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Epigenetics and Disease

Pharmaceutical Opportunities
Preface

The field of epigenetics seeks to explain how cell-type specific controls over gene expression are maintained during self renewal and differentiation and are altered by environmental events. The field covers the biochemistry of genome organization, its expression, inheritance, as well as controls over translation, message stability, and the relationship of all these with external signals. Epigenetics is now at an exciting stage, one comparable to the field of genetics before the elucidation of the structure of DNA and the genetic code. We know most of the players, but are still unclear as to how they work together to maintain gene expression states faithfully. As our understanding of epigenetic inheritance expands, the field intersects increasingly with fields focused on human health and disease. The topics most directly addressed are control over stem cell status and modulation of cell differentiation, which are at the heart of degenerative disease and cancer. Finally, from the study of chromatin and miRNA modifications, we can expect to identify targets, biomarkers, and diagnostic tools relevant for biomedical application.

What new medical opportunities are opened by the field of epigenetics? We note that pharmaceutical developments have in the past avoided targets that control mammalian gene expression, because controls were thought to arise from combinatorial protein–DNA and protein–protein interactions that are difficult to interfere with. Advances in epigenetics have identified enzymes that modify histones, DNA, and other proteins, that collectively control the compaction and organization of chromatin domains, to regulate gene expression. They influence events ranging from transcription, splicing, and mRNA stability to translation. The definition of these molecular modifications and the enzymatic machinery that controls them render gene regulation “targetable” in ways that were not possible in the past. Given that these epigenetic targets guide gene expression both during development and in adult tissues, they become of particular biomedical relevance to a broad range of diseases, including developmental disorders, aging, cancer and tissue degeneration. They are expected to impact the discovery and development of novel treatments.
The field is still exploring what are reasonable goals for an “epigenetics” program in relation to novel medications or developments that promote human health. Below we list a few of the reasonable targets and goals that justify epigenetic studies within the medical world.

1. **Relevant and “druggable” enzymes.** These include new targets such as histone deacetylases, histone acetyltransferases, histone methyltransferases, DNA methyltransferases, demethylating enzymes that act on either DNA or protein, ubiquitinylating E3 ligases, deubiquination enzymes, Sumoylating and Neddy-lating enzymes, helicases, nucleosome remodelers, as well as subdomains of histone modifying enzymes, such as SET domains. Moreover, there are enzymes that signal and control these enzymes, sending signals to chromatin and the translational apparatus. Many of these have been shown to be reasonable and effective targets in cancer cells, for they are upregulated in and essential to tumor cell growth.

2. **Diseases arising from loss or alteration of epigenetic marks.** Examples include Rett syndrome, Prader–Willi/Angelman Syndrome, Fragile X Syndrome, Beckwith–Wiedermann, ATRX, Hutchison–Gilford progeria, schizophrenia and several leukemias. These diseases are useful as models for proof of concept for drugs targeting more general defects. Mouse models of such diseases are particularly useful for understanding human disease and age-related degenerative phenotypes.

3. **Diagnostics and biomarkers.** This arises from genome-wide profiling of modifications, independent of transcription, but correlated with a disease state or response to stress or oncogenic transformation. By monitoring DNA methylation on promoters, or histone modifications generally, we are able to predict the differentiation state of cells, be it in degenerative disease or cancer. This provides a powerful read-out for toxicity, for changes in cellular state, as well as for patient stratification in clinical trials (see below).

4. **Patient stratification.** Epigenomic profiling is a means to select patients and help identify tumor type, prior to clinical trials. The same read-outs are useful to test drug toxicity on both normal and diseased tissues, or as diagnostics of response spectra.

5. **Regenerative medicine.** Cell differentiation correlates precisely with epigenetic changes on the genome-wide level. These can be monitored with high throughput sequencing and Chromatin-IP-sequencing techniques. Small molecules that alter the differentiation state and potential of cells are being discovered. The restoration of a differentiated or pluripotent status to otherwise normal cells may help treat both degenerative disease and cancer. The goal of reprogramming cell fate is within reach, as is intervention to prevent aberrant responses that might alter gene expression profiles in a heritable manner.

We note that particularly cancer and neurological disorders can be traced to misregulation of epigenetic marks. Examples are as shown in Table 1 (adapted with permission from Rodenhiser, D and Mann, M. (2006) CMAJ 174(3), pp 341–348).
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Note: PWS Prader–Willi syndrome; AS Angelman syndrome; BWS Beckwith–Weidemann syndrome; SRS Silver–Russell syndrome; UPD14 uniparental disomy 14; PHP pseudohypoparathyroidism; AHO Albright hereditary osteodystrophy; MAS McCune–Albright syndrome; ICF immunodeficiency, centromeric instability, and facial anomalies; ATRX a-thalassemia/mental retardation syndrome, X-linked; FraX Fragile X syndrome; FSHD facioscapulohumeral muscular dystrophy, CHM complete hydatidiform mole, BiCHM familial biparental CHM

The current book aims to explore novel ideas about diagnostics, treatments, and the power of exploiting regenerative medicine for diseases that have long been inaccessible to medicine. We do not aim at being comprehensive, but rather
forward looking and innovative as we explore the current questions facing biomedical epigenetic research. The goal of this edition is to help define the field of epigenetics in relation to human disease, in order to benefit the medical world, the pharmaceutical industry, and the academic research scientist. Indeed, there are only a few cases in which small molecule inhibitors of epigenetic enzymes have made it to clinical trials. These are the start of a dynamic interaction of epigenetics and drug discovery and will be presented here.

