Cell Research (2011) 21:502-517.

www.nature.com/cr

REVIEW

Cancer epigenetics: linking basic biology to clinical medicine

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Cancer evolution at all stages is driven by both epigenetic abnormalities as well as genetic alterations. Dysregulation of epigenetic control events may lead to abnormal patterns of DNA methylation and chromatin configurations, both of which are critical contributors to the pathogenesis of cancer. These epigenetic abnormalities are set and maintained by multiple protein complexes and the interplay between their individual components including DNA methylation machinery, histone modifiers, particularly, polycomb (PcG) proteins, and chromatin remodeling proteins. Recent advances in genome-wide technology have revealed that the involvement of these dysregulated epigenetic components appears to be extensive. Moreover, there is a growing connection between epigenetic abnormalities in cancer and concepts concerning stem-like cell subpopulations as a driving force for cancer. Emerging data suggest that aspects of the epigenetic landscape inherent to normal embryonic and adult stem/progenitor cells may help foster, under the stress of chronic inflammation or accumulating reactive oxygen species, evolution of malignant subpopulations. Finally, understanding molecular mechanisms involved in initiation and maintenance of epigenetic abnormalities in all types of cancer has great potential for translational purposes. This is already evident for epigenetic biomarker development, and for pharmacological targeting aimed at reversing cancer-specific epigenetic alterations.

Keywords**:** cancer epigenetics; DNA methylation; polycomb proteins; cancer stem cells; biomarkers; epigenetic therapy
Cell Research (2011) **21**:502-517. doi:10.1038/cr.2011.24; published online 15 February 2011

Introduction

Normal biological functions in a multicellular organism rely on intricate orchestration between the basic cellular features preset by genetic constitution, and a sophisticated network of cellular RNA expression patterns governed by epigenetic regulation. Epigenetics refers to the establishment of heritable changes in gene expression without alterations in primary DNA sequences. Such gene patterns play an essential role in various biological processes including embryonic developmental events, adult cell renewal, gene imprinting, and X-chromosome inactivation [1, 2]. Regulation of these essential biological functions depends on the interplay between at least three major epigenetic mechanisms, discussed in great deal in other reviews in this issue (Gasser, Kouzarides, Crabtree, Zhu), including DNA methylation, histone modifications, and nucleosome remodeling. These controlling mechanisms for chromatin organization act coordinately to modulate expression of canonical coding and non-coding RNAs. If these processes are dysregulated, they may lead to many human diseases, including cancer, autoimmune diseases, and neurological disorders [3-6].

In this review, we would like to provide an overview, based on current knowledge, of how epigenetic components are involved in the pathogenesis of cancer. In this context, we especially explore the relationships between epigenetic control events in normal development, and regulation of normal stem/progenitor cells, and that in oncogenesis. These areas are particularly relevant to the "cancer stem cell" hypothesis and its importance to tumorigenesis and to key aspects of cancer clinical management. The latter clinical applications of epigenetic mechanisms in cancer are explored and discussed, particularly in two major areas – biomarker development and therapies targeting epigenetic abnormalities.

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Epigenetic alterations in cancer development

At multiple stages of tumor evolution, cells escape normal physiological regulation of proliferation, differentiation, and cell death, leading to uncontrolled cell growth [7]. This progression course involves abnormal activation of oncogenes, inactivation of tumor suppressor genes, and altered expression of non-coding RNAs, which can also harbor these functions [8-10]. The abnormal gene function states are driven by both genetic abnormalities, through mutations and genomic instability events, and epigenetic alterations involving the epigenetic machinery mentioned in this issue and which we review directly below.

DNA methylation

Of the epigenetic mechanisms dysregulated during oncogenesis, the best studied are those for DNA methylation. This chemical modification of DNA, as reviewed elsewhere in this issue (Zhu and colleagues), involves addition of a methyl group to the 5′ position of cytosines, predominantly in the context of "CpG" dinucleotides for the mammalian genome. The involved enzymatic step utilizes *S*-adenosyl-methionine as a methyl donor and is carried out by three separate DNA methyltransferases (DNMT1, DNMT3a, and DNMT3b) [11, 12].

