations as well as multiple other drugs including the MEK inhibitor GSK1120212 or cisplatin. As demonstrated for the KDM5A^{high} subpopulation [74], IDTCs re-gained drug sensitivity upon 7 days of drug withdrawal. On the molecular level IDTCs displayed elevated expression of drug efflux genes including *ABCB5*, *ABCA5*, *ABCB8* and *ABCB4*, as well as melanoma stem cell markers *NGFR*, *SOX10*, *CD44*, *SOX2* and *SOX4*, suggesting the transition into an undifferentiated state [91]. These molecular changes were accompanied by a profound decrease of histone marks H3K4me3, H3K27me3 that were decreased and H3K9me3, which was increased. Accordingly, expression of several histone-modifying enzymes including the H3K27-specific demethylases, KDM6A, KDM6B and the H3K4-specific demethylases, KDM1B, KDM5A and KDM5B was increased at the IDTC state [91]. Interestingly, a similar transition into an H3K4me3^{low}/H3K27me3^{low}/H3K9me3^{high} state was triggered by hypoxia and nutrient starvation and IDTCs generated by these stressors exhibited tolerance to BRAF inhibitors or cisplatin treatment, suggesting an epigenetically regulated drug-independent generic stress response that allows cells to cope with difficult environmental conditions [91]. Similar to our proposed IDTCs, a slow cycling, reversible NGFR^{high} state that displays features of de-differentiation has also been described, which has been shown to be susceptible to inhibition of epigenetic modifiers as bromodomain inhibitors, that block recognition of acetylated histones, suppressed the slowly cycling NGFR^{high} state [92].

Multiple studies proposed strategies to target the slow cycling drug-tolerant phenotype. Sharma, et al. showed that the KDM5A^{high} subpopulation that emerged after exposure to very high drug concentrations was susceptible to histone deacetylase (HDAC) inhibitors [74] because KDM5A is associated with histone decatylases during removal of histone modification marking active transcription [93]. HDAC inhibitors induced apoptosis in this subpopulation and combination of HDAC inhibitors with other drugs prevented the emergence of acquired resistance. Interestingly, HDAC inhibitors have to be present during the cytotoxic treatment as pre-treatment with histone deacetylase inhibitors followed by exposure to cytotoxic drugs alone was not sufficient to block acquired resistance [74]. This is important as it suggests that drug resistance is not mediated by a pre-existing subpopulation that carries intrinsic resistance mechanisms like additional mutations that can be eradicated, but by a dynamically regulated adaptive response that allows cancer cells to withstand unfavorable and toxic conditions. Roesch, et al. found that the KDM5B^{high} population enriched upon drug treatment in melanoma is dependent on oxidative phosphorylation as several members of the electron transport chain, including NADH dehydrogenase, ubiquinol cytochrome c reductase, cytochrome c oxidase and ATP synthase are highly expressed in these cells [90]. They further described that inhibition of the mitochondrial respiratory chain using oligomycin,

rotenone or phenformin blocked endogenous KDM5B expression and decreased the drug-induced enrichment of KDM5B^{high} cells. Furthermore, combination of orally available NADH dehydrogenase inhibitor phenformin with BRAF inhibitor vemurafenib increased the tumor suppressive effects *in vivo* [90]. In the same year, Yuan, et al. showed AMPK-dependent synergistic cytotoxicity of combining BRAF inhibitors and phenformin which also suppressed the emergence of a drug-resistant phenotype [94].

The IDTC phenotype described by us is characterized by elevated expression of several histone-modifying enzymes showing no specific susceptibility to combinations of BRAF inhibitors with HDAC inhibitors, AKT inhibitors or oligomycin [91]. In accordance with previous studies, knockdown of KDM5B-sensitized melanoma cells to BRAF inhibition, but the surviving cells again displayed the IDTC phenotype. Exposure of established IDTCs to different drugs including MEK, AKT and HDAC inhibitors showed that these compounds effectively suppressed their target pathways within 3 days of treatment. However, slow cycling melanoma cells were able to adapt to this additional stressor and re-activated the respective pathways within 12 days of drug exposure. In the case of HDAC inhibitors, methylation patterns of histone 3 lysine 4 and 9, which have been shown to be co-regulated with histone acetylation via transcriptional regulation of histone methyltransferases and histone demethylases [95, 96] were re-established to resemble the H3K4me3^{low}/H3K9me3^{high} pattern seen in the slow cycling multidrug-tolerant cells [91]. A possible explanation for the discrepancy between the discussed studies in regards to the different strategies to target heterogenous slow cycling populations could be that the KDM5A^{high} or KDM5B^{high} cells are stringently selected subtypes of the slow cycling phenotype whereas IDTCs are characterized by multiple epigenetic modifiers, most likely including multiple subtypes that contribute to the same phenomenon. The dynamic signaling rewiring observed in the IDTC phenotype is reminiscent of the diverse drug resistance mechanisms that have been reported to emerge from slow cycling EGFR inhibitor addicted lung cancer cells [75], which suggests that an adaptive response as described for IDTCs in melanoma might be present in multiple cancer types. One key feature of all slow cycling drug-tolerant cell populations that emerge after 3–12 days of drug exposure is the reversibility upon drug withdrawal. However long-term exposure (90 days) of melanoma cells to BRAF inhibitors resulted in loss of the IDTC markers NGFR as well as KDM5B [91]. Interestingly, these cells displayed no multidrug resistance but maintained resistance to BRAF inhibitors despite drug withdrawal, suggesting the emergence of permanent resistance [91].

