Review TRENDS in Genetics Vol.23 No.9

Unraveling the epigenetic code of cancer for therapy

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Alterations in the genome and the epigenome are common in most cancers. Changes in epigenetic signatures, including aberrant DNA methylation and histone deacetylation, are among the most prevalent modifications in cancer and lead to dramatic changes in gene expression patterns. Because DNA methylation and histone deacetylation are reversible processes, they have become attractive as targets for cancer epigenetic therapy, both as single agents and as 'enhancing' agents for other treatment strategies. In this review we discuss our current view of the mammalian epigenome, this view has changed over the years because of the availability of novel technologies. We further demonstrate how the profound understanding of epigenetic alterations in cancer will help develop novel strategies for epigenetic therapies.

Historical overview of epigenetics

A single mammalian genome, which encodes ~30 000 genes, needs to have the capacity to program gene expression patterns in ~200 different cell types at numerous developmental stages. It is now evident that an additional layer of information required for proper gene expression is encoded in the genomic sequence and exceeds the information of the four bases: adenine, thymine, guanine and cytosine. This is achieved in the form of epigenetic modifications, which in their entirety represent the 'epigenome' (from the Greek prefix epi-, meaning 'on' or 'over'). Epigenetic modifications are heritable and can be transmitted to daughter cells during cell divisions. Most importantly, they leave the option for reprogramming in the context of development and differentiation. Epigenetic modifications change the chromatin structure without altering the DNA sequence, and they can affect gene expression. This has implications for our understanding of tumorigenesis, where epigenetic changes have recently become a major research focus. Because of the reversible nature of these modifications, chemicals and small molecules are being devised to impede this process. Of note, human malignancies of all kinds have displayed alterations in the epigenetic signatures, indicating that all cancer patients could potentially benefit from epigenetic therapy. This review will highlight the importance of understanding epigenetic variation, both in normal and in malignant genomes, and will demonstrate how epigenetic modifiers have the potential to affect patient care.

DNA methylation and its distribution in the genome

In mammals, DNA methylation is the enzymatic addition of a methyl group to cytosines in CG dinucleotides. This reaction, mediated by DNA methyltransferases (DNMTs) in the presence of the methyl donor S-adenosylmethionine (SAM), results in 5-methylcytosine (5 meC). *De novo* methylation of previously unmethylated sequences is catalyzed by DNMT3a and DNMT3b, whereas maintenance methylation by DNMT1 occurs along newly synthesized DNA; the parental strand is used for the DNA methylation template along the newly synthesized daughter strand. Historically, owing to the limitations of whole-genome scans, scientists have evaluated specific gene sequences or chromosomal segments by using either methylation-sensitive restriction enzymes or sodium bisulfite conversion. This work highlighted the importance of tightly regulated DNA-methylation patterns in differentially methylated regions both in the control of imprinted gene expression [1] and in the maintenance of X-chromosome inactivation, which allows for proper gene dosaging in females [2]. It was also realized that dense DNA methylation is seen in repetitive sequences (including satellite sequences, centromeric repeats, rDNA and interspersed repetitive sequences), which contain the vast majority of CG dinucleotides [3]. In contrast to those methylated sequences, GC-rich promoter sequences characterized as CpG islands are generally described as unmethylated [4].

A total of 2.8×10^7 CG dinucleotides in the human genome are potential methylation targets. Early studies using high-performance liquid chromatography (HPLC) demonstrated that in a normal genome ~1% of all cytosines, or 70%–80% of CG dinucleotides, are methylated [5]. Assessing DNA methylation in a sequence-specific and comprehensive manner seems a daunting task, and no current method allows the simultaneous measurement of the DNA methylation of all CpG dinucleotides. Commonly utilized methods are summarized in Table 1 [6,7].