In normal mammalian cells, the CpG dinucleotide is under-represented because it has been depleted over evolution via deamination of methylated cytosines and inaccurate repair of the deaminated product to thymines [13, 14]. The majority of these remaining CpG dinucleotide sites are methylated and found in the non-coding repetitive elements and gene bodies. When distributed in CpG poor gene promoters, the sites are methylated in tissue-specific patterns, which often correlate with decreased transcriptional activity [2, 15]. In contrast to the above, a small percentage of "CpG" dinucleotides are clustered in regions termed "CpG islands", many of which surround gene promoters, the transcription start sites, and/or first exons [16]. Some 85-90% of such islands remain constitutively free of DNA methylation with the remainder associated with transcriptional silencing involved in X-chromosome inactivation [2, 17], genomic imprinting [18], and transposable elements inhibition [2]. Some promoter islands in other gene types can also be mosaically methylated in normal tissues, especially those with less CpG density (or what have been referred to as "intermediate density" islands) [1]. Abnormal DNA methylation of promoter CpG islands is a fundamental abnormality of cancer, which is discussed extensively below in this review.

There are two major altered methylation patterns observed in cancer – global DNA hypomethylation and the above mentioned promoter DNA hypermethylation [4, 19-21]. Multiple lines of evidence have indicated that global loss of methylcytosine is correlated with different stages of cancer progression and metastasis in various tumor types, including prostate, cervical, hepatocellular, and brain cancers [22-25]. Moreover, this change may appear at earlier stages such as in pre-invasive colon polyps [26]. The potential cellular consequences of global DNA methylation loss are diverse, ranging from chromosome instability, genetic mutation, to reactivation of various cancer-related genes including cancer testis antigen MAGEA1, inserted viral oncogenes, or imprinted genes related to growth such as IGF2 [25, 27, 28].

In virtually every type of cancer, in the same cancer cells that harbor genome-wide DNA hypomethylation, hundreds of genes simultaneously exhibit DNA hypermethylation of promoter CpG islands [29, 30]. In point of fact, this epigenetic event affects more genes than do mutations [5, 31]. The alteration is associated with very stable states of transcriptional silencing, and for many tumor suppressor genes such as the von Hippel-Lindau (*VHL*) gene [32], cyclin-dependent kinase inhibitors 2A (*CDKN2A*) [33, 34] and 2B (*CDKN2B*) [35-38], and others, this change can serve as an alternative mechanism to mutation for tumor suppressor gene inactivation. For such genes, as embodied in the Knudson two-hit model, the above epigenetic silencing can provide the first hit to inactivate one, or both, alleles of a gene or can co-exist with a mutation in the opposite allele [39].

Epigenetic silencing of key genes can affect virtually all pathways, which participate in the development of cancer at different stages [4] – cancer initiation, progression, invasion, and metastasis. Examples include dysfunction of DNA repair genes, such as *hMLH1* (DNA mismatch repair protein), which can be an early event in the development of endometrial and colon cancer [40, 41] and associated with a microsatellite instability phenotype [42]. Another DNA repair gene, O^6-methylguanine-DNA methyltransferase (*MGMT*), if inactivated by DNA hypermethylation, can predispose tumors to mutations of a specific type in critical genes including *TP53* [43] and *K-Ras* [44]. Loss of cell cycle control can be involved with silencing of the aforementioned *CDKN2A* gene, and changes in cell migration and invasion can involve epigenetically mediated silencing of genes such as *CDH1* [45]. Most recently, pathways modulated by microRNAs (miRNAs) have also been identified as involved with promoter DNA hypermethylation silencing of these non-coding transcription products [46-49]. Also, downregulation of miRNA expression has been linked to overexpression of DNMTs and, thus, facilitation of gene promoter DNA hypermethylation in cancer [50, 51].

