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REVIEW

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Epigenetic regulation of immune escape genes in cancer

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Abstract According to the concept of immune surveillance, the appearance of a tumor indicates that it has earlier evaded host defenses and subsequently must have escaped immunity to evolve into a full-blown cancer. Tumor escape mechanisms have focused mainly on mutations of immune and apoptotic pathway genes. However, data obtained over the past few years suggest that epigenetic silencing in cancer may be as frequent a cause of gene inactivation as are mutations. Here, we discuss the evidence that tumor immune evasion is mediated by non-mutational epigenetic events involving chromatin and that epigenetics collaborates with mutations in determining tumor progression. Since epigenetic changes are potentially reversible, the relative contribution of mutations and epigenetics, to the gene defects in any given tumor, may be a factor in determining the efficacy of treatments. We review new developments in basic chromatin mechanisms and in this context describe the rationale for the current use of epigenetic agents in cancer therapy and for a novel epigenetically generated tumor vaccine model. We emphasize that epigenetic cancer treatments are currently a 'blunt-sword' and suggest future directions for designing chromatin-based programs of potential value in the diagnosis and treatment of cancer.

Keywords Epigenetics · Chromatin · Immune escape · Tumor immunity · Tumor vaccine · Clinical trials

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Abbreviations ChIP: Chromatin immunoprecipitation · CIITA: Class II transactivator · DNMTi: DNA methyltransferase inhibitors · HAT: Histone acetyltransferase · HDACi: Histone deacetylase inhibitor · Treg: Regulatory T cell · HMT: Histone methyltransferase · HP1: Heterochromatin protein 1 · MBP: Methyl binding protein · Pc: Polycomb complex · PRMT: Protein arginine methyl transferase · RIT: RNAi transcriptional complex

Introduction

Most discussions of immune escape in cancer have centered on mutations and on the potential relation of structural defects in genes to immune function in vivo. However, in only select cases have the mutations have been definitively shown to be responsible for an in vivo immune defect in function [1] and for most tumors the relation between the mutant gene and the escape process is uncertain. As will be discussed in detail here, in addition to the mutations, epigenetic silencing of genes is also potentially important in cancer and has recently been recognized in multiple tumor types. Although hard to precisely quantify, epigenetic silencing of immune genes in cancer may be as frequent a cause of gene repression as are mutations. Numerous studies have identified human and mouse genes epigenetically regulated in cancer [reviewed in 2], and several excellent reviews have focused on the epigenetics of immune genes particularly in regulating T and B cell differentiation [3–5]. Table 1 lists some of the major immune genes and processes that are epigenetically regulated. The evidence for epigenetic regulation has come primarily from both the direct analysis of changes in chromatin structure in relation to gene transcription and from the determinations of the covalent modification of histones and transcription factors that regulate the expression of specific genes. Chromatin immunoprecipitation (ChIP) assays have identified histone modifications at promoters and

Table 1 Examples of epigenetic regulation of immunity

VDJ recombination
Ig expression—μ and κ chain enhancers, isotype switching
Th1 and Th2 differentiation
Allelic silencing
B cell maturation
Cytokine gene expression IFN-γ, IL-12, IL-10, others
MHC class I, II, CIITA expression
Costimulatory genes (CD40, CD80, CD86, others)
NKG2D ligand expression

determined the factor and co-factor binding patterns to promoter sequences associated with activation or repression of genes. Monitoring gene effects following treatment with chemical agents that alter the covalent signature of histones and DNA (epigenetic agents) has furthered our understanding of the potential role of epigenetics in cancer and other diseases.

The rationale for the current clinical trials in cancer with histone deacetylase inhibitors (HDACi) has primarily focused on their ability to inhibit growth and to induce cell differentiation [6]. Trichostatin A (TSA), one of the first HDACi described [7] was isolated from *Streptomyces hygroscopicus* and subsequently a variety of natural and synthetic HDACi have been studied in vitro and in various clinical models (reviewed subsequently and in Table 2). DNA methylation is also epigenetically regulated and the underlying principle in using DNA methyltransferase inhibitors (DNMTi) in cancer treatment is to promote re-expression of epigenetically silenced tumor suppressor genes. Several inhibitors of DNA methylation are available for experimental and clinical studies (Table 2). Here, we consider an additional mechanism for epigenetic agents and present evidence suggesting that these agents can restore expression of silenced immune escape genes in cancer cells and enhance tumor immunity. This was first suggested by studies showing the activation of silenced MHC genes in several tumor cells by TSA [32]. We discuss a novel epigenetic vaccine produced by treatment of tumor cells in vitro with HDACi. With the current systemic administration of epigenetic agents in clinical trials, host immune responses could potentially be enhanced by correcting the negative affects of the tumor on host immunity. As will be emphasized, the epigenetic agents currently in use affect numerous genes and pathways in tumors, as well as normal cells, and may vary in their effects on different tumor types and even in individual tumors of the same general type. The bottom line is that, at this point, we are only just beginning to understand the underlying chromatin mechanisms and how epigenetic inhibitors can beneficially be employed to alter the course of cancer. In this regard, the information potentially obtainable from a high resolution human epigenome project, now being considered, could greatly accelerate progress and possibly lead to unforeseen findings of benefit to cancer and other diseases [33]. This review will focus on three major topics: (1) the role of epigenetics in the regulation of immune genes involved in evasion of immunity (immune escape); (2) chromatin structure and (3) the potential for designing epigenetic programs which may be useful in immunotherapy and tumor vaccine development.

Tumor immunity

It is now well established that tumors can induce host tumor-specific immunity and in certain models, procedures, which activate adoptive and innate immune responses, can be effective therapies. However, in some mouse models and in most human cancers, the immunotherapies currently employed have not been successful. This may be attributed to a failure of adequate stimulation of appropriate components of immunity and/or to the ability of the tumors to evade the host's response which will be discussed in some detail subsequently in the context of epigenetic alterations designed to boost immunity and prevent escape. Tumor antigens are predominantly self-proteins but also mutated proteins, which as a result of genomic instability, are abundant in cancer cells (as many as 10,000 mutations/cell) [34]. The quantitative levels of tumor antigens, MHC, and costimulatory genes are important in determining T cell priming in immunotherapy. It has been suggested that antigen-specific T cell levels approaching 1% of the $CD8^+$ cells may be required for an effective antitumor response [35], whereas most vaccine procedures in patients attained levels of less than 0.01%. Studies performed under stringently controlled conditions have shown that the strength of the immune response against tumor-specific antigens determines the number of lung metastases and tumor clearance [36]. Analyses of various predictive factors following cancer vaccination indicate that the in vitro percentage of antigen-specific $CD8^+$ T cells after restimulation best correlates with tumor regression [37].

Thirty years ago, cross-presentation was described [38, 39] and much evidence has accumulated that tumor antigens are transferred to host professional antigen presenting cells (APCs) for presentation by the MHC class I pathway (cross-presentation). Moreover, current data suggest that most tumor cells do not present antigen, and if presentation is detected it is weak and does not prime an immune response. A somewhat different view of tumor antigen presentation will be subsequently discussed which encompasses both cross- and direct-presentation. Major issues which remain with cross-presentation include: the nature and route of transfer of cross-presented antigens, the efficiency of live versus apoptotic cells and whether mature proteins and/or soluble fragments or peptides bound to heat shock proteins are involved. Additionally, the recent recognition that tumors, similar to self-antigens, can also induce peripheral tolerance has opened new therapeutic dimensions addressing the nature of the immunosuppressive

Table 2 Types of epigenetic inhibitors used in pre-clinical and clinical trials for anti-cancer therapy

Inhibitor	Target	Tumor type/cell line	Trial phase [references]
HDAC inhibitors			
Aliphatic acids	HDAC		
Phenylbutyrate (PB)	Class I, IIa	AML, MDS, glioma	I [8, 9]
AN-9	Class I, IIa	NSCLC	II [10]
Valproic acid (VA)	Class I, IIa	Cervical cancer	I [11]
Sodium butyrate (SB)	Class I, IIa	Squamous cell carcinoma	Pre-clinical [12]
Benzamides			
CI-994	Class I	Solid tumors	I [13]
MS-275	Class I	Solid tumors, lymphoma	I [14]
Cyclic peptides			
Depsipeptide[a]	Class I	CLL, AML	I [15]
Apicidin[b]	Class I	Leukemia	Pre-clinical [16]
Hydroxamates			
SAHA	Class I, IIa/b	Advanced cancer	I/II [17]
LAQ824, LBH589	Class I, IIa/b	Hematological cancer	I [18, 19]
Pyridoxine	Class I, IIa/b	Solid tumors	I [20]
PXD101	Class I, IIa/b	Solid tumors	I [21]
TSA[c]	Class I, IIa/b	Breast tumor	Pre-clinical [22]
CHAP compounds[d]	Class I, IIa/b	Melanoma	Pre-clinical [23]
DNMT inhibitors			
Nucleoside analog			
5-Aza	DNMT-1, -3a, -3b	MDS	Approved [24]
Decitabine	DNMT-1, -3a, -3b	MDS, CML	II [25]
Gemcitabine	DNMT-1, -3a, -3b	Transitional cell carcinoma	I/II [26]
Zebularine	DNMT-1	Ovarian cancer	Pre-clinical [27]
Others			
MG98	DNMT-1	Solid tumors	I [28]
Hydralazine	DNMT-1, -3a	Cervical cancer	I [29]
Procaine	DNMT-1	Breast tumor	Pre-clinical [30]
EGCG[e]	DNMT-1	Esophageal carcinoma	Pre-clinical [31]

[a]Isolated from *Chromobacterium*
[b]Isolated from two *Fusarium* species
[c]Isolated from *Streptomyces hygroscopicus*
[d]Hybrid of TSA and trapoxin (isolated from *Helicoma ambiens*)
[e]Epigallocatechin-3-gallate, the major polyphenol in green tea

networks inherent in the tumor environment [reviewed in 40–42]. Peripheral tolerance has classically been viewed as a mechanism of preventing self-reactive T cells which escape central tolerance in the thymus from inducing autoimmunity. An important contribution was the discovery of the $CD4^+CD25^+$ regulatory T cell subset (T reg) by Sakaguchi [43] and the finding that elimination of these cells produces a diffuse autoimmune syndrome which is reversed by administration of T reg. T regs are also marked by *FOXP3* and CTLA-4, and are not only involved in normal self-tolerance, autoimmunity and allergy, but also in cancer [44]. T regs, likely generated in the periphery, can produce suppressive cytokines, IL-10 and/or TGF-β, and recent evidence links T reg content of human tumors to patient survival [45]. In lung and late-stage ovarian tumors T regs secrete TGF-β [46] which is known to be produced upon activation of the CTLA-4 inhibitory T cell receptor [47]. TGF-β has been shown to inhibit NK cell production of IFN-γ and Th1 helper cell development [48]. Other types of suppressor cells, including those of myeloid origin, have been described in mice and humans [41, 49]. $CD11b^+$ suppressor cells function normally to limit the $CD8^+$ T cell memory pool [50]. These and other data suggest that tumor antigen presentation may result in tolerance rather than priming and that the mechanisms of tolerance may be similar to those normally employed to prevent autoimmunity to self-antigens. A major focus then would be for therapies to shift the balance toward immunity by positively enhancing priming and also limiting the inhibitory elements responsible for tolerance. Thus various strategies, many of which have been identified as initiating autoimmunity, are being employed together with procedures that directly activate tumor immunity [40, 51, 52]. Many of the agents used as adjuvants (CpG, IL-12, CD40L, etc.) in combination with tumor vaccines elicit inflammation which usually promotes immunity [40], while others, including radiation and chemotherapy, induce apoptotic cells which can enhance inflammation and additionally provide tumor antigens. Anti-T reg and anti-TGF-β are being explored together with adjuvants to abrogate tolerance and enhance T cell as well as NK anti-tumor activity [53, 54]. In relation to this review, there is, as yet, little information on the epigenetic regulation of T regs as a mechanism of immune escape. However, since Th1/Th2 subsets [5] and IL-10 expression in T cells [55] are known to be regulated at the level of chromatin, it seems likely that epigenetic mechanisms are involved in T reg suppression. Moreover, we have recently shown that

suppression of MHC class II and CD40 in brain macrophages (microglial cells) by TGF-β is reversed by HDACi suggesting that epigenetic mechanisms are involved [56].