We thank the contributors to this volume for their readiness to submit their visions of the field at short notice. We thank our collaborators who have helped review and discuss the many aspects of epigenetics and disease, and SG wishes to thank her assistant, Nicole Jascur for exceptional support in this project.

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DNA Methylation and Cancer

Phillippa C. Taberlay and Peter A. Jones

Abstract  DNA methylation acts in concert with other epigenetic mechanisms to regulate normal gene expression and facilitate chromatin organization within cells. Aberrant DNA methylation patterns are acquired during carcinogenic transformation; such events are often accompanied by alterations in chromatin structure at gene regulatory regions. The expression pattern of any given gene is achieved by interacting epigenetic mechanisms. First, the insertion of nucleosomes at transcriptional start sites prevents the binding of the transcriptional machinery and additional cofactors that initiate gene expression. Second, nucleosomes anchor all of the DNMT3A and DNMT3B methyltransferase proteins in the cell, which suggests a role for histone octamers in the establishment of DNA methylation patterns. During carcinogenesis, epigenetic switching and 5-methylcytosine reprogramming result in the aberrant hypermethylation of CpG islands, reducing epigenetic plasticity of critical developmental and tumor suppressor genes, rendering them unresponsive to normal stimuli. Here, we will discuss the importance of both established and novel molecular concepts that may underlie the role of DNA methylation in cancer.

1 Overview

The eukaryotic genome is complex and has evolved to enable large amounts of DNA to be contained within the boundary of the nucleus. The structural organization of DNA into chromatin involves several orders of compaction and creates an environment that is generally repressive for gene transcription. However, chromatin is a highly dynamic structure that must be modified to accommodate the transcriptional

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machinery when gene expression is required, to facilitate DNA repair mechanisms, or to allow DNA replication [1]. Epigenetic regulation of these processes is typically driven in a cell type-dependent manner during and following differentiation from totipotency. It has also been established that epigenetic mechanisms, such as DNA methylation, govern many aspects of embryonic growth from conception and are necessary for the survival of mammals. Since several enzymatic systems coordinate epigenetic modifications, a high level of combinatorial control must be maintained to ensure the correct chromatin conformation and identity of each cell. To this end, it is now apparent that alterations to normal epigenetic processes deregulate biological signaling pathways, contributing to carcinogenesis and disease. Here we will discuss mechanisms that may be involved in establishing aberrant DNA methylation patterns in carcinogenesis.

2 Mechanisms of Silencing by DNA Methylation

The biology of DNA methylation events in cancer is currently the best characterized epigenetic aberration in disease [2]. DNA methylation is a relatively stable modification that occurs in the context of CpG dinucleotides in mammalian cells. The presence of CpG sites within the genome is irregular, with some regions containing a high frequency of CpG dinucleotides (CpG islands) in contrast to areas where this dinucleotide is underrepresented. The distribution of CpG sites throughout the genome has implications for cellular gene expression profiles. First, CpG rich regions are often situated in promoters that are proximal to the transcription start sites of many genes while the remainder of the genome is relatively CpG poor, including sites of viral integration as well as intergenic and intronic regions [3]. Second, not all CpG sites in the genome are methylated. CpG islands are resistant to de novo methylation in normal cells [4, 5], while CpG poor regions are predisposed to this process [6].

Distinct methylation patterns are established during embryonic development and are mitotically heritable through many cellular divisions. The faithful maintenance of normal DNA methylation patterns is disrupted in cancer, where CpG islands become susceptible to methyltransferase activity and CpG poor regions undergo hypomethylation during transformation. Consistent with this, the overall level of genomic 5-methylcytosine is decreased in cancer cells [7, 8]. Hypomethylation of bulk cellular DNA might result in genomic and chromosomal instability [9, 10] and is perhaps suggestive of a global switch mechanism that directs changes in chromatin structure concomitant with aberrations in DNA methylation patterns. The change in DNA methylation patterns is considered to be common in most cancers [11], with significant effects on gene expression patterns, cellular growth, and selective advantage. These changes can be the result of silencing of tumor suppressor genes and alterations to associated downstream pathways [2, 12], such as repression of the p53 tumor suppressor pathway [13]. It is important to emphasize
that epigenetic mechanisms act in concert to coordinate normal gene regulatory processes and that cellular deregulation in disease involves many systems.