The initial models that proposed a relatively static epigenome have recently been challenged by genome-scanning approaches. In *Arabidopsis thaliana*, the presence of DNA methylation does not indicate that transcription is turned 'off' [8]. Instead, it has been noted that different levels of DNA methylation are responsible for modulating levels of expression and can interfere with transcription,

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Available online 6 August 2007.

 0168-9525/$ – see front matter doi:10.1016/j.tig.2007.07.005

Table 1. Considerations and methods of DNA-methylation assessment

	Candidate gene approach	Global DNA methylation approach
Definition	Assessment of DNA methylation in genes previously identified as methylated. Analysis is performed on known genes	Assessment of DNA methylation as it pertains to sequences that may or may not have been identified as methylated previously. Not dependent on sequence identity
Points to consider when selecting an assay	Number of CpGs being analyzed	Assay cost
	Assay difficulty	Coverage of the genome
	Assay cost	Distribution of assayed sequences
	Assay reproducibility	Sensitivity of assay
	Assay sensitivity	Expected false positive and false negative rates
	Quantitation depends on technique applied	Support in data analysis
	DNA quality does not affect technique	DNA quality needed
	Amount of input DNA required	Ability for high-throughput
Common techniques	Combined Bisulfite Restriction Analysis (COBRA)	Restriction landmark genomic scanning (RLGS)
	Bisulfite sequencing	Array-based
	MassARRAY	Differential methylation hybridization (DMH)
	Quantitative methylation-specific PCR (MSP)	Tiling arrays
	Southern hybridization	Methylated CpG island recovery assay (MIRA)
	Pyrosequencing	5meC antibody purification of methylated sequences (meDIP)
		Large-scale bisulfite sequencing
		Amplification of inter-methylated sites (AIMS)
		Global re-expression of silenced genes

even outside of classical 'promoter' regions [8,9]. Methylation of sequences outside the promoter region, which results in a reduction of transcript, is thought to block the elongation process of transcription [9].

Similar comprehensive data are not yet available for the human or mouse genome. However, evidence from studies addressing inter-individual DNA methylation distinctions indicates that the picture of a static mammalian epigenome needs to be changed to a more dynamic and complex picture, where epigenetic modifications along the DNA are not independent of one another and may act simultaneously to exude transcriptional changes. For instance, by studying familial methylation patterning along Alu elements [10], and from work in monozygotic twins [11], differences in methylation along seemingly genetically identical regions can vary in families, even in identical twins, demonstrating that environmental factors partly influence the epigenetic program and that this influence leads to changes in DNA methylation, and in this way creates dynamic patterns. The Human Epigenome Project (HEP), a European-led consortium, was the first to recognize the need for a normal reference epigenome in multiple tissues and individuals [12]. In the pilot study, seven different tissues were evaluated in 32 individuals. This study investigated the methylation status of both promoter and intronic gene regions. Surprisingly, 10% of the analyzed regions showed tissue-specific differences in DNA methylation. Out of those, only 31% were localized in the upstream regions of genes. The HEP follow-up study for chromosomes 6, 20, and 22 indicated that 69.8% of the loci on these chromosomes were either largely methylated or unmethylated. However, an astonishing 30.2% showed heterogeneous methylation, with a frequency between 20% and 80% most likely because of mosaic methylation patterns in the tissue studied [13]. These data highlight the heterogeneity of DNA-methylation patterns in different cell types, as previously described [14,15]. These findings raise the possibility that alterations in epigenetic patterns in diseases such as cancer might pre-exist in normal cells. This 'normal methylation' might reflect why particular sequences are susceptible to acquiring additional epigenetic modifications observed during cancer progression.