There is substantial evidence for the role of epigenetics in B cell development and differentiation and in the formation of antibodies (VDJ recombination and isotype switching) but, thus far, not for direct regulation of antibody responses following epigenetic treatments. In this review we will focus on T and NK cell mediated responses to tumors and the potential impact of epigenetically active agents in tumor immunity. $CD8^+$ effector T cells can be potent cytotoxic lymphocytes (CTLs) and their activation and optimal activity depend on the cooperation of $CD4^+$ T helper cells. Studies in experimental models and in humans have demonstrated the importance of these T cells in anti-tumor immunity [57]. $CD4^+CD25^+$ T regs suppress both T cell and NK cell mediated immune responses and have been shown to inhibit both the initiation and effector phases of tumor immunity [53, 58]. However, in certain circumstances and specific tumors, T regs have recently been reported to enhance immunity and their presence in the tumor has been correlated with a good prognosis [59]. Secretion of cytokines by $CD4^+$ and $CD8^+$ T cells also play a key role in recruiting and activating other anti-tumor innate effectors, such as NK cells and macrophages, as well as inhibiting angiogenesis [60]. Stimulation of innate effectors and the production of inhibitors of angiogenesis enable $CD4^+$ T cells to eliminate MHC class II negative tumors [61]. Innate immune responses can also contribute to the activation of adaptive immunity [62]. Activation of innate effectors, such as NK cells, in addition to $CD4^+$ and $CD8^+$ cells results in secretion of IFN-γ which initiates a cascade of cytokine and chemokine expression leading to macrophage activation. Maturation of dendritic cells (DC) by innate cells, cytokines and danger signals enhances MHC class II, costimulatory molecules and antigen presentation to naïve $CD4^+$ and $CD8^+$ T cells and strategies to elicit maximally effective immunity against tumors require the activation of both types of T cells. Activation of naïve T helper cells and CTL is achieved primarily through cross-presentation of tumor antigens by professional APCs [63] but also, as discussed subsequently, direct antigen presentation by tumor cells can contribute, provided the tumor cells can deliver an MHC-restricted antigen-specific signal together with appropriate costimulatory signals [64–66].

Although cross-presentation of tumor antigens delivered to APCs by ingestion of soluble antigens or apoptotic tumor cells is well established [63, 67], direct antigen presentation by tumor cells has been controversial. This is an important issue since the conversion of cancer cells in tumor sites to APCs could potentially provide an alternative or additional pathway to establish tumor immunity. When tumors are first visible by current diagnostic procedures, $\sim 10^9$ tumor cells [68] may be present which could potentially represent a substantial pool of APCs for local presentation or following migration to regional nodes. MHC class I mediated direct priming of CTLs has been observed in an engineered tumor model and is dependent on the density of MHC/peptide complexes and the expression of B7 costimulatory molecules on tumor cells [69]. Interestingly, low levels of B7-1 have been correlated with enhanced tumor escape, while high levels of B7-1 activate immunity. This may result from the much greater affinity of the CTLA-4 receptor for B7-1 compared to CD28 (100-fold to 1,000-fold) leading to T cell anergy when B7-1 is low [70]. The role of MHC class II expression on tumor cells in elicitation of tumor immunity has not been well defined. Studies correlating MHC class II expression in human tumors with invasiveness have, in general, shown a better prognosis in HLA-DR positive tumors, although there are ample exceptions [71]. Transfection of MHC class II negative tumors with MHC class II and B7-1 genes produces a cellular vaccine capable of eliciting immunity to challenge with wild type tumor cells and provides evidence for direct presentation [72]. Furthermore, MHC class II positive tumor cells have been reported to be effective APCs in vivo and, in fact, may present novel endogenous antigenic peptides not presented by host APCs [64]. Transfection of tumors with the MHC class II transactivator (CIITA) elicits MHC class II expression and can restore the ability of certain tumor cells to present intact antigen [73]. However, expression of MHC class II is not always associated with enhanced tumor immunity and, in the absence of costimulatory factors, may promote tumor progression by inducing T cell anergy [74]. Human anergic T cells may act as suppressor cells and inhibit the antigen presenting function and/or survival of host DCs and therefore inhibit cross-presentation [75]. Thus, the differences noted in the functional effects of expression of MHC class II may be related to associated defects in costimulation and other factors required for the generation of immunity.

Constitutive expression of MHC class II is largely restricted to professional APCs such as B cells, DC and macrophages. Expression of class II on B cells is developmentally and therefore epigenetically controlled; class II genes are suppressed in pro-B cells, actively expressed on most pre-B and B cells, and then silenced again in plasma cells [76]. Many cell types are MHC class II negative but expression can be induced by IFN-γ. However, there are some normal cells that are class II negative and non-inducible, such as plasma cells and trophoblasts. Plasma cell tumors usually express MHC class I, but not class II, and this is associated with an absence of CIITA [76]. Recent studies have suggested that Blimp-1, a zinc-finger DNA-binding protein, recruits a co-repressor complex containing histone deacetylases (HDACs) to the CIITA promoter and this may be responsible for the failure of the plasma cells and related tumors to express class II [77]. Agents that inhibit HDAC can de-repress CIITA and enhance the expression of class II on plasma cell tumors [32, 78]. Importantly, in several tumor cell types and in normal

mouse kidney epithelial cell cultures, HDACi treatment has been shown to activate MHC class II by a mechanism that is apparently independent of CIITA induction [78]. The earliest description of the activation of silenced immune genes in tumor cells by treatments with epigenetic agents focused on the MHC and CD40 genes induced by HDACi [32]. Extension of this work has led to the initial report of an epigenetic tumor vaccine using HDACi in a mouse plasmacytoma model [65]. HDACi can induce the expression of class II and costimulatory molecules and convert plasma cells to APCs capable of presenting antigen and peptide to activate $CD4^+$ T cells [65, 78]. While these experiments have demonstrated the ability of HDACi to induce MHC class II expression on several tumor cell types, additional studies are needed to establish the epigenetic profiles in various primary tumors. HDACi treatment allows the MHC activation on class II negative tumor cells that are unresponsive to IFN-γ. IFN-γ induction of CIITA is also involved in MHC class I expression and HDACi treatment has been found to upregulate MHC class I on multiple tumor types. For example, class I is upregulated on J558 plasmacytoma cells and B16F10 melanoma by HDACi [32; A. N. H. Khan et al., unpublished data]. The significance of epigenetic regulation of MHC genes will be discussed further in the Tumor Escape section.

Chromatin structure and epigenetic gene regulation

The eukaryotic genome is composed of arrays of nucleosomes in which 146 bp of DNA are wrapped in almost two left-handed helical turns around a central core of four basic histones (H3, H4, H2A and H2B). Each nucleosome has eight histone proteins arranged in a tripartite structure with one (H3H4) tetramer and two H2AH2B dimers. The nucleosomes are separated by linker DNA and repeated about every 200 bp throughout the genome. This 10 nm 'bead on a string' structure can be compacted about 30-fold to 40-fold into higher ordered 30 nm structures by histone H1 [79]. The chromatin fibers condense further at interphase and are compacted yet again in the metaphase structure. While providing a mechanism of inserting several meters of DNA into a single nucleus this structural compaction can also restrict the access of regulatory proteins to DNA. High resolution crystal structure analysis of the nucleosome has led to a model in which the *N*-terminal tails of all four histones protrude between the DNA gyres and extend outward from the core histones and are therefore more accessible to histone modifying enzymes [79]. Many studies over the past decade have shown that multiple covalent modifications (acetylation, phosphorylation, methylation, ubiquitination, sumoylation, ADP-ribosylation) occur on histone tail residues and, as more recent data demonstrate, also in the body of the histone proteins. These covalent histone modifications can establish marks that are recognition signals for non-histone proteins which are the downstream mediators of chromatin structure and gene activity. Nucleosomes have been found to be depleted at promoters in active regulatory regions throughout the genome [80] consistent with earlier studies showing an increased nuclease hypersensitivity and loss of histone-DNA contacts following gene activation. This could result from sliding of nucleosomes on DNA in *cis*, which exposes the underlying DNA, or disassembly of nucleosomes in *trans*. A number of chromatin mechanisms, summarized in Table 3, target histones, modify accessibility and are key regulators of gene expression. These will be discussed in more detail subsequently.

The histone code

The histone code hypothesis suggests that a dynamic constellation of post-transcriptional modifications determines the binding of chromatin remodeling factors to the nucleosome [81]. These factors, by altering chromatin structure, regulate the accessibility of transcription factors, co-factors and the general transcriptional machinery to DNA and ultimately gene expression. Although substantial evidence has accumulated for a histone code, the potential combinations are becoming progressively more complex and the nature of the code is perhaps less clear as more players enter the picture. Moreover, as critics of the code hypothesis have pointed out, the overall charge on the histone tails, which is independent of the position of the covalent modification, may be an important element in gene expression [82]. It seems likely at this point that both global effects as well as position-dependent histone code(s) at specific residues are involved in gene expression and the issue now is their precise position and combinations and relative importance in different gene expression systems.