DNA methylation is a mediator of long-term silencing [6] and contributes to the regulatory mechanisms of tissue-specific gene expression in normal cells. The covalent addition of a methyl group to DNA can influence gene transcription [14] by varying the binding of transcription factors [15, 16] or through the recruitment of methyl-binding proteins [17, 18] and chromatin modifiers such as histone deacetylases [14, 19]. These studies provided some of the first evidence that there was interplay between different epigenetic modifications. Despite this evidence, the exact mechanism for gene silencing by DNA methylation is still debated. Foremost, while covalent histone modifications are sufficient to repress gene expression, they are considered to be a less stable and reversible process [3, 20]. Therefore, it is likely that there are additional determinants that specify the establishment of permanently silenced and hypermethylated CpG islands in cancer.

CpG islands remain unmethylated in normal cells and are frequently sites of DNase hypersensitivity on a global scale [21–23]. DNase hypersensitivity has been used as a marker for genomic regions that are free of nucleosomes, suggesting that the extent of nucleosome occupancy may be correlated with gene silencing and expression. Given these data, it has been hypothesized for many years that inactive genes exhibit a closed, compact chromatin structure in contrast to active gene promoters that are less condensed to allow for the binding of transcriptional machinery. Technological advances have confirmed and extended these data, and it is now clear that the role of nucleosome positioning in gene silencing through DNA methylation is critical for gene control.

DNA accessibility is a requirement for transcription [1, 24] and can be considered to be either a constitutive state [25, 26] or one that is generated following extensive chromatin remodeling, as demonstrated for the PHO5 promoter in yeast [27]. Genome-wide screens in several organisms have shown that the regions upstream of many transcriptional start sites are devoid of nucleosomes [28–32], indicative of the specific gene expression patterns in these eukaryotes. In support of a model whereby nucleosomes are central to gene control, it has been shown that a nucleosome depleted yeast PHO5 promoter is maintained through DNA replication [33]. The inheritance of a nucleosome depleted state was shown to be independent of coactivator complexes, and transcription of PHO5 was not required to maintain the nucleosome depleted region [33]. Despite such striking associations, the significance of these findings was not directly correlated with events of gene silencing during carcinogenesis until recently [24].

Extensive analyses of the MLH1 promoter, which is frequently hypermethylated in cancers, reveal that the formation of a nucleosome depleted region is required for gene expression [24]. The precise positioning of nucleosomes can be determined at individual promoters by using a high-resolution single-molecule assay called methyltransferase-based single-promoter analysis (M-SPA) [34]. The M-SPA assay has also been utilized to confirm the requirement for a nucleosome depleted region at the GRP78 [35] and BRCA1 [24] promoters. By extension, a nucleosome depleted region is likely to be characteristic of expressing genes containing a CpG
island promoter. A nucleosome is inserted immediately upstream of the transcriptional start site of an inactive MLH1 promoter, which becomes permanently silenced by DNA methylation in cancer cell lines [24]. These data suggest that changes in nucleosome occupancy contribute to the epigenetic silencing of CpG islands during transformation (Fig. 1). The mechanisms that then ensure that CpG islands remain permanently silenced are unclear, but it is feasible that this process involves protein complexes that facilitate the addition and removal of other epigenetic marks.

3 DNA Methylation, Covalent Histone Modifications, and Histone Variants

3.1 Histone Variants

Beyond the physical positioning of nucleosomes, the composition and posttranslational modification of these core particles must also be considered. Histone variants, including H2A.Z and H3.3 (Fig. 2a), have altered amino acid sequences compared with the canonical histone proteins [36] and have been shown to have profound effects on gene expression [32, 37, 38] as well as being associated with distinct chromosomal regions [39, 40]. Importantly, H2A.Z is enriched at transcriptional start sites of both active and inactive genes [41], suggesting that H2A.Z also has roles that are independent of transcription. One such function may be to maintain genes in a poised state [42] and to prevent the permanent silencing of these loci by DNA methylation in cancer [40, 43]. Alternatively, H2A.Z may contribute to the over-expression of oncogenes or cell cycle regulators during transformation. It has recently been demonstrated that the over-expression of H2A.Z is linked to the progression of estrogen-responsive breast cancers [44]. In this study, c-MYC was shown to bind to the H2A.Z promoter in response to estrogen, increasing H2A.Z protein expression [44]. This observation correlated with altered proliferation properties of MCF7 cells [44]. Despite this, a conclusive mechanistic link between H2A.Z and cancer progression remains to be established.