Gains and losses of DNA methylation in cancer

DNA-methylation patterns are disturbed in cancer, both by gain of methylation (hypermethylation) and by loss of DNA methylation (hypomethylation) as compared with the normal genome. The underlying mechanisms that cause these changes are unknown, but there is evidence that DNA-methylation changes occur early in cancer development and might even initiate tumorigenesis [16]. DNA hypomethylation contributes to cancer because it promotes chromosomal instability, oncogene activation, transposon activation and loss of imprinting (LOI) [17]. However, the detailed aspects of how DNA hypomethylaiton affects gene expression are unclear. DNA methylation can be removed either by an active process involving a DNA demethylase or passively by subsequent replication without DNMT1 maintenance activity [18]. Emerging evidence supports the involvement of DNA-repair mechanisms in the removal of DNA methylation; such repair mechanisms might replace stretches of DNA containing the modified cytosine and thus remove 5-methylcytosine [19].

Environmental influences greatly affect methylation levels; the methyl donor, SAM is largely synthesized in the folate cycle, which can be affected by nutritional intake. Dietary compounds that provide SAM include vitamins B12 and B6, choline and methionine [20,21]. It has been demonstrated in the ApcMin mouse model for colon cancer that the knockout of DNMT1, in combination with a low-folate diet, results in hypomethylation and reduced tumorigenesis [22]. However, mice carrying a hypomorphic DNMT1 allele, which reduces DNMT1 expression levels to 10% of wild-type levels, leads to the development of aggressive T-cell lymphomas [16]. These findings indicate that a stable DNA-methylation pattern is crucial for cancer prevention and that the contribution of DNA-methylation changes to tumorigenesis depends on both cell type and environmental influences.

Concordant with hypomethylation events, gene-specific DNA hypermethylation has been shown to occur in all investigated cancer types, with the exception of seminomatous germ cell tumors [23]. The methylation signature seen in cancers has also been described as non-random [24,25]. In fact, it has been estimated that up to 10% of CpG island sequences are targets of DNA methylation [24]. The descriptions of these methylation events in the promoters of genes have often correlated with repressed transcription of tumor-suppressor genes and other cancer-related genes, fostering cancer at all stages of development.

Loss of regulated DNA methylation in cancer undoubtedly poses consequences for tumor initiation and progression. Initial investigations implicated the overexpression of DNMTs as the initiating event resulting in aberrant DNA methylation, as these enzymes are often found to be overexpressed in human malignancies [26]. However, this explanation doesn't resolve the question of why certain sequences are targeted for methylation, whereas other regions of the genome are demethylated, and why other tumors with minimal increases in DNMT expression continue to demonstrate aberrant methylation patterns [27]. Insight into directed DNA methylation has been eloquently provided by the finding that DNMTs directly associate *in vitro* with oncogenic transcription factors, such as the fusion protein product of promyelocytic leukemia protein-1,retinoic acid receptor (PML-RAR) and MYC; this association results in the recruitment of these factors to target sequences [28,29]. However, data from primary human leukemia cells could not demonstrate the interaction of onco-fusion proteins with the RAR promoter binding sequence [30]. Furthermore, there are differences between methylated (methylation-prone) and unmethylated (methylation-resistant) pools that were independent of CpG island length, CG content and the number of Sp1 sites [31]. This 'methylation sensitivity' is consistent with the hypothesis of targeted DNA methylation. Alternatively, gene-expression changes caused by loss of transcription-factor binding might trigger a cascade of epigenetic events, including histone-tail modifications and nucleosome repositioning, resulting in DNA methylation [32–34]. In addition to the mechanisms mentioned above, a strong selection process can provide growth advantage to cells with DNA methylation and gene expression patterns, can be active.

Histone modifications in normal cells and cancer cells

Compacting the 3 billion bases that comprise the linear human genome, histone octamer cores are found centrally within ~140 bp of DNA, known as the nucleosome. Histone octamer cores consist of two copies of each histone protein (H2A, H2B, H3 and H4). Although variants of these core histone proteins exist, the vast majority of versatility in chromatin effect is attributed to post-translational modifications of these histone proteins. Histones can be modified by acetylation, methylation, phosphorylation, sumolyation and ubiquitination [35]. These modifications result in dramatic changes in the accessibility of the DNA to transcription factors and in the subsequent protein interactions which determine the chromatin structure [21].