The combinatorial possibilities of histone modifications are enormous. Recently, certain site-specific combinations have been identified and correlated with activation or repression. The sites currently recognized to be specifically targeted by epigenetic changes, the enzymes involved in the modifications and whether they correlate generally with activation or repression are shown in Table 4 and summarized in Fig. 1. Modifications can occur on both histone tails and on core histone residues and changes on one histone can require a

Table 3 General mechanisms of modifying chromatin

Transcription factors and cofactors target chromatin modifiers to specific genes
MicroRNAs target chromatin modifications to specific genes or groups of genes to mediate transcriptional gene silencing
Variant histone exchange
Direct phosphorylation of histones via MAPK activated kinases
Nucleosome remodeling complexes (SWI/SNF, etc.) slide or displace nucleosomes
Global chromatin modifications, e.g., charge effects
Histone code

Table 4 Histone methylation (Me) or acetylation (Ac) by various enzymes in transcription regulation

Effect on transcription	Site-specific modification	HMTs	HATs	References
Activation	H3-R2-Me	CARM1		[83, 84]
	H3-K4-Me	SET1, SET9/SET7, ALL, MLL		[85, 86]
	H3-R17-Me	CARM1		[83, 84]
	H3-R26-Me	CARM1		[83, 84]
	H3-K36-Me	SET2 (NSD1)		[86, 87]
	H3-K79-Me	Dot1L		[86, 88]
	H4-R3-Me	PRMT1		[89]
	H3-K9-Ac		SRC1, CBP/p300	[90]
	H3-K14-Ac		CBP/p300, GCN5, PCAF, TAFI, SRC1	[91]
	H3-K18-Ac		CBP/p300	[91]
	H3-K23-Ac		CBP/p300	[92]
	H4-K5-Ac		CBP/p300, ATF2	[91, 93]
	H4-K8-Ac		CBP/p300, ATF2, PCAF	[91, 93]
	H4-K16-Ac		ATF2	[93]
	H-3/H-4-Ac		CIITA	[94, 95]
Repression	H3-R8-Me	PRMT5		[96]
	H3-K9-Me	Suv39h, G9a		[85, 86]
	H3-K27-Me	EZH2, G9a		[85, 97]
	H4-K20-Me	SET7		[85, 86]

modification on another histone. Modification of one histone residue can also prevent binding on other residues in *cis*; for example, the activation marker H3K4 trimethylation (H3K4me3) inhibits both the binding of the NURD silencing complex to H3K4 and the repressive methylation of H3K9 [98]. It seems therefore that covalent modifications occur on all of the histones on both the tails and the core regions and their interactions determine gene expression. It should be noted that the histone modifications in Table 4 are specified as being activating or repressive according to the major effects observed in the references selected. However, in some cases (e.g., H3K36), conflicting evidence has been found in regard to the in vivo effects of specific modifications and they cannot always be easily explained by technical or species differences. It seems likely that, as yet unidentified, changes in the interplay between associated histone residues and the marker being examined, as well as the overall chromatin environment, may influence the outcome of a specific modification.

The combinations of histone modifications found in cells result from the nature of histone targeting processes and the substrate specificity of the enzymes involved. The enzymes that carry out chromatin modifications are under intense study and several reviews on this topic have recently been published [85, 86, 99, 100]. All of the epigenetic alterations currently recognized on histones are reversible and separate sets of enzymes for removing these marks have been identified (Fig. 2). It is the balance between the opposing activities of enzymes that add

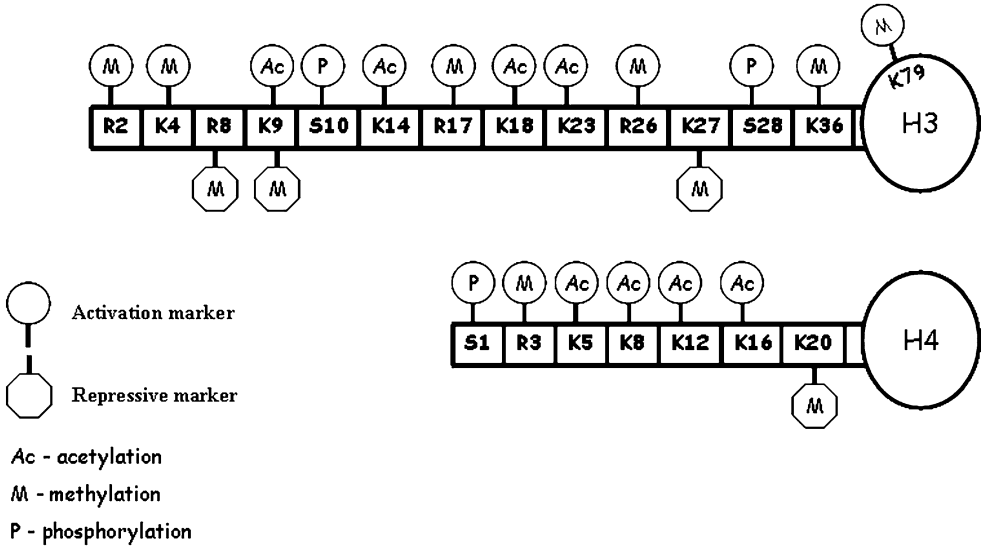

Fig. 1 Covalent modification of histone tails

and remove each of the epigenetic marks that determine local changes in chromatin structure at the gene level and gene expression patterns. In addition the relative levels of total histone acetyltransferases (HATs) and HDACs determines the global status of acetylation in the genome of a cell and these levels may also regulate the cell's response to endogenous and exogenous stimuli. For example, whether a gene is repressed or enhanced by TGF-β correlates with the cell's global HAT/HDAC balance [56, 101]. This concept may be important in the clinical setting where substantial levels of HDACi are attained following treatments and might be expected to induce broad increases in acetylation which could alter the cell's response to external stimuli. It should be emphasized that although histone acetylation is generally correlated with activation and deacetylation with repression, this is not always the case. For example, the 'master regulator' of MHC class II, CIITA, is activated by STAT1 and this requires HDACs [78] as does interferon stimulated innate immunity [102]. Genome-wide studies in yeast also indicate that HDACs are required for both transcriptional activation and repression [103].

The enzymes that acetylate and methylate histones

In humans, 7 HATs and 18 HDACs, the latter divided into three classes, have been identified [104]. Seventeen histone methyltransferases (HMTs) are known [85, 86] and, recently, a single demethylase (LSD-1) has been characterized. Of the HMTs, there are nine that methylate histone lysines and eight that modify arginines. Some of these enzymes appear to be specific for a particular histone residue. For example, the HMT Set9 is quite specific for lysine 4 on H3. However, this same enzyme also methylates a single lysine residue in the p53 protein stabilizing the protein and thereby demonstrating that some histone modifying enzymes can functionally alter non-histone proteins [105]. Other enzymes have broad residue specificity. For example, the HAT enzyme CBP/p300 alters acetylation in a wide range of genes and proteins at multiple sites. Lysine residues can be mono-, di- or trimethylated and these modifications have different activities, probably related to their stability and perhaps to the structure of the modification. Trimethylation of lysines appear to be the most stable and are not removed by the LSD-1 demethylase. Therefore, it is possible that the progressive conversion of mono- and dimethylation to the fully trimethylated lysine state may represent a more long term and heritable chromatin imprint. The strongest functional correlations with gene expression thus far are: H3K9me3, H3K27me3 and H4K20me3 with repression and H3K4me3, H3K36me3 and H3K79me3 with activation. H3K9, H3K14 and H4K16 acetylation are often associated with activation. Acetylation of H4K16 is a particularly prevalent histone modification which has been uniquely shown to decondense higher order chromatin (30 nm fibers) which enhances the accessibility of transcription factors and promotes gene expression [106].

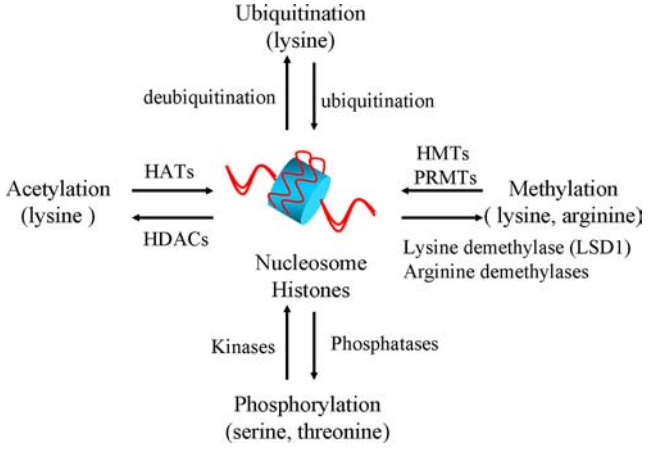

Fig. 2 Epigenetic marks on histones

In addition to histone–lysine covalent modifications, histone–arginines can be methylated. Arginine methylation occurs in low abundance and has been largely overlooked until the recent description of the arginine protein methyltransferases [reviewed in 107]. The arginine residues on histones and other proteins can be epigenetically altered by enzymes (protein arginine methyltransferases or PRMTs). PRMTs have been implicated in cell signaling, DNA repair, apoptosis and transcriptional regulation. PRMTs, like HMTs, have protein targets other than histones, such as CBP/p300, which when arginine methylated shows enhanced HAT activity [108]. The role of arginine specific methylation in immune gene regulation has only recently been addressed. PRMT4 (CARM1) is involved in MHC class II gene expression induced by IFN-γ [109] and it is likely, in view of a recent report demonstrating the binding of CIITA to the SRC-1 cofactor [110], that a complex of CIITA, SRC-1 and CARM1 activates the MHC class II promoter (see below). From information currently available, it appears that, while arginine methylation is undoubtedly an important regulator of cell growth and development its role in immune gene regulation is just beginning to be mapped. To our knowledge, there are no reports on the effects of HDACi on arginine methylation. This could be a fertile area for future investigations.

Histone phosphorylation and kinase pathways in chromatin remodeling

Kinase pathways are positioned as responders to environmental changes and their direct connection with chromatin would provide a rapid route to transcription and gene regulation. In mammalian cells histone H3 is rapidly phosphorylated following exposure to a variety of factors and stresses that activate the mitogen activated protein kinases (MAPK) and the stress activated protein kinases (SAPK). Both pathways lead to the

activation of MSK1 and MSK2 kinases which phosphorylate serine 10 and 28 of histone H3 and are associated with changes in chromatin accessibility [111]. MSK1 and MSK2 are downstream kinases of Ras-Raf-MEK-ERK signaling which activates the immediate early gene stress response of c-fos and c-jun. Allis and colleagues have reported that H3S10 phosphorylation precedes and greatly enhances (tenfold) the acetylation of H3 supporting their code hypothesis and suggesting that histone modifications may be synergistically coupled [112]. Inflammatory stimuli that activate the MAPK pathways (microbial products, CD40L, LPS, etc.) induce H3 phosphoacetylation (at H3S10 and H3K14) and enhance transcription of selected cytokine and chemokine target genes including IL-6, IL-8, IL-12 and macrophage chemoattractant protein (MCP-1). As indicated earlier, inflammation may have an adjuvant affect on immunity, at least in part, mediated by cytokine release. These cytokine genes are activated by MAPK signaling and NF-κB and histone phosphorylation appear to be important mechanisms of recruiting NF-κB to gene targets involved in inflammation. Interestingly, histones that are phosphorylated at H3S10 appear to be much more sensitive to lysine acetylation by the histone deacetylase inhibitor TSA [113]. This has potential clinical relevance, since 'stressed' tumors and those having Ras oncogenes might be expected to have the H3S10/28 phosphorylated phenotype at certain genes and therefore would be more sensitive to HDACi. It is presumed, as discussed earlier, that the phosphoacetylated cluster at H3 10–14 disrupts histone–DNA charge interactions, decondenses chromatin and facilitates transcription.