DNA methylation and H2A.Z are mutually exclusive epigenetic marks in plants [40]. Altered DNA methylation patterns are mirrored by changes in H2A.Z localization and vice versa [40], suggesting a high level of interaction between the mechanisms underlying these two epigenetic modifications. Specifically, genomic regions that exhibit a loss of DNA methylation become enriched for H2A.Z [40], which is proposed to be a direct effect of DNA hypomethylation events rather than changes in the levels of transcription [40]. The insertion of H2A.Z into nucleosomes is reliant on the Snf-2-related CREB-binding protein activator (SRCAP) chromatin remodeling complex in humans [45, 46]. A mutation in plants of the equivalent complex, PIE1, results in genome-wide DNA hypermethylation in Arabidopsis thaliana [40]. While the distribution of DNA methylation patterns
Nucleosomes contribute to the epigenetic silencing of genes in concert with DNA methylation in cancer cells. In normal cells (above), active promoters are depleted of nucleosomes immediately upstream of the transcriptional start site. Nucleosome-depleted regions are flanked by nucleosomes that are enriched for active marks and are permissive for transcription, such as H3K4me3. In addition, these nucleosomes contain histone variants shown to correlate with transcription, such as H2A.Z. During the silencing process (below), a nucleosome is inserted into the nucleosome depleted region, physically interfering with the process of gene expression. DNA methylation and the acquisition of repressive histone marks, such as H3K9me3, permanently silence genes in cancer cells. DNMT3A and DNMT3B are anchored to nucleosomes associated with methylated DNA. Removal of DNA methylation leads to the eviction nucleosomes from reactivated loci after treatment of cancer cells with DNMT inhibitors such as 5-Aza-CdR (not shown; [24]) agent.

Small white circle: unmethylated CpG site; small black circle: methylated CpG site; large circle: nucleosome; X: silenced transcriptional start site; 4 trimethylation of histone H3 at lysine 4 (H3K4me3); 9 trimethylation of histone H3 at lysine 9 (H3K9me3); DNMT: DNA methyltransferase; 5-Aza-CdR: 5-Aza-2′-deoxycytidine demethylating agent.

Fig. 1
remained similar, an increase in the total level of DNA methylation was observed [40]. Increased DNA methylation correlated with loss of H2A.Z from these loci [40], further suggesting that nucleosome composition contributes to the DNA methylation process.

3.2 Posttranslational Histone Modifications

In addition to composition of the nucleosome, histone proteins may be posttranslationally modified, acquiring active or repressive marks (Fig. 2b). A combination of up to 17 modifications, including previously characterized marks such as the trimethylation of lysine 4 on Histone 3 (H3K4me3) [47], has been proposed to correlate with highly expressed genes [48]. The H3K4me3 modification marks the 5' regions of genes [37, 49] and is anticorrelated with DNA methylation [50]. The
trimethylation of Histone 3 at lysine 27 (H3K27me3) is mediated by the Polycomb Repressive Complex 2 (PRC2) and is associated with gene repression [51, 52]. Interestingly, H3K27me3 enrichment is also anticorrelated with DNA methylation [53, 54]. It is possible that the presence of PRC2 prevents the binding of DNA methyltransferase enzymes (DNMTs), similar to the mechanism by which DNMT 3-Like (DNMT3L) is inhibited by H3K4me3 in germ cells [55]. Perhaps H3K27me3 colocalizes with another histone modification or histone variant and prevents the aberrant hypermethylation of PRC2 target genes in normal cells.

After cellular transformation there are several characteristic marks and protein complexes that accompany DNA methylation. Accessory proteins, such as heterochromatin protein 1 (HP1) [17, 56], are proposed to contribute to the permanent silencing of DNA methylated genes since they are also associated with heterochromatic regions of the genome [57, 58]. The enrichment of H3K9me2 [59] and H3K9me3 [57, 58] is associated with genes silenced by DNA methylation in cancer. Interestingly, H3K9me2 can create a binding site for HP1 [60, 61] and is removed from promoters that have been demethylated and reactivated in cancer cells [59].

Taken together, these data indicate that DNA methylation patterning can be dependent on nucleosome placement and composition, particularly near the 5’-regions of genes that may encompass CpG islands. DNA methylation can result in, or be the result of, the exclusion of histone variant containing nucleosomes near transcriptional start sites. Therefore, a lack of epigenetic marks that specify transcriptional competence, or alternatively a permissive but repressed state, may result in a feedback mechanism that progressively silences CpG island promoters by DNA methylation in cancer.

4 Epigenetic Switching in the Cancer Genome

CpG island promoters often become hypermethylated during cancer progression, while the remainder of the genome exhibits a reduction in DNA methylation [7, 8]. Genes containing CpG islands that were PRC2 targets in embryonic stem cells appear to be predisposed for hypermethylation [62–66]. These genes are critical for development and are normally repressed by PRC2 following differentiation from pluripotency.

DNA methylation and PRC2 occupancy at gene promoters are typically considered to be mutually exclusive events [53, 54]. This may be explained partially from the results of embryonic stem cell studies, which suggest that PRC2 and DNA methylation have the potential to regulate different subsets of genes [67]. However, it is also evident that PRC2 occupied gene promoters are preferentially methylated during the transformation of somatic cells [53, 62, 63, 65, 66], suggesting that there are genes that may be regulated by both PRC2 and DNA methylation.