Particular histone-tail modifications have been directly linked to active or repressed transcription. For example, acetylation of N-terminal lysine residues along the histone tails by histone acetyltransferases (HAT) has been associated with actively transcribed regions, and in general heterochromatin is fundamentally hypoacetylated and associated with heterochromatin protein 1 (HP1), whereas euchromatic regions are largely acetylated [36]. The acetyl group is negatively charged and counters the overall basic nature of histone tails, which reduces the binding to DNA and thus improves the accessibility of the chromatin during transcription. The reverse process of histone deacetylation, which is catalyzed actively by histone deacetylases (HDACs), restores the overall affinity of histone linkage with DNA, preventing transcription [37].

Other histone modifications cannot be separated distinctly as transcription 'activating' or transcription 'inactivating'. For example, the addition of methyl groups along histone tails can be considered both activating and inactivating, depending on the lysine residue in question [38]. Lysine residues of histone-3 lysine-9 (H3K9) act as the target of mono-, di-, or tri-methylation, which results largely in condensed chromatin in heterochromatic regions, including centromeres and telomeres, and along promoters of genes on the inactive X chromosome in females [39]. In fact, studies modeling loss of H3K9 methylation resulted in enhanced cancer susceptibility, a loss of heterochromatin and increased genomic instability [40]. However, when lysine-4 or -27 of the histone-3 (H3K4 or H3K27) becomes methylated, gene transcription is active [38]. Binding of the heterochromatin protein 1 (HP1) along transcriptionally repressed regions of the chromosomes appears to be what distinguishes them from regions that are transcriptionally active in the presence of methylated lysine [41].

Although the selection process of placement of histone marks throughout the genome is still not completely deciphered, it is clear that these modifications and the enzymes responsible for them are actively participating in carcinogenesis. In fact, H3K9 and H4K20 trimethylation are reduced in many human malignancies and can be used as diagnostic markers for tumor progression [39,42]. Recent data indicate the existence of long-range combinatorial modification states (e.g. coexistence of H3K4me3 and H3 hyperacetylation) on the same residues, providing the potential to target different cellular activities to selected regions in the genome [43].

The role of DNA methylation and histone modifications in gene silencing

Euchromatin is largely associated with a lack of DNA methylation and acetylated histones, whereas heterochromatin is associated with deacetylated histones and methylated DNA [44]. The epigenetic regulation of gene transcription was initially depicted as a cascade in which the DNA became hypermethylated at CpG dinucleotides and subsequently bound to methyl-binding proteins which acted as recruiters for co-repressor complexes containing HDACs and resulted in repressed chromatin and gene silencing [20]. In support of this theory, DNA hypomethylation in repetitive regions has been shown to be an initiating event leading to histone-modification changes [42].

Hormone signaling pathways have also been recently associated with histone modifications that result in gene silencing. Leu *et al*. have shown that loss of estrogen-receptor signaling in breast cancer results in the failure to activate downstream targets and is accompanied by histone marks associated with transcriptional repression. The promoter of the progesterone receptor then becomes methylated to ensure the 'expression signature' is passed on to daughter cells [45,46]. Further support that histone modifications precede DNA methylation in the process of gene silencing comes from *Arabidopsis* and *Neurospora*, where H3K9 methylation is observed first [47,48]. Also, the addition of epigenetic modifying agents, such as decitabine and trichostatin A (TSA), indicates that changes in either histone modifications or DNA methylation might be sufficient to alter gene expression, but there are examples of genes, such as MutL protein homolog 1 (*MLH1*), cyclin-dependent kinase inhibitor 2A (*CDKN2A*) and tissue inhibitor of metalloproteinase 3 (*TIMP3*), where the addition of these agents in combination result in a synergistic change in gene expression, indicating a requirement for both [49].