The CBP/p300 HAT enzymes are phosphoproteins which contain a consensus motif preferred by the AKT kinase. The p300 protein can be phosphorylated on serine 1834 in vitro and in vivo by the AKT kinase. This specific phosphorylation stimulates the HAT activity of p300 significantly and enhances transcription at the ICAM-1 promoter [114]. In non-small cell lung cancer (NSCLC), TSA has a limited ability to induce apoptosis resulting from the NF-κB mediated protection of tumor cells from apoptosis. The mechanisms of apoptotic resistance in NSCLC involve AKT mediated phosphorylation of CBP/p300 which increased NF-κB transcriptional activity, likely by enhancing its access to the promoters of anti-apoptotic target genes [115]. These results suggest potential synergistic effects between specific kinase pathways and HDACi that may be exploited clinically. Additionally, the H3S10/28 phosphorylation and H3 acetylation status of tumors could be markers for in vivo HDACi sensitivity.

Heterochromatin

An important consideration is how long term, stable chromatin states are maintained. Are different sets of chromatin modifying enzymes responsible or are the same enzymes used for euchromatin, perhaps in different combinations or with other chromatin factors, to specify the stable modifications of genes? We do not know the complete answer yet but certain patterns of more stable and potentially heritable chromatin configurations have been found. For example, in heterochromatin, heterochromatin protein 1 (HP1) binds to H3K9me3 in the absence of trimethylated H3K4. Repression of genes by the polycomb (Pc) complex during development has been shown to be mediated by the EZH2 HMT resulting in H3K27me3 which binds the Pc protein and promotes stable silencing of genes [97]. The Pc protein EZH2 has been shown to be involved in progression of prostatic cancer and may be a risk factor for metastatic disease. Interestingly, and of potential clinical relevance, HDACi attenuates gene silencing by EZH2 [116]. Thus, heterochromatin silencing by both HP1 and Pc is associated with fully methylated histone sites at different locations on histone H3 tails and, as discussed subsequently, gene silencing by histone methylation is often accompanied by promoter DNA methylation.

DNA methylation and chromatin silencing

Methylation of CpG dinucleotides in DNA is an important regulator of chromatin structure and gene expression. Methylation of DNA is associated with inactive chromatin in multiple and diverse biological processes including development, X-inactivation, gene imprinting, recombinations and the maintenance of genomic stability [117]. Silencing by DNA methylation occurs in a large portion of the normal genome, especially in repeat sequences, and in locations of viral insertion sequences and transposons. Although cancer cells are generally hypomethylated compared to normal counterparts, specific genes in cancer cells are often hypermethylated [118]. Methylation in cancer usually occurs at CpG islands, often in promoter regions, and is especially common at tumor suppressor genes. Both the general hypomethylation and the tumor suppressor gene hypermethylation may be involved in cancer etiology. Three enzymes that catalyze DNA methylation have been identified: DNMT1, DNMT3a and DNMT3b. DNMT1 is the maintenance methylase and the major factor maintaining the methylated DNA of human cells. DNMT3a and DNMT3b are de novo DNA methylases and these enzymes interact with DNMT1 and contribute to gene silencing in cancer. DNMTs also interact directly with HDACs and can be recruited (by gene-specific transcriptional repressors) to promoters to silence transcription [119]. These findings suggest that complexes involving both DNMTs and HDACs may be recruited to promoters, by oncogenic transcription factors, to induce DNA methylation and silencing. Indeed, this has been reported by DiCroce et al. [120].

Silencing by DNA methylation is carried out primarily by binding of proteins (methyl binding proteins or MBPs) to the methylated CpG. CpG islands are

regions of about 500–1,500 bp with > 55% GC content. Five MBPs are known, all of which have similar methyl binding domains (MBDs). The various MBPs may each associate with different co-repressor complexes, regulate distinct sets of genes and respond to different developmental and environmental signals. MeCp2, the first MBP to be described, was shown to bind and recruit the Sin3-HDAC co-repressor to DNA and repression is relieved by the HDACi, TSA [121]. The interrelations between histone modifications and DNA methylation are schematically summarized in Fig. 3. These findings clearly establish a link between DNA methylation, histone modification and chromatin structure. They also suggest potential targets for therapeutic intervention with epigenetic agents that could be more specific than the global methods currently being employed. For example, targeting specific MBD proteins or co-repressor complexes in different types of cancer with RNAi (see below). There is presently much interest in epigenetic therapies directed at the methylation of suppressor genes common to many cancer types but, as yet, none have focused on abrogating silencing of immune escape genes in particular tumors which may be more specific epigenetic targets.

RNA interference in gene silencing

A new paradigm for gene silencing and heterochromatin formation has recently arisen with the finding that eukaryotic cells use RNA to silence transgenes, transposons and other genomic parasites presumably as a defense against genomic invasions. dsRNAs with specificities to a large number of genes are processed in cells into small RNAs (21–23 nucleotides [nt]) which are capable of mediating RNA interference (RNAi). Two major precursors of small RNAs have been described; one is a long, unstructured, single strand RNA that folds back to form a hairpin structure of about 70 nt which is generated in the nucleus by Drosha and cleaved by Dicer in the cytoplasm to 22 nt microRNAs (miRNA). The other precursor is a classical dsRNA mostly or entirely derived from external sources, for example, following viral infection or transfections, and is also cleaved to a 22 nt siRNA. The same Dicer complex is probably involved in producing miRNA and siRNA and both mediate silencing. MiRNA genes in eukaryotic cells are involved in gene silencing at the post-transcriptional level (PTGS) as well as transcriptional gene silencing (TGS) in the nucleus [reviewed in 126]. The founding members of these non-coding RNAs are the lin-4 and let-7 gene products which are involved in development [127]. Currently, about 250 miRNAs have been described which are thought to regulate about 10% of all protein coding transcripts. However, some projections suggest that this figure may increase substantially perhaps to as many as 1,000 miRNA [128].

One model suggests that a higher-order looping of chromatin which involves small RNAs and chromatin silencing factors, including HDACs and HMTs, which are recruited to sites of miRNA–DNA binding mediate TGS (Fig. 4) [reviewed in 129]. Targeting of the nuclear RNAi transcriptional complex (RITs) by miRNAs promotes H3K9 methylation and HP1 mediated heterochromatin formation [130]. Recruitment of DNMT1 together with the factors described earlier may mediate RNAi associated DNA methylation in addition to chromatin silencing [131]. In addition to their

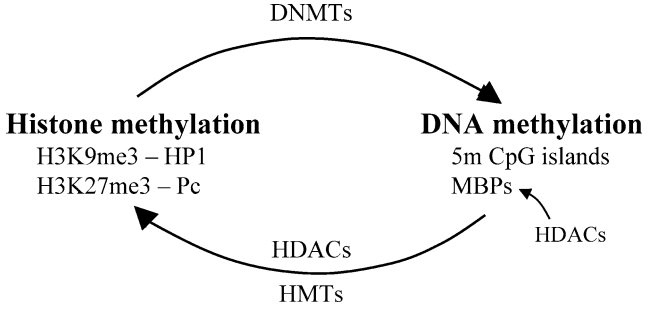

Fig. 3 Epigenetic repression by cross talk between DNA and histones. Repression is associated with specific histone signatures, such as of H3K9me3 and H3K27me3, which are binding sites of HP1 and Pc, respectively. It is likely that deacetylation of these histone residues precedes and is required for their methylation. DNA methylation (by DNMT1) is often associated with histone methylations suggesting that histone methyltransferases may be required for DNA methylation [122]. In addition, HDAC1 binds to DNMT1 [123, 124] and HDACi can inhibit DNA methylation [125]. These findings indicate cross talk between histone modifications and DNA methylations. This is consistent with the observation that, while both HDACi and DNA demethylating agents can separately enhance expression of certain silenced genes in tumor cells, they often synergize in enhancing gene expression

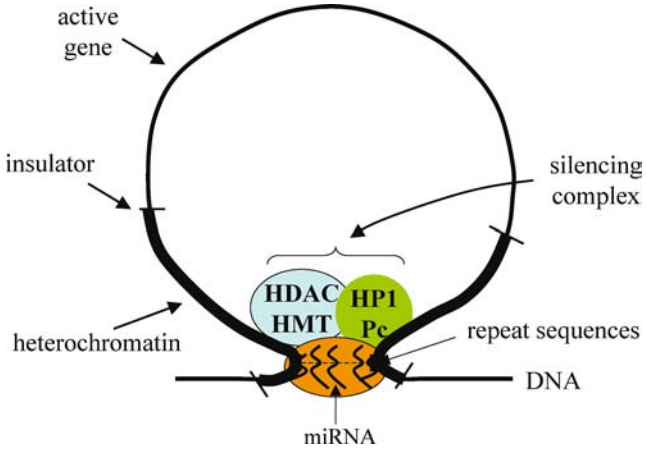

Fig. 4 miRNA mediated transcriptional gene silencing. miRNA interaction with genomic repeat sequences may initiate a looping of chromatin and recruitment of a repressive complex to mediate transcriptional gene silencing in regions of heterochromatin [129]. Insulator sequences within the genomic DNA prevent the spreading of heterochromatin beyond defined boundaries and can preserve the activity of genes within the looping structure. The silencing complex may also contain DNMTs (not shown) which mediate DNA methylation

well-established role in normal development, naturally occurring miRNAs have been shown to protect against viruses and potentially other invaders and thus, in a sense, constitute an 'RNA immune system'. In human tumors, specific miRNAs, often in clusters, are preferentially located at fragile sites which are common breakpoint regions involved in cancer. Some cancers, such as chronic B cell leukemias, have been shown to have a distinct signature of miRNAs suggesting that miRNA may be involved in the pathogenesis of these cancers [132]. As yet, to our knowledge, no immune escape genes are known to be regulated by miRNA. However, since a large number of genes are potential targets of miRNAs (estimated to be ~30% of the genome), miRNAs are candidate 'epigenetic' repressors of genes silenced in immune escape. Moreover, there are recent examples of possible tumor escape mediated by miRNA; miRNA-21 is overexpressed in glioblastoma cells and its knockdown triggers caspase induced cell death suggesting that an anti-apoptotic factor is involved in tumor escape [133]. We consider it likely that immune escape genes will be regulated by miRNAs. The potential for treatment of cancer with siRNA and 'antagomirs' against miRNAs, to modulate immune genes in cancer, is discussed in the section on Future Directions.