Genome-wide comparisons of normal prostate epithelium with a prostate cancer cell line revealed that there are three distinct subsets of genes that are silenced or
repressed by epigenetic processes as a consequence of oncogenesis [53] (Fig. 3). First, there are genes that are expressed in normal tissue, but are silenced by DNA methylation in cancer due to a process termed 5-methylcytosine reprogramming. A second group of genes are expressed in normal prostate epithelium and become repressed by PRC2 in cancer [53, 68]. This type of event is termed PRC reprogramming. Finally, there are those developmentally important genes that are repressed by PRC2 in normal cells, but are hypermethylated in cancer [53]. This type of change is termed “epigenetic switching,” but does not result in gene expression changes. Epigenetic switching may reduce the capacity of PRC2 regulated promoters to respond to signals that may otherwise reactivate these genes.

The mechanisms responsible for epigenetic switching are not yet clear. One hypothesis suggests that DNMTs are actively recruited to PRC2 occupied gene promoters during the carcinogenic process [69, 70]. The concept of active DNMT recruitment contradicts evidence that DNA methylation and PRC2 occupancy are typically mutually exclusive epigenetic marks [53, 54]. A model whereby DNMTs

![Fig. 3](image.png)

**Fig. 3** A model of epigenetic switching of PRC2 target genes in cancer cells. In normal cells, genes contained within CpG islands are usually unmethylated and can be active (left) or repressed by PRC2, which mediates the addition of a trimethyl group to lysine 27 of histone 3 through EZH2 (27me3; right). Following cellular transformation, an active gene may undergo 5-methylcytosine reprogramming or PRC2 reprogramming. In addition, genes regulated by PRC2 in embryonic stem cells appear to be predisposed to become DNA hypermethylated in cancer and can undergo epigenetic switching upon transformation (dashed box). Small white circle unmethylated CpG site; small black circle methylated CpG site; large circle nucleosome; 4 trimethylation of histone H3 at lysine 4 (H3K4me3); 9 trimethylation of histone H3 at lysine 9 (H3K9me3); 27 trimethylation of histone H3 at lysine 27 (H3K27me3); X silenced transcriptional start site; DNMT DNA methyltransferase; EZH2 Enhancer of Zeste 2
are actively recruited to gene regulatory regions is therefore insufficient to explain why the majority of embryonic PCR2 targets become hypermethylated in cancer. Alternatively, the process of epigenetic switching could encompass a series of passive events that gradually result in the hypermethylation of CpG islands in cancer. Could a change in histone composition, such as the removal of variants associated with transcriptional activation from gene promoters, mediate some of the alterations that we observe in the cancer epigenome? Ultimately, DNA methylation may be a cause or consequence of changes in nucleosome occupancy, both of which occur during transcriptional silencing and the “locking” of the genome by acquisition of specific histone methylations (such as H3K9me2). Several unique events are likely to be intricately involved in epigenetic switching, resulting in reduced epigenetic plasticity and the silencing of tumor suppressor genes in cancer. The dissection of such events may be possible using newly available high-resolution genome-wide DNA methylation assays [71].

5 Mechanisms of DNA Methylation Inheritance

DNA methylation is a mitotically heritable epigenetic modification. The classical model for the maintenance of DNA methylation patterns has recently been reviewed [72]. A revised model has now been proposed, which encompasses new data and addresses unexplained and previously inconsistent observations regarding DNA methylation inheritance [72]. There are two key characteristics of DNA methylation that partially explain the mechanisms through which it could be inherited. First, patterns of DNA methylation exist, and second, these patterns are distinguishable in somatic cell types [73, 74]. These observations, together with accumulating data, gradually led to the conclusion that mammalian cells had two classes of enzymes, de novo and maintenance methyltransferases, that use DNA as a substrate for methylation.

The DNMT family has now been well characterized. DNMT1, DNMT3A, and DNMT3B are the only enzymes to be shown to have indispensable roles in DNA methylation in somatic cells [75, 76]. DNMT3A/3B are required for de novo methyltransferase activity [76], both having an equal preference for hemi-methylated and unmethylated DNA in vitro [77]. In normal somatic cells, the expression of DNMT3A/3B is reduced compared with embryonic stem cells. Complete abrogation of DNMT3A results in viable litters; however, these mice die approximately 4 weeks after birth [76]. DNMT3B−/− mice are not viable and do not survive embryogenesis [76]. DNMT3L (DNMT3-Like) is a regulatory protein that acts to enhance the activity of DNMT3A/3B. DNMT3L is expressed primarily in gametogenesis to establish parental origin methylation patterns [78, 79]. DNMT3L mice are viable [80], suggestive of its complementary role in establishing DNA methylation patterns. In contrast to DNMT3A/3B, DNMT1 is the “maintenance methyltransferase,” though it also exhibits de novo methyltransferase activity [81]. DNMT1 is preferentially targeted to hemi-methylated DNA [82] and determines
the steady-state level of methylation [83]. DNMT1 is localized to the replication fork during DNA synthesis through interactions with PCNA, a p21-dependent nuclear antigen involved in DNA replication and repair [84] and UHRF1, a DNA repair protein that also has a role in cell cycle progression [85, 86]. Once tethered to the replication fork, the patterns of methylation that were established by DNMT3A/3B are faithfully copied to the newly synthesized daughter strand of DNA by DNMT1 [87]. Like the de novo methyltransferases, abolishment of DNMT1 results in embryonic lethality [88]. It is interesting to note that DNMT1 is also required for cancer cell survival [89, 90], suggesting that this protein has essential roles in both early development and in cancer.