Chromatin looping adds an extra layer of complexity in epigenetic regulation

The epigenetic complexity of the human genome does not end at the level of DNA methylation and histone modifications. An additional layer of the chromatin configuration pertains to its position in the nucleus and how the location and interactions with loci *in trans* results in transcriptional changes [50]. Chromosomal loops positioned spatially within the nucleus are believed to be sites where distant sequences are brought together in proximity for gene regulation, and understanding how they function might shed light on tissue and cell-type specific differences in gene expression [51]. These 'chromosomal territories' often comprise sequences that converge from multiple chromosomes, and they affect patterns of imprinting and expression [50]. The proximity of chromosomal domains might have key roles such as enabling transcription-factor binding sites to be present in specific locations, which is important for required timing and proper gene expression. Furthermore, chromatin loops enable DNA to be processed efficiently by chromatin-remodeling complexes [52]. Both mechanisms act crucially to maintain proper expression in 'nuclear pockets' and might reflect, in part, what has gone awry in cancer cells.

Epigenetic modifiers as single-agent therapies

Although we are just beginning to understand the complexity of epigenetic gene regulation, epigenetics has become an attractive therapeutic area as a result of the reversibility of these modifications. Most studies examining the effect of epigenetic modifying agents in patients to date are still in the realm of clinical trials. The most commonly used agent for reversing DNA methylation in cells is 5-aza-2′-deoxycytidine (decitabine), a nucleotide analog of cytosine with a carbon replaced at position 5 by a nitrogen atom. Once decitabine is incorporated and targeted for DNA methylation, the methyltransferases become irreversibly bound to the DNA and are prevented from methylating other sites, resulting in a decrease in DNA methylation [37]. Expression-profiling experiments in a colon cancer cell line highlighted the complexity of the response after decitabine treatment. As expected, numerous genes were upregulated after treatment, but a similar number of genes demonstrated reduced expression after demethylation treatment [53]. Interestingly, several cyclins including cyclin B2 (*CCNB2*) and cyclin 25B (*CDC25B*), as well as c-Myc-binding protein (*MYCBP*), were among those found to be downregulated [53]. Although the presence of a canonical CpG island was demonstrated upon examination of these genes, the underlying molecular mechanisms for downregulation are unclear but could be due to secondary effects of genes that became upregulated or DNA methylation changes in sequences that regulate gene clusters (e.g. imprinting control regions).

Nevertheless, decitabine has recently been approved by the Food and Drug Administration for treatment of myelodysplastic syndrome and acute myeloid leukemias as a single-agent therapy [37,54,55]. In solid tumors, although high levels of DNA methylation have been observed, use of decitabine as a single agent has yet to prove effective, although stabilized disease has been noted [56–58]. Dosage and delivery regimens are being examined so that the administration in patients with solid tumors. Because of the requirement of demethylating agents to incorporate into replicating DNA in the S phase of mitosis, it is likely that prolonged exposure at lower doses than originally administered might increase the effective delivery to cells [37]. There have been no reports that using demethylating agents increases tumor incidence in treated patients [37].

Beyond attempts to simply destroy the cancer cells with a toxic agent [59], the focus of epigenetic therapy involves re-expression of genes that had been epigenetically silenced in cancer, allowing cancer cells to be 'reprogrammed' (Figure 1). This approach indicates that there is also potential for demethylating agents and histone modifying agents to act in conjunction with conventional chemotherapy at lower dosages and thus provide enhanced efficacy along with lower toxicity and enhanced patient response. Unlike decitabine, use of MG98, a specific inhibitor of *DNMT1* mRNA, might reduce toxicities associated with generalized demethylating agents [37,60]. Because it is likely that other compounds with demethylating activity exist, one major challenge is to quickly determine the activity spectrum of these agents and identify the most promising molecules for clinical studies. An attempt to evaluate several cDNA methyltransferase inhibitors, including 5-azacytidine, decitabine, zebularine, procaine, epigallocatechin gallate (EGCG) and N-Phthalyl-1-tryptophan (RG108), in parallel was recently published and highlights the diverse molecular activities of these drugs [61].