Chromatin modifiers as transcriptional co-factors

The DNA sequence and nucleosomal architecture at the gene promoter are of fundamental importance in gene regulation and determine transcription factor (TF) binding to specific DNA motifs in the promoter. The signal for transcription of a gene (up or down) usually is initiated by a cellular receptor which activates or represses a specific set of TFs which bind and recruit co-factors, often as multiprotein complexes, to promoters. The precise timing and sequence of binding of transcription factors and chromatin modifying factors to the promoter of the IFN-β gene have been determined [134]. There are, however, many variations of this basic transcriptional theme. One example relevant to tumor immunity and escape is the IFN-γ induced expression of CIITA. IFN-γ elicits CIITA by activating the transcription of IRF-1 and inducing phosphorylated STAT1. pSTAT1 homodimers enter the nucleus and, together with IRF-1, activate the CIITA promoter. CIITA does not bind DNA but has HAT activity and is a major determinant in the formation and stability of the MHC class II enhanceosome illustrated in Fig. 5. Noteworthy is that MHC class II gene activation by IFN-γ is initiated by a co-factor not by a transcription factor. At least seven transcription factors are present on the CIITA activated class II promoter. All of these factors are constitutively expressed and bind to DNA sequences in the X and Y boxes of the class II promoter. We have recently found that activation of class II is accompanied by demethylation and acetylation of H3K9 and trimethylation of H3K4, H3K36 and H3K79 [78, S.-D. Chou et al., unpublished]. Previous studies using ChIP assays have shown that multiple co-factors are present at the activated class II promoter which includes five co-factors having HAT activity (CBP, p300, PCAF, SRC-1, CIITA) and the Pol II complex factor, TAF1, which also has HAT activity. The arginine–methylase CARM1 and BRG1, the ATPase subunit of the SWI/SNF remodeling complex, are also enhanceosome components [reviewed in 135]. In addition to the histone alterations, direct protein modification of transcription factors and co-factors can occur and, in some cases, by the same enzymes that modify histones. For example, direct phosphorylation, acetylation and ubiquitination of CIITA can substantially affect its activity [136, 137]. Importantly, some tumors have silenced MHC class II genes which are not activated by IFN-γ but can be induced using HDACi [32, 78]. In these tumors, MHC expression has been shown to be mediated by a pathway which is likely to be independent of CIITA [78]. Thus HDACi can apparently replace the functions of multiple HAT enzymes at the class II promoter, a topic which will be further discussed in relation to the development of epigenetic treatments and vaccines.

Histone H1 and variant histones

In addition to the H2A, H2B, H3 and H4 histones, human nucleosomes contain H1 (one molecule per nucleosome), an important transcriptional regulatory molecule. Histone H1 is a complex family of proteins (H1 variants) that bind to the DNA of nucleosomes with no known sequence specificity. H1 binding inhibits

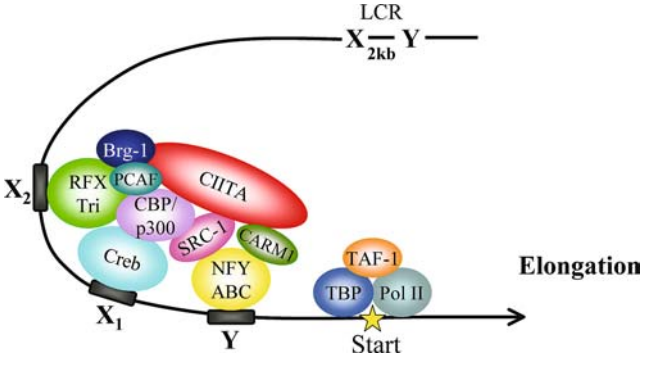

Fig. 5 The MHC class II enhanceosome. Critical DNA sequences for transcriptional regulation of the MHC class II genes are represented by the *X1, X2 and Y boxes* in the proximal promoter. Similar *X and Y boxes* have been found upstream and may represent a locus control region (LCR). Seven basal transcription factors bind these *cis* elements—the trimeric (Tri) RFX complex, cAMP-response element binding protein (Creb) and the trimeric (ABC) NFY complex. These transcription factors interact with the basal transcription machinery (TBP, TAF-1 and PolII), the ATP-dependent chromatin remodeling component BRG-1, the histone methyltransferase CARM-1 and several other coactivators as shown in the figure. The MHC class II transactivator, CIITA, is considered the 'master regulator' and coordinates the interactions at this enhanceosome. Six of the enhanceosome components have HAT activity—CBP, p300, PCAF, SRC-1, TAF-1 and CIITA

nucleosome sliding, condenses chromatin and represses transcription [reviewed in 138]. While, at a specific point in time, most of the nucleosomes in the chromatin fibers are occupied by H1 (~80%), different nucleosomes may be either occupied or unoccupied by H1. The residence time of H1 on chromatin can be regulated by competing transcriptional proteins and by epigenetic modifications [139]. These findings suggest that H1 may act as a modulator of chromatin condensation perhaps by altering the chromatin accessibility of remodeling proteins, such as the SWI/SNF ATP-dependent remodeling complexes.

The canonical histones are primarily incorporated into nucleosomes during S phase and subsequently undergo the covalent histone code modifications discussed earlier. Recently, the regulatory functions of variant histones have been described and the important observation made that certain variants are incorporated into the nucleosome outside of the S phase [140]. The histone variant H3.3 can replace the major form of the histone in the nucleosome (H3.1), and this substitution is found mainly in actively transcribing genes. The H3.3 variant has an activation phenotype with predominantly acetylated H3K9 and trimethylated H3K4. The levels of H3.3 have been reported to be sufficient for packaging all of the transcribed genes of the cell [141]. These data suggest that replication may assemble mainly silent (repressed) chromatin and that, during transcription, these histones are replaced in 'active' nucleosomes. The above considerations invite the speculation that chromatin alterations are associated with the assembly of variant histones and that treatment with epigenetic agents, such as HDACi, may enhance the expression of certain genes (perhaps including immune escape genes) by promoting histone exchange.

The H2AX histone variant has been implicated in the maintenance of genome stability and in the repair of DNA double-strand breaks (DSB). H2AX, phosphorylated on serine 139, is found over a large region around DSB in nuclear foci which appear within minutes following stress [142]. DSBs are generated by external stresses, including certain drugs, radiation and DNA damaging agents as well as 'physiological' programmed DSBs seen in meiotic recombinations and VDJ rearrangements, both of which require H2AX in the repair process [143 and reviewed in 144]. The kinases transducing the DNA damage response to stress are most often members of the phosphatidylinositol-3 kinase related kinases (PIKKs) and include ATM, ATR and DNA-PK [145]. A recent review of chromatin changes in DNA damage discusses the possibility of an epigenetic code for DNA damage repair pathways [146]. The ATM/ATR DNA damage pathway also induces NKG2D ligands (NKG2DL) in cancer cells [147] and could, by directly activating NK and $CD8^+$ T cells, be an important component in early tumor immunity. TSA has been shown to upregulate NKG2DL [147] presumably via activation of ATM although this has not been established. These data suggest that the effectiveness of HDACi in clinical treatments could be related, at least in part, to the activation of the DSB/ATM/H2AX pathway leading to NK and $CD8^+$ T cell activation. The role of NKG2DL is being explored in the epigenetic vaccine model discussed subsequently.

We have attempted, in this section, to outline some of the basic chromatin mechanisms which may be important in the regulation of tumor immunity and escape. Other aspects of chromatin structure, not detailed here, will very likely become important to tumor immunity and cancer therapy in the near future [see 148, 149 for further discussion of basic mechanisms]. The above discussion does, however, illustrate the enormous complexities that are beginning to be uncovered in the epigenetic regulation of gene expression. How they apply to the new treatment protocols being initiated for tumors is currently uncertain but obviously important to determine. This is further discussed in the Therapy and Future Directions sections.

Tumor escape

The concept of tumor immunosurveillance implies that the unmanipulated immune system is capable of recognizing and eliminating primary tumors, at an establishment phase. When they do successfully grow, tumors are said to have "escaped" from immunosurveillance and the escape variants are thought to have a selective advantage that allows them, over time, to become a major population in the tumor [150, 151]. The concept of immunoediting is a view of immunosurveillance that recognizes the role of the immune response as a double-edged sword with the potential to kill tumor cells on the one hand and to select tumor cells resistant to immune recognition and/or destruction on the other [150]. An alternative view to the classical clonal selection model has been proposed (the epigenetic progenitor model) suggesting that cancer cells arise from stem cells by polyclonal epigenetic alterations in tumor progenitor genes that are inherited through cell division [reviewed in 152]. In this model, genetic clonal selection may also occur but later during tumor progression. The failure of immunosurveillance and the escape of tumors have been attributed to a variety of factors that have been extensively reviewed elsewhere [150, 151]. Here, we will focus on epigenetic regulation as a basis of escape and for designing systemic therapy and tumor vaccines.

The use of epigenetic agents in the treatment of tumors in vitro has demonstrated the expression of a variety of immune genes in tumor cells (Table 5). This data suggest that, in order to escape immune destruction, tumor cells may exploit, in addition to deletions and mutations, epigenetic repression of immune genes. It is interesting to consider the potential connections between epigenetic regulation of gene expression and mutations inherent in tumorigenesis. It is likely that the interplay of genetic and epigenetic modifications influences not only developmental processes of carcinogenesis but also

Table 5 Tumor immune escape mechanisms: effect of epigenetic agents

Mechanisms used by tumors to evade immune responses	Gene expression changes mediated by HDACi and/or DNMTi treatments		References
Loss of tumor antigens	↑MAGE 1–4 ↑ GAGE 1–6 ↑ RAGE 1 ↑ NY-ESO 1		[153–155]
Defective antigen processing and presentation pathways	↑ MHC class I and II		[32, 156–164]
Lack of costimulation	↑ CD80 ↑ CD86 ↑ CD40		[32, 156]
Down-regulation of NKG2D ligand	↑ NKG2D ligand		[147, 165, 166]
Defective death receptor pathways	↑ Fas ↑ FasL ↑ TRAIL ↑ DR4 and DR5 ↑ Bax and Bak	↓ c-FLIP ↓ Bcl-2 ↓ XIAP ↓ Survivin	[167–170]
Repression of growth inhibition pathway	↑ TGFβR I, II		[171–174]

selective pressures involved in immune escape. This has been clearly demonstrated in an analysis of several melanoma cell lines derived from patients who underwent successful immunotherapy and recurrence [175]. Characterization of cells from different stages of progression and relapse demonstrated evolution of HLA and β_2m mutations that may facilitate immune escape. Reconstitution of β_2m in one melanoma tumor having a mutant gene led to recovery of HLA surface expression and immune recognition [175]. We envision several potential scenarios of the interplay between mutation and epigenetic alteration. Using MHC class I as an example:

(1) An MHC mutation is identified and the defect is repaired by transfection of a wild type gene and normal function is restored (i.e. antigen presentation or susceptibility as a target)—this signifies a mutational escape mechanism as described earlier [175].
(2) A mutation may be identified in an MHC gene but transfection of the wild type does not restore function. However, treatment with an epigenetic agent demonstrates that epigenetic silencing of associated genes (such as TAP, LMPs) in fact caused the cellular defect. This is an epigenetic mechanism which is unrelated to the mutation or related by as yet unknown mechanisms.
(3) A mutation may be identified but to restore function both transfection of the wild type gene and treatment with an epigenetic agent are required—this signifies both mutational and epigenetic mechanisms.