6 Nucleosomes and DNA Methylation Patterns in Cancer

What is the significance of the nucleosome in maintaining accurate DNA methylation patterns and how are these altered in cancer? The classical model of DNA methylation events involves DNMT3A/3B as de novo methyltransferases, together with DNMT1 as the maintenance enzyme. However, several experimental observations do not completely fit with this model of DNA methylation maintenance [72]. Of importance to our understanding of how DNA methylation patterns are inherited is the structure of chromatin, which was largely ignored in the classical model of DNA methylation maintenance [72]. As discussed previously, nucleosomes are absent from the transcriptional start sites of active genes whose promoters are located in CpG islands (Fig. 1). The physical placement of the nucleosome is sufficient to impede transcriptional initiation and correlates strongly with DNA methylation of CpG islands [24] (Fig. 1). Several lines of evidence suggest that chromatin structures contribute to DNA methylation establishment and maintenance. DNMT3 proteins have been shown to associate with heterochromatin [91, 92], which has a higher density of nucleosomes than euchromatic regions. Indeed, DNMT3A has been shown to generate a structure with DNMT3L that could physically encompass a nucleosome [55], and DNMT3A/3B are known to interact strongly with nucleosomes within methylated CpG islands and repeat sequences [93]. However, it is excluded from this interaction by the active H3K4me3 mark. These data strongly suggest that the physical anchoring of DNMT3A/3B to nucleosomes is necessary for the maintenance of DNA methylation patterns (Fig. 1).

It has been suggested that a component of PRC2 recruits DNMT3A to gene regulatory regions, yet this occurs without subsequent de novo methylation [69], indicating that this process may not be a widespread mechanism for the mistargeting of DNMTs and the resultant methylation of CpG island loci in cancer.

The expression of variant DNMT3 isoforms is increased during oncogenesis, resulting in more random patterns of DNA methylation. Altered expression of wildtype DNMT proteins, together with delta DNMT3 isoforms (ΔDNMT3) and catalytically inactive forms of DNMT3, is expressed in a tissue-specific manner [94] and is linked to several types of cancer [48, 95–97]). The ΔDNMT3 isoforms
are a family of seven transcriptional variants that are generated by alternative splicing [98]. Truncated and inactive DNMT3 isoforms can compete with wildtype proteins to alter DNA methylation patterns [99] in a promoter-dependent manner [100].

Increasing the expression of altered DNMT3 isoforms is one way that aberrant DNA methylation patterns could be established in a cancer cell. Alternatively, both wildtype and ΔDNMT3 isoforms could be mistargeted to chromatin during cellular transformation. Chromatin structure is integral to the mechanisms underlying the process of DNA methylation, and the anchoring of wildtype DNMT3A/3B proteins to nucleosomes is dependent on the N-terminal regulatory region of the proteins [93]. This might ensure that unbound DNMT3A/3B does not cause aberrant hypermethylation. Some ΔDNMT3 variants are not anchored to nucleosomes because they lack the N-terminal domain [93]. Therefore, it is possible that the unbound mutant DNMT3 proteins contribute to the establishment of aberrant DNA methylation pattern at CpG islands, irrespective of nucleosome occupancy. Another possibility is that the insertion of the nucleosome into the previously depleted region upstream of transcriptional start sites (Fig. 1) redirects wildtype DNMT3A/3B to CpG islands during oncogenesis. These two possibilities are not likely to be mutually exclusive.

7 Epigenetic Regulation of miRNAs

Small, noncoding microRNAs (miRNAs) have the potential to regulate gene expression by modulating mRNA stability or translation [101–103]. The activity of miRNAs occurs in a cell type-dependent manner [104], contributing to crucial cellular processes such as proliferation and differentiation [105, 106], and it is not surprising then that miRNA expression is altered in cancer cells [107, 108]. The genome-wide profiling of miRNAs has now been conducted for several cancers [109], revealing that most of these ~22 nt noncoding RNA molecules function as tumor suppressors in somatic cells. It has been noted that miRNAs may also be used as biomarkers for various types of cancers [110], predicting outcome and treatment options for patients. This has been correlated with variable miRNA expression within cancer subtypes at different stages of the disease [109].