Like the demethylating agents, inhibitors of histone deacetylases have some promise as effective therapeutic agents. Histone deacetylase inhibitors (HDACi) induce differentiation, cellular growth arrest, and apoptosis and inhibit angiogenesis [62–68]. One of the best studied HDAC inhibitors is valproic acid, which was originally used as an anti-seizure medication but was shown to be effective against cancer [69,70]. Another example of an HDACi with clinical applications is phenylbutyrate therapy, which results in re-expression of tumor-suppressor genes and a

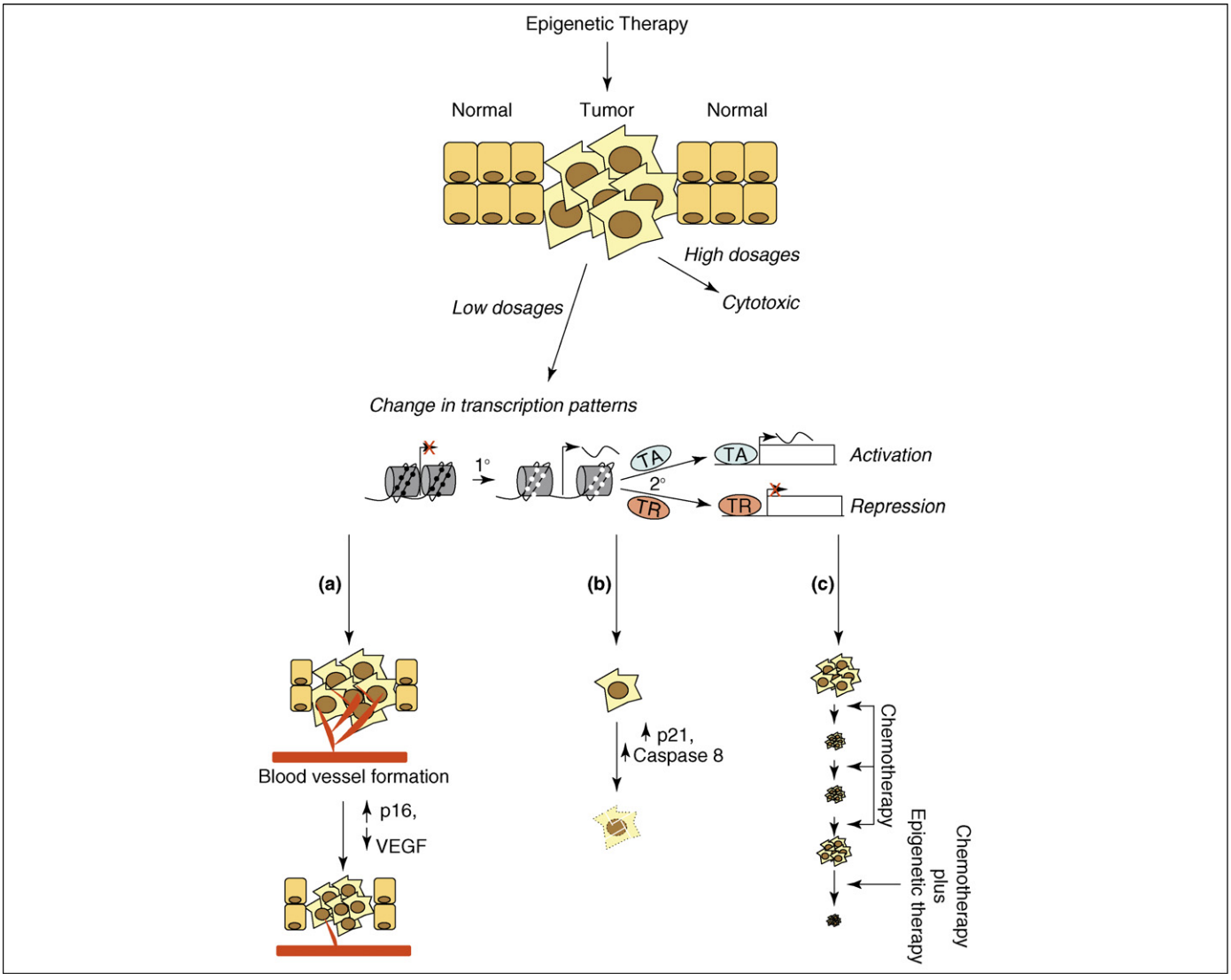

Figure 1. Effects of epigenetic therapy in cancer. Studies performed in laboratory and clinical settings have demonstrated a wide utility of treating cancer cells with histone- and DNA-methylation-modifying agents, so-called epigenetic therapy. Although there have been few or no effects observed in normal cells, primarily because of their limited replication rates, measurable effects have been noted in malignant cells and their environments. One of the earliest mechanisms of action for epigenetic therapy was believed to be on changing the transcription profiles, although, at high doses, demethylating agents have been shown to exhibit cytotoxic effects, which result in generalized cellular death. However, under conditions where prolonged exposure to lower doses of epigenetic therapies has been conducted, cellular reprogramming through changes in transcription has been observed. In the case of transcription-factor activation after epigenetic therapy as the primary (1°) event, both secondary (2°) transcriptional activation (TA) and transcriptional repression (TR) can occur. As a result of these changes in transcription, multiple downstream events can occur in malignant cells. (**a**) Gene reactivation of *p16*, and downregulation of *VEGF* secondary to treatment, results in reduced angiogenesis and defects in endothelial cell