Tumor antigenicity broadly covers several of the categories in Table 5 since loss of MHC class I and/or II, antigen processing machinery, altered expression of tumor antigens and lack of costimulation all affect T cell activation and the immune response to tumors. It has been found that tumors can downregulate the expression of tumor-associated antigens; in CT antigens and in MAGE, this has been shown to be due to epigenetic modifications at the antigen promoter which are reversed by DNMT or HDAC inhibitors [153–155]. As mentioned, tumors have been shown to carry mutations or deletions in genes encoding MHC class I molecules and/or components of the class I antigen processing and presentation machinery and these tumors are resistant to effector mediated cytolysis. However, the loss of MHC class I and II in some tumors results from altered transcriptional regulation and MHC expression can be recovered by treatment with HDAC inhibitors [32, 65, 78]. We have also shown that components of the class I antigen processing machinery (TAP1, TAP2, LMP7, Tapasin) can be epigenetically regulated in certain tumors (unpublished data). Furthermore, tumors with normal capacities for antigen expression, processing and presentation may downregulate costimulatory molecules leading to the induction of anergy in tumor-specific T cells [176]. Epigenetic regulation of costimulatory molecules has not received as much attention as other components of the immune response but it has been reported, in several human and murine tumor cell lines, that tumor cell surface expression of CD40, and B7-1/2 costimulatory molecules, can be induced by treatment with HDAC inhibitors [32, 65, 177]. Thus, escape can potentially occur at the level of antigen presentation and initiation of the immune response or, later, at the effector stage. Innate effector activation may also be compromised in tumors and HDACi have been shown to induce the expression of NKG2D ligands [165, 166; Gregorie et al., in preparation]. It is clear that tumors have evolved the means to evade immunity by targeting numerous genes critical to productive antigen processing, presentation and effector functions and, as will be discussed in the therapeutic section, some of these targets are susceptible to re-expression by HDACi and demethylating agents and form the basis for epigenetic immunotherapy and vaccines.

Tumors have also been shown to downregulate the expression of various pro-apoptotic and death inducing pathways and some of these can be reactivated by HDACi treatment (Table 5), although others are

resistant likely because of mutations. For instance, HDACi treatment can induce Fas, DR4 and DR5 expression causing tumors to become sensitive to killing by FasL and TRAIL, respectively [167]. Similarly, tumor cells can become insensitive to TGF-β-mediated growth suppression, and downregulation of TGF-βRII in breast cancer cell lines can be reversed by HDACi treatment, thus restoring TGF-β sensitivity [171]. This may be another example of a double-edged sword where activation of TGF-β signaling enables TGF-β to have a direct affect on inhibiting tumor proliferation or alternatively, TGF-β may downregulate T cell and NK cell anti-tumor immunity [178]. It has been suggested that, early in tumor growth, TGF-β directly inhibits tumor progression, while late in the disease it enhances growth and metastases likely via the T reg inhibition of host immune responses [179].

In order to transform, cells must escape the normal mechanisms that regulate the cell cycle, senescence and apoptosis. Some of the same processes that allow tumors to evade normal cellular lifespan controls can interfere with immune effector mechanisms. For instance, cFLIP and other anti-apoptotic molecules may be overexpressed by tumors allowing them to escape both programmed cell death and receptor mediated cell killing by CTL [180]. Several groups have reported the ability of multiple HDAC inhibitors to downregulate cFLIP expression and enhance tumor sensitivity to Fas mediated killing [168, 169]. This is an important reminder that HDACi treatments silence nearly as many genes as they activate and may thereby reverse specific gene induction critical to tumor growth and/or immune escape. The mechanisms involved in HDACi mediated downregulation have not been defined but may be related to activation of a transcriptional inhibitor or possibly an miRNA repressor.

Therapy with inhibitors of epigenetic regulators

HDAC inhibitors

Naturally occurring and synthetic HDACi are a new generation of chemical agents being used to develop therapy against cancer and other diseases including AIDS [181, 182]. Some of the more general in vitro and in vivo effects of HDACi are shown in Table 6. Expression profiling of cells cultured with HDACi and analyzed by DNA microarrays demonstrates that the expression of 2–5% of the genes are altered (activated or repressed) depending on the cell type and the HDACi analyzed. Upregulation of $p21^{waf}$, p16 and p27 and downregulation of Cyclin A, Cyclin D, CDK4 and dephosphorylation of pRb are common features of HDAC inhibition and are necessary for cell cycle arrest and growth inhibition of tumor cells [183, 184]. Altered expression of genes involved in the mitochondrial and death receptor apoptotic pathways is also associated with induction of apoptosis by HDACi [185].

Deacetylase inhibitors directly interact with the catalytic site of HDAC, thereby blocking substrate access to the active Zn^+ or NAD^+ at its base [6, 186]. Inhibitors of Zn^+—dependent HDAC (class I, class IIa and class IIb) have been the focus of intense research, whereas inhibitors of Zn^+—independent HDAC (class III) have recently been implicated in regulation of the cell cycle and aging [24, 186]. Class I and II HDAC inhibitors can be divided into four main structural classes (hydroxamates, cyclic peptides, benzamides and aliphatic acids) and representative members from each class are listed in Table 2. Among these compounds, TSA is widely used in functional studies because of its high HDAC reactivity [187]. The design of many synthetic HDACi has been modeled after TSA. Despite a variety of distinct structures, most of the presently known HDACi have three basic components: a hydrophobic cap that blocks the entrance to the active site, a hydroxamic acid zinc-binding active site and a hydrophobic linker region between them [187]. Many of the HDACi described (>30 published to date) have broad specificity but some do in fact selectively target particular HDAC family members. Studies on class I HDAC inhibitors have shown that MS-275 inhibits HDAC1 and HDAC3 but is inactive against HDAC8 [188]. Furthermore, depsipeptide can inhibit HDAC1 and HDAC2 (class I) but not HDAC 4 and HDAC6 (class II) [188]. Other recently identified HDACi have shown some degree of specificity, such as Scriptaid and Tubacin against HDAC8 and HDAC6, respectively [188]. Generally, HDACi cause the concentration dependent induction of differentiation, growth arrest and apoptosis in a broad spectrum of transformed cells including both hematological (leukemias, lymphomas and myelomas) and epithelial (such as breast, bladder, ovarian, prostate and lung) tumors. Although HDACi have shown significant anti-tumor effects in preclinical models and some are currently in phase I/II clinical trials (Table 2), the precise molecular pathways involved in the anti-tumor effects have not been fully determined. This is not surprising in view of the earlier discussion of the complexities of the regulatory networks that control chromatin.

As shown in Table 5 and discussed earlier, HDACi can activate or repress a number of genes involved in immune escape. Several publications show that treatment with HDACi can enhance expression of MHC, CD40, B7-1/2, ICAM-1 genes in various human (e.g., neuroblastoma, squamous cell carcinoma, acute myeloid leukemia) and mouse tumor (e.g., plasmacytoma, adenocarcinoma) cell lines [32, 65, 156, 189]. Additionally HDACi can inhibit angiogenesis and are thought to induce tumor regression by upregulation of tumor suppressor genes (p53 and VHL) and downregulation of HIF-1 and VEGF genes [190, 191]. These findings support the proposal that enhanced acetylation by HDACi leads to activation or repression of the transcription of a select group of genes resulting in inhibition of immune escape. HDACi given systemically are generally well tolerated but accumulation of acetylated histones in

Table 6 Some major effects of deacetylase inhibitors

In vitro
Alters expression of 2–5% of genome—approximately an equal number of repressed and activated genes
Cell cycle and growth arrest—enhances p21 expression; G1 arrest in most cells, some cells G1/S and G2M
Differentiation—certain cells
Senescence—at low concentration
Apoptosis—at high concentrations
Transcriptional activation and repression associated with acetylation of histone lysines
Reduces the production of inflammatory cytokines (TNF-α, IL-1, IL-12, IL-6)
Corrects aberrant expression of cytokines in lupus T cells (inhibits CD40L and IL-10)
In vivo
Anti-tumor activity in animal models and human trials
Reversal of polyglutamine repeat mediated neurodegeneration in *Drosophila*—a model for Huntington's disease
Inhibits lupus-like disease in NZB and MRL-1pr/1pr mice
Reduces graft versus host disease
Increases life span in yeast and *Drosophila*
Inhibits eye abnormalities in *Drosophila* resulting from defects in Hsp90

normal tissues may induce some toxicity depending on the dose, route and specific drug [186, 192].

DNMT inhibitors

Inhibitors of DNA methyltransferases, which were originally developed as nucleoside analog chemotherapeutic agents, have been used for treating cancer and other diseases [181, 193]. Similar to HDACi, repressed genes, including p16, p14, p21, Apaf-1, caspase 8 and other suppressor genes, can be re-expressed by treatments with DNMTi [24, 118]. Additional experiments addressing the potential importance of repression of immune genes silenced by methylation could provide useful information in crafting more effective clinical trials. To date very little attention has been focused on immune escape genes following these treatments. Table 2 lists most of the DNMT inhibitors employed in current anti-cancer therapy. These are divided into two groups on the basis of mechanism of action [194]. 5-Azacytidine (5-aza) and 5-aza-2′-deoxycytidine (Decitabine) were the first two DNMTi to be synthesized. Nucleoside analog DNMTi, after incorporation into DNA, covalently bind and inactivate the DNMTs resulting in significant demethylation. Other non-nucleoside DNMTi use alternative mechanisms, for example antisense oligonucleotide MG98 can hybridize to the 3′ untranslated region of DNMT1 mRNA and deplete DNMT1. Treatment with a variety of DNMTi has been shown to induce growth inhibition and differentiation of several tumor types in pre-clinical models and has beneficial effects in clinical cancer trials (Table 2). Although this strategy has the potential to improve outcomes in human leukemia, treatment with DNMTi have shown limited efficacy against human solid tumors [193]. Several problems have been observed with the use of currently available DNMTi, the most serious being dose limiting toxicity. Incorporation of DNMTi into the DNA of host cells may be responsible for bone marrow and other host toxicities. Most DNMTi are not specific for a particular DNMT, which could also contribute to toxicity. Additionally, demethylation by decitabine increases the expression of MDR1, a gene whose expression enhances drug resistance, and uPA, a pro-metastatic gene in non-metastatic breast tumor cells [24, 195]. Because of these limitations, and since DNA methyl binding proteins recruit HDACs and are an important component of repression by DNA methylation, combined treatments have used HDACi with DNMTi.