4.3. Epigenetic alterations and immunotherapy

Epigenetic regulation is a key mechanism for maintaining immune cell identity and differentiation. For example, CD8 positive cytotoxic T lymphocytes undergo dynamic changes of DNA methylation and histone modification patterns following infection that are important for regulation and maintenance of their differentiation states [97]. Therefore, it is important to consider that epigenetic targeting drugs will not only affect tumor cells but also influence immune cells and other cells of the tumor microenvironment. Herein, the effects of epigenetic alterations within cancer cells, specifically melanoma, and how these changes affect the therapeutic effect of immunotherapy will be discussed.

The most promising immunotherapies currently in clinical use are anti-PD-1 and PD-L1 therapies [98]. Analyses of 52 immunotherapy-naïve stage III melanomas specimens in regard to the PD-L1 expression suggested that PD-L1 negative status is associated with worse prognosis and a poor immune response gene signature. PD-L1 positive melanomas showed a significant association with the TCGA hypomethylation cluster suggesting that upregulation of immune checkpoint inhibitors is found in cancer cells with altered gene expression. Another study showed that treatment with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine activates a viral defense pathway. Expression levels of these viral defense genes grouped different cancers including melanoma into separate categories where high expression was associated with the TCGA immune reactive (IMR) tumors with a good prognosis [99]. Melanoma patients with high levels of the viral defense signature correlated with response to anti-CTLA-4 for more than 6 month and combined treatment of 5-aza-2′-deoxycytidine and anti-CTLA4 immune checkpoint therapy in a B16-F10 mouse melanoma model enhanced tumor responses [99]. Another important factor for the successful immunotherapy response is the expression of tumor-associated antigens [100]. Along this line, it has been shown that the expression of high molecular weight-melanoma associated antigen (HMW-MAA) is regulated by DNA methylation as its expression correlates with promoter methylation. As such it is induced by treatment with 5-aza-2′-deoxycytidine [101].

Multiple studies reported the importance of histone modifications for the regulation of immunogenic factors. For example, H3K4me3 dependent PD-L1 expression has been observed in pancreatic cancer [102] or H3K27me3 and DNA methylation-mediated silencing of Th1-type chemokines CXCL9 and CXCL10 in ovarian cancer cells [103], suggesting an important role for histone modifications in the regulation of immunomodulatory factors across different cancer types. Further evidence of epigenetically regulated PD-L1 expression is provided by studies using HDAC inhibitors in melanoma cell lines. Specifically, treatment with class I HDAC inhibitors resulted in increased acetylation of histone 3 in PD-L1 and PD-L2 promoter regions, which resulted in increased PD-L1 expression *in vitro* and *in vivo* [104].

5. Conclusion

Keeping in mind the wealth of data describing epigenetic alterations during melanoma development and also in relation to the therapeutic response targeting or co-targeting these epigenetic events appears to be a very promising strategy for improving melanoma management. This is especially true in light of the highly heterogeneous and adaptive nature of melanoma which cannot be explained only by stable genetic events. While epigenetic biomarkers have not yet been put to clinical use, there is an overwhelming number of clinical trials utilizing and testing epigenetic drugs in different cancer types. These trials investigate the use of general epigenetic inhibitors targeting histone deacetylases, bromodomain and extra-terminal (BET) proteins (histone acetylation binding proteins) and more specific inhibitors targeting DNMT1, IDH1 and IDH2 (affect TET enzyme function), EZH2, DOT1L (histone H3K79 methyltransferase) or KDM1A [105].

Additionally, epigenetic drugs are tested in combination with already established chemo-, targeted- and immunotherapies. Besides synergistic effects of these drugs, this approach could also result in prevention or reversion of drug resistance, a concept that has already been shown *in vitro* more than 15 years ago [106]. In melanoma, one clinical trial is currently investigating the combination of the BRAF/MEK inhibitors vemurafenib and cobimetinib with the DNA hypomethylating agent decitabine (NCT01876641). However, the main focus in the field appears to be the combination of epigenetic drugs, especially DNA methyltransferase and histone deacetylase inhibitors with immunotherapy, which is currently tested in numerous clinical trials [107] and the outcome of these promising approaches is highly anticipated.

While these current clinical trials hold great promise, improved understanding of detailed epigenetic mechanisms, identification of new key players in epigenetic remodeling and the subsequent development of specific inhibitors, which modulate and target epigenetics have the potential to shape the future of melanoma therapy.

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