miRNAs have the potential to act as epigenetic modifiers. A mechanism for the widespread effects of miRNAs in carcinogenesis may therefore be partly explained by their abilities to regulate the translation of DNMTs through epigenetic modification. For example, a reduction in DNMT3A/3B mRNA is observed concomitant with decreased expression of the miRNA-290 cluster [111]. Similarly, overexpression of the miRNA-29 family, which occurs frequently in lung cancer, correlates with reduced transcription of DNMT3A/3B [112]. Aberrant miRNA expression or activity may therefore specify a mechanism by which DNMTs are deregulated in cancer.
In a similar manner, miRNAs have been shown to alter other epigenetic pathways that are disrupted in cancer, such as PRC2 [113, 114]. As mentioned previously, many genes that were PRC2 targets in embryonic stem cells become hypermethylated in cancer due to epigenetic switching [53]. A second group of genes are not de novo target genes of PRC2, yet became repressed in prostate cancer cell lines due to PRC2 reprogramming [53]. Indeed, Enhancer of Zeste 2 (EZH2), the catalytic component of PRC2, is overexpressed in prostate [115], breast [116], and bladder cancers [117]. The overexpression of EZH2 has now been linked to reduced expression of miRNA-101 in cancer cells [113, 114]. Exemplifying the role of miRNAs in regulating epigenetic pathways, it was demonstrated that the restoration of miRNA-101 expression resulted in the reexpression of genes that had been repressed by EZH2 in cancer cells [113]. Further, reduced H3K27me3 levels are observed at known target gene promoters, FAM84 and DDIT4 [113] as well as RUNX3 and WNT1 [114] after EZH2 knockdown in cancer cells. These data indicate that complex networks are involved in epigenetic switching, 5-methylcytosine reprogramming, and PRC reprogramming. Moreover, it is clear from these results that the deregulation of key epigenetic pathways can be mediated by miRNAs.

In addition to acting as epigenetic modifiers, miRNAs themselves can be epigenetically regulated [118]. miRNA-127, located in a CpG island, is silenced by DNA methylation in tumors [118]. A widespread reduction in histone acetylation of miRNA-127 was also detected in these tumors [118], suggesting that both DNA methylation and posttranslational histone modifications play a role in epigenetically regulating miRNAs. The epigenetic deregulation of miRNAs in additional cancers has now been described, such as the hypermethylation of miRNA-34b/c in colorectal cancer [119]. Thus, the hypermethylation of CpG islands can contribute to carcinogenesis not only by silencing tumor suppressor genes, but also by silencing miRNAs.

8 DNA Methylation at CpG Poor Regions

The focus in the field thus far has been on hypermethylation of CpG island promoters in cancers. As stated previously, CpG islands constitute approximately 2% of the genome, while the remainder is CpG poor. Some promoters with intermediate CpG dinucleotide content exhibit tissue-specific patterns of expression, which can be associated with the DNA methylation status of these regulatory regions [120]. There are also examples whereby a single methylation site can influence gene activity at non-CpG islands. For example, repression of the CpG poor Interferon-β promoter has been correlated with the methylation of one CpG site, due to inhibitory effects on transcription factor binding [16].

CpG poor regions are generally methylated in normal tissue [121]. During the initiation and progression of cancer, the DNA methylation landscape is altered such that the genome-wide hypomethylation of CpG poor regions accompanies the hypermethylation events at CpG islands [2]. Such profound changes in DNA
methylation have now been linked to increased chromosomal rearrangements [122], leading to genomic instability and changes in nucleosome positioning throughout bulk chromatin. These events have been correlated with an increase in the incidence of tumors [123] and constitute potential gain of function epigenetic effects by increasing the access of transcription factors and coactivator complexes to DNA [124]. Of particular interest is the resultant reactivation of proto-oncogenes, such as c-Myc [125]. Activation of signaling pathways is also commonly reported, including Wnt/β-catenin [126], several components of which are linked to many types of cancers [127]. Effects on cellular behavior due to the activation of oncogenes and their associated signaling pathways include disruptions to the cell cycle, DNA repair mechanisms, and altered gene expression profiles, to name a few.

The genome-wide hypomethylation of a cancer cell has additional implications. Since retrotransposons and repetitive elements constitute much of the genome, demethylation of these can result in an increased frequency of chromosomal rearrangements and insertional mutagenesis [128], in addition to chromatin instability. Sense and antisense transcripts may also occur, which directly interfere with transcription of proximal genes [124].

Similar to hypermethylation of tumor suppressor genes, the hypomethylation of CpG poor gene promoters and CpG rich repetitive sequences, such as Alu or LINE-1 elements [129], can be correlated with patient prognosis. For example, the long-term survival of patients with ovarian cancer can be linked to hypomethylation of the MAL gene [130]. Interestingly, this is also indicative of resistance to current standard of care treatment with platinum reagents [130]. It could be speculated from these data that the hypomethylation of genes may determine cellular responsiveness to drugs, including epigenetic therapies.