Epigenetic therapy in combination with other agents

Several studies have shown synergy of DNMTs and HDACs in silencing gene transcription in cancer [reviewed in 118]. Such findings have encouraged investigation of HDACi in combination with DNMTi in anti-cancer treatment. Due to tumor selectivity and relatively low toxicity of these agents compared to most front-line cancer therapies, HDACi or DNMTi have been used in conjunction with a variety of novel and conventional anti-cancer drugs. Such drugs include topoisomerase II inhibitor (etoposide), tyrosine kinase inhibitor (imatinib), proteasome inhibitor (bortezomib), apoptosis pathway activator (TRAIL), Flt-3 kinase inhibitor (PKC412), Hsp90 antagonist (17-AAG), retinoic acid and several cytotoxic agents. Various combinations of these drugs are outlined in Table 7. In general, these combinations have shown some synergistic effects in inhibiting tumor growth and inducing differentiation and/or apoptosis compared to either agent alone and have allowed the use of lower doses of conventional drugs. The precise mechanisms of the anti-tumor effects by these combination therapies are not well understood, since, as outlined earlier, the pathways affected by the individual agents have not been completely defined. Nevertheless, these findings have implications in the development of future anti-tumor therapies and, based on promising pre-clinical data, several combined therapies are currently in clinical trials (Table 7). For example, treatment with CI-994 in combination with gemcitabine achieved partial response (PR) or stable disease (SD) in 70% of patients [200] and combination treatments with CI-994 and capecitabine resulted PR or SD in 40% of patients [220], while treatment with CI-994 alone achieved PR or SD only in 7% of patients with advanced solid malignancies [13]. These ongoing clinical studies will determine the efficacy of current epigenetic combination therapies and studies of chromatin mechanisms will contribute to the design of future therapeutic approaches.

Table 7 HDACi in combination with DNMTi or other agents in pre-clinical and clinical anti-tumor treatments

Combination treatment	Tumor type/cell line	Trial phase [references]
TSA or depsipeptide + decitabine	Myeloid leukemia	Pre-clinical [196]
Scriptaid + decitabine	Breast cancer	Pre-clinical [197]
PB + 5-Aza	Solid and hematological	I [198]
VA + decitabine	MDS, AML	I/II [199]
CI-994 + gemcitabine	Advanced cancer	I [200]
LAQ824 + taxotere, transtuzumab, gemcitabine or epothilone	Breast cancer	Pre-clinical [201]
TSA + tamoxifen	Breast cancer	Pre-clinical [202]
TSA or CBHA + retinoic acid	APL, neuroblastoma	Pre-clinical [203, 204]
TSA or SAHA + etoposide, camptothecin, ellipticine, cisplatin, doxorubicin, 5-flurouracil or cyclophosphamide	Breast cancer, neuroblastoma, colon cancer, leukemia	Pre-clinical [205, 206]
SAHA or apicidin + imatinib	AML	Pre-clinical [207, 208]
SAHA, SB or LBH589 + 17-AAG	Leukemia	Pre-clinical [209, 210]
SAHA or decitabine + TRAIL	Melanoma, glioblastoma	Pre-clinical [170, 211]
SAHA + bortezomib or flavopiridol	Multiple myeloma, leukemia	Pre-clinical [212, 213]
MS-275 + fludarabine	Leukemia	Pre-clinical [214]
LAQ824 + PKC412	AML	Pre-clinical [215]
PB + 5-fluorouracil, cytarabine, etoposide or topotecan	Colon cancer, lymphoma, CLL, multiple myeloma	Pre-clinical [216]
PB + retinoic acid	APL	I [217]
AN-9 + docetaxel	Advanced NSCLC	I [218]
VA + retinoic acid	MDS, AML	I [219]
CI-994 + capacitabine, carboplatin or paclitaxel	Advanced cancer	I [220, 221]
Decitabin + cisplatin, carboplatin, temozolomide or epirubicin	Ovarian and colon cancer	Pre-clinical [222]
Gemcitabine + cisplatin	NSCLC	I/II [223]

Epigenetic tumor cell vaccines

By exploiting naturally occurring defense systems, immunotherapy could potentially be a relatively non-toxic method of evoking tumor-specific immune responses against residual or recurrent tumors. Similar to vaccine development for infectious diseases, tumor vaccination strategies are designed to mount an effective immune reaction against TAAs expressed by tumor cells. Although preclinical and clinical evidence have shown the induction of tumor immunity by several vaccination techniques, at present no human anti-cancer vaccines have been recommended for treatment [224]. One of the concerns in vaccine trials is the nature of the TAAs. Although expression of well-characterized TAAs has been identified in several types of tumors, the nature of the TAAs in most cancers, including some with high recurrence rates (e.g., pancreatic and renal carcinoma), is unknown. In addition, studies with well-characterized TAAs have indicated that immunization with a single type of TAA molecule may not suffice. This may result from selection pressures, which foster the appearance and expansion of tumor cells with low or no expression of the specific TAA [150, 151]. Therefore, effective tumor vaccines are thought to require the inclusion of several TAAs—i.e., polyvalent vaccines [225]. Among various polyvalent tumor vaccines (whole cell, tumor lysate, shed antigens and heat shock proteins), whole tumor cell vaccination has been investigated extensively in animals and humans. This vaccination approach does not require TAA identification and involves a repertoire of TAAs that can be unique to the individual tumor. Tumor cells inactivated by irradiation were the first employed as whole cell vaccines. Subsequently, various vaccination strategies involving autologous and allogenic whole tumor cells have been developed and many of these approaches have entered clinical trials [226, 227]. These studies have suggested the potential usefulness and show the safety of whole tumor cell vaccines in humans; however, tumor eradication utilizing this strategy has not been reported in humans. In an effort to enhance immune reactivity, different groups have developed other strategies, such as IL-12 or CD40 ligand transfected and formalin-fixed tumor cell vaccines [228–230]. While each of these additions has enhanced the protective potential of whole cell vaccination in animal models, none of the combined vaccine procedures have been evaluated in clinical trials. Although strong evidence has been presented for cross-presentation of tumor antigens in several experimental systems, a significant problem is the availability of sufficient soluble and apoptotic tumor antigens for optimal activation of both $CD4^+$ and $CD8^+$ T cells [231, 232]. Therefore, to induce a more effective anti-tumor immune response using the whole tumor cell vaccination approach, in addition to cross-presentation of tumor antigen by APCs, direct antigen presentation by tumor cells might be advantageous.

Another important factor in the lack of success of current cancer vaccines is the ability of the tumor cells to evade immune destruction. Although current systemic therapies have not been specifically designed to target

immunity this should be considered in subsequent trials since, as discussed, tumor cells treated with HDACi and/or DNMTi can upregulate silenced immune genes and initiate immune responses. Effective tumor-protective immune responses have been achieved in murine melanoma and plasmacytoma models utilizing epigenetically altered tumor cell vaccination [65]. In these studies, significant numbers of animals showed tumor-specific durable immunity and developed cytotoxic T cells after vaccination with TSA treated tumor cells that expressed MHC and costimulatory molecules. $CD4^+$, $CD8^+$ T cells and NK cells were involved in immunity. Vaccine inocula containing ~50% apoptotic cells were the most effective [65] and recent studies in MHC class I and II knockout chimeric animals suggested that direct antigen presentation by TSA treated tumor cells was a component in the induction of immunity [A.N. Khan et al., in preparation]. Since the tumor cell was converted to an effective antigen-presenting cell after HDACi treatment [78], we suggest that the immunity observed resulted, at least in part, from direct antigen presentation [69, 72] in addition to the cross-presentation mechanisms which have been shown in other vaccine models. The finding, as discussed earlier, that HDACi treatment activates the ATM/ATR pathway and induces NKG2D ligand on tumor cells [147, 165, 166] suggests that the epigenetic tumor cell vaccine may also be capable of directly activating NK cell mediated innate responses which can also enhance tumor-specific adaptive immunity. Furthermore, the apoptotic and necrotic components of this vaccine may augment the anti-tumor immunity by activating inflammatory regulators [233, 234] and have the potential to activate the Toll-like receptors that play central roles in stimulating innate immune responses [235]. Although further work is required to dissect the mechanisms involved in epigenetic vaccination, these studies suggest that autologous tumor cells treated with agents that alter chromatin in vitro could be used, perhaps in combination with other agents, to create effective epigenetically designed whole tumor cell vaccines. This strategy would perhaps be most effective in preventing metastatic and recurrent cancers following surgical reduction. The effects of prior and concomitant chemotherapy on subsequent epigenetic vaccine responses are currently under study. In addition, the effect of systemic epigenetic agents on activation of tumor immunity should be explored in future studies. As we understand more of the mechanisms of epigenetic regulation, it may be possible to select specific therapeutic combinations based on gene profiling and other characterizations of the tumor. These issues are discussed further in the subsequent sections.

Future directions

Characterization of epigenetic modifications is now providing tools for detection, diagnosis and prognosis of cancer and other 'epigenetic diseases'. Several groups have identified histone and DNA methylation modifications that correlate with the presence of cancer or the prognosis for response to chemotherapy [236–238]. For example, Fraga et al. [239] have described the early loss of H4K16 acetylation and H4K20 methylation as a common hallmark of cancer. Detection of these markers in patient biopsies has been demonstrated and assays appropriate for patient screening have been reported [236, 237]. Optimal sensitivity was achieved by combination of histology and methylation analysis. In addition to tumor biopsies, these methylation markers for the presence of cancer and tumor progression can be detected in the DNA isolated from patient's serum and urine. Thus these tests may offer earlier, non-invasive detection and allow monitoring of therapeutic efficacy [237]. Patterns of specific gene methylation may be developed as diagnostic markers in other tumor types and assays of additional epigenetic markers are likely to contribute to diagnosis. Future markers might include epigenetic marks on histones including H1, variant histones, methyl binding proteins and miRNA expression patterns. A recent report, using a bead-based flow cytometric miRNA profiling technique, found that human cancers, in general, show a downregulation of miRNAs compared to normal tissues and importantly the patterns reflect the developmental and differentiation state of tumors. This technique was also more successful in classifying poorly differentiated tumors than messenger RNA profiles [240] and may be an important method of evaluating tumor cell epigenetic profiles regulated by miRNAs. Recently, miRNA signatures have been described for human solid tumors and some of their predicted targets include the TGF-β receptor II gene [241]. This study clearly demonstrated that aberrant expression of miRNAs (either up or down) regulate cancer genes. Since epigenetic regulation is central to many processes of development and differentiation, it will be important, as a component of clinical trials, to evaluate the effects of epigenetic agents not only on the disease site, i.e. tumor, but also on healthy cells and particularly on the patient's immune response.