proliferation. (**b**) Increased levels of p21 and Caspase 8 have been correlated with induced programmed cell death in malignant cells treated with epigenetic modifiers. (**c**) A tumor, which might initially respond to conventional chemotherapy, but no longer responds, and might even continue to grow, has been shown to become 'sensitive' to the chemotherapeutic agent once again in the presence of these epigenetic modifiers.

blockade of histone deacetylases *in vitro*. In patients, phenylbutyrate treatment resulted in partial responses in hematologic malignancies, but it has not demonstrated efficacy in solid tumors, although many patients had stabilized disease [71]. Depsipeptide, an HDACi that causes an increase in acetylated H3 and H4, which is associated with increased transcription of apoptotic proteins, has been used to treat chronic lymphocytic leukemia and cutaneous T-cell lymphoma/leukemia and is being explored in clinical trials involving a variety of solid tumors [72,73].

Epigenetic inhibitors have also been directed toward angiogenesis [63], the process whereby a tumor develops its own blood supply and is thus able to grow and to metastasize [74]. Studies using decitabine have demonstrated upregulated *p16*, which in turn downregulates vascular endothelial growth factor (*VEGF*), an important pro-angiogenic factor [67], whereas HDACi administration of suberolylanilide hydroxamic acid (SAHA) resulted in downregulation of *VEGF* by an unknown mechanism [66]. Therefore, future anti-angiogenic therapies might include decitabine and HDAC inhibitors to further impede the growth of endothelial cells important for generating tumor vasculature [63,65,68].

Epigenetic therapy as modifiers of conventional regimens

Conventional cancer therapies often result in genetic and epigenetic alterations that result in resistant tumor cells [75]. Because of the reversibility of these epigenetic modifications, combination therapy including demethylating