9 Epigenetic Therapy

DNA methylation is integral to the epigenetic silencing process, and aberrations in this process occur at high frequency across a range of cancer types. DNA methylation is an attractive target from a therapeutic standpoint, particularly because CpG island promoters are seldom regulated by DNA methylation in normal cells. Several potent nucleoside analogs have been derived and have been the subject of thorough investigation, including two that have now been approved for clinical use by the Food and Drug Administration, 5-Azacytidine (5-Aza-CR) and 5-Aza-2'-deoxycytidine (5-Aza-CdR) [131]. Of the remainder, 5-Fluro-2'-deoxycytidine (FCdR) is currently undergoing clinical trials [132]. Zebularine is a highly stable inhibitor of DNA methylation [133] and is effective when administered orally. Treatment can result in the reactivation of p16 in bladder cancer xenografts in nude mice [134] and long-term enteral administration of zebularine to cancer prone mice prevented intestinal tumors, having minimal side effects [133].

Interference of the DNA methylation process in cancer cells can promote gene reactivation, together with cellular differentiation [135], similar to observations
first made using immortalized mouse embryonic stem cells [136]. While cancer cells respond to treatment with DNMT inhibitors such as 5-Aza-CdR, it has been demonstrated that normal cells are more resistant to the effects of these compounds [134, 137, 138]. This observation is critical for the therapeutic potential of these agents because it suggests that cancer cells can be selectively targeted.

Clinical trials have demonstrated that 5-Aza-CR and 5-Aza-CdR are effective in the treatment of myelodysplastic syndrome and myelogenous leukemias. However, the administered dosage is critical and must be within a low, narrow range in order for epigenetic effects to be apparent because the drugs become cytotoxic at high concentrations [136, 139]. It was this characteristic that prompted low-dose clinical trials in elderly patients with myelodysplastic syndrome [140]. A low-dose regimen of 5-Aza-CR has approximately 50% efficacy, increasing long-term survival, remission rates, and a general improvement in quality of life [141]. These studies were the first of many demonstrating that an epigenetic agent could delay disease progression [142], although it has been noted that 5-Azanucleosides are particularly unstable and must be given as part of a chronic treatment program to avoid reversal of DNA methylation patterns.

In addition to their instability, nucleoside analogs, such as 5-Aza-CdR, may not be ideal candidates for epigenetic therapy since they become incorporated into DNA and are thus cytotoxic. It has been speculated that the use of nucleoside analogs may result in the formation of secondary tumors that arise due to chromosomal instability after drastic changes in DNA methylation [123, 143]. These hypotheses were drawn from studies in genetically manipulated mice that developed a higher number of tumors following genome-wide DNA hypomethylation [123, 143]. However, the mice in question were born with genetic aberrations in DNMT1, exhibiting phenotypic changes at birth [123, 143]. Moreover, the studies mentioned here did not specifically address problems arising from use of DNMT inhibitors in these animal models [123, 143]. To address this, an investigation into the cytotoxic actions of nucleoside inhibitors was undertaken in leukemic patients [144]. Secondary tumors were not detected in any patient undertaking a nucleoside analog treatment program, though it was not ruled out that they could possibly occur. Alternative DNMT inhibitors are also being investigated and developed [145]. SGI-1027, RG108, and MG98 are small molecule inhibitors that are proposed to decrease DNA methylation by impairing binding sites for cofactors, inhibiting the catalytic activity of DNMTs or acting much like miRNA sequences that inhibit mRNA translation [146, 147].

For the most part, it is assumed that DNA methylation itself specifically results in gene silencing. In agreement with this tenet, many genes are reexpressed in cancer cell lines after treatment with 5-Aza-CdR, including MLH1 in the RKO colorectal carcinoma cell line [24]. However, it must be emphasized that the reactivation of MLH1 is dependent on the eviction of a nucleosome from the promoter. Using the high-resolution MSPA technique, it was noted that approximately 50% of individual DNA molecules became demethylated after 5-Aza-CdR treatment [24]. Of the demethylated molecules, 50% exhibited a nucleosome depleted region immediately upstream of the transcriptional start site [24]. The
remaining promoters, still occupied by a nucleosome in the promoter region, are likely to represent the molecules that are demethylated but transcriptionally repressed [24]. These data illustrate the importance of the nucleosome in the process of transcriptional reactivation after demethylation and suggest that the nucleosome may serve as a mechanism for the epigenetic inheritance of DNA methylation patterns in cancer.

10 Conclusions and Future Directions

The importance of DNA methylation in cancer establishment is clear, though the specific order of events that result in transformation remains to be conclusively established. The advent of sophisticated epigenetic studies has allowed us to integrate our knowledge and study the interplay between histone modifications and nucleosome positioning together with DNA methylation. All of these processes are integral to normal cellular stability, and the initiation of cancer is likely to involve disruptions to each simultaneously. We are now in a position to fully understand the roles of aberrant DNA methylation in cancer establishment and progression. There are exciting new links between stem cell behavior and cancer cells, and perhaps thorough investigation of developmental epigenetics will direct us to the key events that are deregulated in carcinogenesis. Increased information about these mechanisms will allow us to yield novel and specific epigenetic therapies that will ensure lower toxicity and better patient outcomes.

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