Epigenetic therapeutics in tumor immune escape

In addition to the possibility of reversing immune escape, epigenetic agents may also be able to enhance the utility of other therapeutics. For instance, Rituxan has demonstrated success in B cell lymphoma treatment but a small number of CD20 low or negative variants are not susceptible to this therapeutic antibody [242, 243]. If epigenetic therapy can enhance CD20 expression, as it does certain other receptors (e.g., CD40, TGF-βRII), these tumors may be targeted by Rituxan. Since TSA, in mouse studies, and SAHA, in human studies, were tolerated at doses that achieved micromolar plasma concentrations and our in vitro studies demonstrate immune gene induction in primary murine kidneys between 100 and 250 nM, and in splenocytes and thymocytes at low

nanomolar concentrations, we consider it to be likely that systemic epigenetic therapy will modify gene expression in normal patient cells [78, 244, 245]. Therefore, evaluations should consider the impact of systemic epigenetic therapy on MHC, costimulatory molecule, NKG2D and other immune genes in normal cells as well as tumors obtained from systemically treated patients. Furthermore, epigenetic regulation is critical to the development of T helper cells [5] and it will be important to monitor patient Th1/Th2 ratios in HDACi clinical trials. HDACi treatment has also been shown to inhibit the production of certain cytokines, as well as T cell proliferation, and could potentially interfere with cancer immunotherapy [246, 247]. Thus, following systemic treatment with HDACi, it would be prudent to consider effects on normal differentiation, and altered patient immune homeostasis and possibly susceptibility to infection. We should be aware that current epigenetic therapeutics have complex effects and may be double-edged swords. With these caveats in mind, current and future epigenetic therapeutics likely have great potential but the combinations, route and form of therapy may be critical considerations in order to maximize patient benefit and minimize side effects.

In addition to the diagnostic testing described earlier, it may be possible to test patient circulating tumor cells and tumor biopsies in vitro for sensitivity to specific epigenetic agents. As studies more clearly identify genes critical to immune escape and their response to various epigenetic agents alone or in combination with chemotherapeutics we may be able to correlate specific gene expression patterns on patient tumor samples with clinical response. Analysis of epigenetic patterns, such as H3K9me3, variant histone composition, DNA methylation, to mention only a few, could potentially predict the success of single or combined treatments with epigenetic agents. With therapeutic choices based on epigenetic markers, similar studies peformed during the course of treatment could monitor progress and perhaps the development of resistant tumors based on new mutations or additional epigenetic alterations. In more general terms, it would be reasonable to study the altered gene expression profiles of a panel of fresh tumor types and their responses to the various combinations of epigenetic agents currently available. This type of approach could potentially identify tumor types most responsive to a specific agent or combination and provide the basis for rational selection of agents for patient treatment. As seen with many of the newer, targeted chemotherapeutics, such as Gleevec, highly beneficial outcomes in specific patient populations may not be identified in studies of broader populations necessitating careful patient selection for therapy [248]. Epigenetic therapeutics are viewed as having broad effects but their clinical efficacy may be restricted by genetic and/or epigenetic characteristics that we still have to define.

New inhibitors are under development to more selectively target HDAC or DNMT and to improve the relatively weak inhibition of the orally available agents such as SAHA [249, 250]. Most current clinical trials utilize broadly effective HDAC inhibitors although newer HDACi are now being studied that show specificity for particular classes of HDAC [188]. These more specific inhibitors will provide additional selectivity in the subset of genes activated by HDACi treatment of tumors and may be useful in the design of more effective epigenetic tumor therapies. A comparable search for selective inhibitors of DNMTs would be useful and the new compounds identified would likely extend our understanding of mechanisms of DNA methylation and provide more specific reagents with less toxicity. It should be feasible to use peptides corresponding to histone *N*-terminal or other sequences as substrates in screens designed to identify novel small molecule inhibitors of epigenetic modifications. This approach is being used for HMT inhibitors [251] and should be adaptable to evaluate arginine-specific methyltransferases and to better characterize other lysine-specific enzymes. Similar strategies could also be developed to evaluate inhibitors of other histone modifications.

As signal transduction pathways that impact epigenetic modifications are further characterized, activators or inhibitors of these signaling pathways (e.g., MAPK, ERK, p38, PI3K/AKT) may prove as significant as the current HDACi, and possibly more specific, and may be used in combination with epigenetic agents. For example, inhibitors of PI3K/AKT downmodulate tumor NF-κB which is responsible for overexpression of certain anti-apoptotic proteins which have been implicated in resistance to HDACi treatment of lung tumors (see earlier). As investigations continue to elucidate these networks, new targets may be revealed and expose roles for specific signaling pathways in overcoming immune escape mechanisms.

Designing future epigenetic tumor vaccines

The strength of the epigenetic tumor vaccination approach lies in the incorporation of multiple endogenous tumor antigens (that do not need to be individually identified) and the elicitation of direct antigen presentation by tumors to assist cross-presentation by host APCs. Ongoing studies with antibody mediated depletion of regulatory T cells and RNAi targeting of specific molecules in immune escape will further help in defining the molecules and pathways critical to an effective immune response.

Beyond more selective chemical inhibitors, RNAi techniques have advanced rapidly and now offer the means to selectively inhibit specific gene expression. For example, recent studies have demonstrated the efficacy of siRNA targeting the multdrug resistance gene MDR1 and specific fusion genes, such as TEL-PDGFβR [252, 253]. While carefully designed siRNA offer target specificity rarely achieved with traditional inhibitors, off-target effects and non-specific effects remain potential difficulties [254]. Nevertheless, careful design criteria and

testing can avoid significant off-target effects and allow effective use of siRNA in vitro and, potentially, in vivo.

Early studies of miRNA in *C. elegans* and *Drosophila* have demonstrated that many miRNAs are silenced or expressed at different stages of development implying that they are epigenetically regulated. Many miRNAs have their own promoter and can therefore be specifically regulated. HDACi and other epigenetic treatments could therefore alter the profile of miRNA expressed by tumor cells and this is currently under study in our laboratory. The recent description of 'antagomirs', mentioned earlier, has opened the potential for in vivo inhibition of specific miRNA [255]. The components of RNAi thus represent tools as well as targets. For example, Cimmino et al. demonstrated that miR-15 and miR-16 negatively regulate the anti-apoptotic protein BCL2 (see Table 5) and that expression of these miRNA are lost due to deletion or translocation in ~65% of B cell CLL patients [256]. Other examples of deletion or downregulation of specific miRNAs in cancer have been noted [reviewed in 257]. MiRNA profiling of tumors can identify targets for siRNA or antagomirs that may compensate for the dysregulated miRNA function. As mentioned, miRNA may be oncogenes or tumor suppressors in various cancers and the use of RNAi must therefore be tailored carefully to individual tumors [258]. Patient treatments, systemically or at the tumor site, with siRNA designed to inhibit one or more of the various immune inhibitory molecules, such as CTLA-4, IL-10 or TGF-β, could diminish the tolerizing activity of T reg and potentially enhance tumor immunity in vivo. We are currently examining a more global reversal of silenced immune genes using siRNA for Dicer and Argonaute genes which are components of the RNAi machinery. This knockdown could interfere with a broad spectrum of miRNA potentially involved in immune escape and may not be applicable to all tumors. The exploitation of RNAi for systemic therapy presents additional significant hurdles beyond those discussed earlier. The issues of delivery, stability, safety and efficacy, among others, were recently reviewed by Uprichard [254]. Adeno-associated viral vectors and other vector types are being developed and tested for stable integration of specific siRNA or replacement of a deleted miRNA (i.e., miR-15/16 in CLL). In an immune escape model, if designed and validated properly, short-term systemic therapy may be sufficient to initiate an immune response or to overcome suppression of an immune response and, especially in combination with vaccine or adjuvant stimulation, may be capable of inhibiting immune escape.

While combinations of treatments can be designed to achieve expression profiles predicted to be maximally immunogenic, the cellular content must also be considered. Recent studies [65] have indicated that the apoptotic/necrotic content of tumor vaccines is a critical component and one that can be manipulated to optimize immunogenicity. This work has shown that apoptotic cells produced by different agents vary in their 'adjuvant' effect in a vaccine setting. Treatments with epigenetic agents alone and in combinations, while altering TAAs, surface protein expression and, perhaps, uptake of potential antigens, will also affect other cellular components, potentially including those that represent 'danger signals' (i.e., heat shock proteins, HMGB1) or inflammatory mediators. The type and degree of cell death induced by these treatments will also affect the immune response to treated tumors.

Several laboratories have described vaccination approaches utilizing exosomes; membrane bound vesicles secreted from cells as products of the normal cellular endocytic pathway [259]. Follicular dendritic cells have been shown to be decorated with exosomes in vivo suggesting that exosome surface proteins, including MHC–peptide complexes, may be directly presented to T cells in the context of an APC surface or they may be internalized and deliver tumor associated antigens to the follicular DC [260]. Several groups have demonstrated the immunogenicity of tumor-derived exosomes in murine tumor models [261, 262]. We propose that treatments of tumor cells with epigenetic modifiers which enhance MHC, costimulatory molecule and, perhaps, tumor antigen expression may be reflected in the exosomes produced by the treated cells. Exosomes could thus be designed to maximize tumor antigenicity/immunogenicity while avoiding many of the issues associated with whole cell vaccination. Exosomes, especially derived from tumor cells treated with HDACi and TLR ligands, may also be 'adjuvants' and could enhance vaccines in a fashion similar to apoptotic/necrotic cells. It also remains to be determined whether exosomes mediate some of the in vivo anti-tumor responses seen with systemic use of epigenetic agents.

Alternatively, an epigenetic vaccine approach could be adapted to ex vivo treatment. For instance, following vaccination, patient derived T cells could be stimulated in vitro with autologous, epigenetically modified tumor cells or exosomes, expanded and transferred back to the patient in an adoptive immunotherapy model. Adoptive T cell therapies for certain tumors, for example melanoma, are now in clinical trials [263]. In vitro direct antigen presentation by the epigenetically modified tumor cells or cross-presentation by autologous APC could effectively stimulate tumor-specific T cells for adoptive therapy. Rapoport et al. [264] recently reported enhanced immune recovery and vaccine responses in myeloma patients after adoptive transfer of autologous T cells. These patients were vaccinated with pneumococcus prior to apheresis and high-dose chemotherapy, their T cells were stimulated in vitro and expanded T cells were returned to the patient by adoptive transfer. Subsequent vaccination with the pneumococcal conjugate vaccine significantly demonstrated enhanced B and T cell responses in those patients who received both vaccination and adoptive transfer of costimulated T cells [264]. An adoptive therapy approach using T cells expanded with epigenetically altered patient tumor cells or exosomes could avoid complications of drug toxicity

while allowing better control of T cell stimulation and selection of reactive T cells based on defined characteristics [263]. Adoptive transfer into lymphodepleted hosts has also been suggested to improve therapeutic outcome by diminished suppression due to T reg cells and other tolerogenic mechanisms [265] and this method could potentially be used with epigenetic vaccines.

Future experiments using RNAi and knockout mice should address each of the suspected immune escape genes in an attempt to determine which of the proposed factors or a combination of factors lead to tumor susceptibility. Additionally, data derived from animal models of spontaneous tumors could be used to correlate tumor development with specific epigenetic profiles. It seems almost certain that, as more is known of the mechanisms that mediate epigenetic regulation, improved therapeutic protocols can be designed which will enhance epigenetic therapies. Additionally, ongoing studies are likely to expose further targets for therapeutic intervention as well as lead to a better understanding of the plieotropic effects of epigenetically targeted therapeutics. Mutations are currently difficult to correct but, in diseases including tumors where gene silencing is mediated by chromatin, the use of epigenetic therapies may allow alternative treatments of these 'genetic diseases'.

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