and/or histone deacetylase inhibitors has been recently explored [76,77]. Currently, there are several examples of epigenetic modifiers that result in either tumor regression or restored chemotherapy re-sensitization. For example, one of the standard treatments for melanoma and renal cell carcinoma is the immune modifier interferon (INF), which induces apoptosis, differentiation, increased anti-tumor immune responses and possibly decreased angiogenesis in cancer cells [78–80]. Defects in the ability of interferon to effectively treat these cancers have been associated with DNA methylation and silencing of genes involved downstream of interferon. For example, interferon regulatory factor 8 (*IRF8)* [81] and XIAP-associated factor 1 (*XAF1*) [78] have been demonstrated to be important downstream contributors to metastasis suppression that become methylated during the process of IFN resistance. However, when decitabine was injected intraperitoneally in nude mice with xenografted melanoma and renal cell carcinomas, the mice tumors were re-sensitized to subsequent interferon treatment, which resulted in a significant reduction in tumor burden *in vivo* [78]. Similarly, a Phase I trial conducted in melanoma and renal cell carcinoma, where decitabine was administered along with IL-2, resulted in measurable patient responses [82]. In patients with refractory chronic myelogenous leukemia, who are resistant to imatinib mesylate treatment, there was a complete hematologic response in 34% of patients when treated with low doses of decitabine [83].

There are also early pre-clinical data suggesting that HDAC inhibitors are effective radiation sensitizers. Several reports suggest that this effect is not through increased expression of selected genes, but rather through decreased expression of genes. For example, investigators demonstrated that DNA repair was diminished in cell lines treated with Vorinostat, presumably through the reduced expression of repair-related genes, including *Ku70*, *Ku80* and *Rad50* [84], and this reduced gene expression might enhance radiation sensitivity. Alternative explanations of the enhancement of radiation sensitivity by pre-treatment with HDAC inhibitors include acetylation of non-histone proteins (including tubulin, p53 and others) and general changes that occur in chromatin structures and enhance radiation induced cell cytotoxicity. These conceptual issues have not yet worked their way into clinical trials, but are an area of active interest.

Concluding remarks

Since the identification of hypomethylation in cancer approximately three decades ago, attempts at 'outsmarting' the aberrant mark in human malignancies have been limited. In part, this reflects the complex and heterogeneous nature of cancer, where likely a single-agent therapy will probably not be effective at 'destroying the masses'. Our basic understanding of DNA methylation as end-point changes in gene expression is expanding exponentially, but our resources for reversing these modifications are limited to non-specific global approaches, leaving several key questions unanswered (see Box 1). In light of all these unanswered questions it is clear that epigenetics as it relates to a translational approach, although demonstrating efficacy *in vitro* and in hematologic malignancies, is limited in regards to the wide use of these agents. It becomes more critical to evaluate how and in what cellular situations the agents function. This evaluation should include cell cycle stage and cellular targets, to most effectively delineate how to utilize their therapeutic potentials. Despite all of these uncertainties, the field is therapeutically promising because of the recently identified synergy between epigenetic inhibitors with more cytotoxic chemotherapies, and we are emerging into a new realm of epigenetic therapy. Previous limitations based on chemotherapy resistance in tumors are now being overcome by epigenetic reversal, building a new era for these agents.

Box 1. Questions to be answered in the future

What are the mechanisms leading to epigenetic silencing of a gene, and how are tumor-type-specific DNA-methylation patterns established in a cancer cell?

How differently do epigenetic modifying agents function *in vivo*, and what consequences to the cells (malignant and nonmalignant, alike) will be encountered?

Will it be possible to develop strategies to reactivate specific genes or groups of genes in a cancer cell?

How can we most effectively administer these agents to patients: alone or in combination treatments?

What are the underlying factors that determine whether a tumor responds to these agents or not, considering how frequently these changes are detected in virtually all tumors?

Why do solid tumors seem more refractory to epigenetic modifiers as opposed to hematologic malignancies?

What assays should be used to assess whether changes in epigenetic patterns have occurred, and in which genes?

Acknowledgements

The authors wish to thank Bjoern Hackanson and Martin Brena for critical discussions of the manuscript. Financial support was obtained for L.T.S by a T32 CA009338–28 fellowship in Oncology, P30-CA16058, PO1-CA101956–01 (CP), The Leukemia and Lymphoma Society of America. C.P is a Leukemia and Lymphoma Society Scholar.

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