Over the past 20 years, a growing number of proven and candidate tumor suppressor genes have been identified, and characterized, by virtue of their being DNA hypermethylated and silenced in cancer. One key aspect of this work is that many of these genes are seldom mutated, or have never been recognized as genetically altered in tumors [5]. Thus, their loss of function in cancer appears to be due solely to epigenetic mechanisms. Examples include, tissue inhibitor of metalloproteinase-3 (*TIMP3*) [52], the secreted frizzled-related gene family, which acts normally to counteract Wnt pathway activity (*SFRP1*, *SFRP2*, *SFRP4*, and *SFRP5*) [53], Ras association gene (*RASSF1A*) [54, 55], and so on. Such gene identification has fueled the development, as will be discussed later in this review, of many new strategies to screen the cancer genome for DNA-hypermethylated genes, and to identify new genes later well proven to have potential to function as tumor suppressor genes [56-60].

How DNA hypermethylation silences genes in cancer cells is a critical aspect of research. Some aspects are known from understanding of basic molecular facets of the DNA methylation machinery and more detailed descriptions of these can be found in the review by Zhu and colleagues in this issue. Briefly, DNA methylation serves as a signal to recruit the methyl CpG-binding domain (MBD) family including MeCP2, MBD1, MBD2, MBD3, and MBD4 [2, 61, 62]. The MBD proteins can recruit histone deacetylases, which are key to many gene silencing protein complexes [2, 63-66]. Importantly, all three biologically active DNMTs also bind these enzymes [67-69]. In addition, MBD proteins are participants in chromatin remodeling complexes, containing transcriptional corepressors such as Sin3A, which are recruited to methylated loci, thereby establishing repressive chromatin architecture leading to transcription repression. The NuRD complex (also known as Mi-2 complex) is one such multi-protein complex involved in methylation-mediated gene silencing, which contains MBD3, histone deacetylases, a chromatin remodeling ATPase, and others [70, 71]. MBD protein-related transcriptional repression can be histone deacetylase-dependent [63, 64, 72] or -independent [73]. The deacetylase-independent mechanisms in transcriptional repression by MBD proteins include steric inhibition of transcription complex assembly, and higher order chromatin structure changes associated with DNA methylation [74].

What is much less understood is how all of the above proteins and complexes and other molecular determinants participate in the initiation of the cancer-specific DNA hypermethylation, and do this in the setting where involved cells are also simultaneously losing normal regions of the same modification. This question is under intense investigation by multiple groups. All clues generated, to date, concern complex interactions of chromatin regulation inherent to developmental biology and cell renewal and interaction of the DNA methylation machinery with modifications of histones, and the proteins that guide these latter modifications. Some features of current hypotheses are discussed in later sections.

Links between abnormal patterns of DNA methylation and chromatin regulation in cancer cells

The active or repressed transcription states of genes are maintained, as reviewed elsewhere in this issue (Kouzarides, Crabtree, Workman) by communications between histone modifications and chromatin-modifying protein complexes. In this regard, polycomb and trithorax, two major groups of chromatin-modifying proteins, have been shown to direct cell fate determination and to preserve gene expression patterns through many rounds of cell division [75-79]. This histone regulation is critical to normal development and adult cell renewal when properly orchestrated and serves in combination with patterns of nucleosome positioning as the key layer of control to establish gene expression patterns [80, 81]. Elucidating these interactions and dynamics is proving critical to the understanding of epigenetic abnormalities in cancer.

In essence, mechanisms that may underlie aberrant gene silencing in cancer, particularly, are being linked to altered patterns of the above chromatin regulatory events as governed by the many different post-translational modifications on histone tails. As reviewed in this issue by Kouzarides and colleagues, there are various types of histone tail modifications, such as acetylation, methylation, phosphorylation, ubiquitination, among others, that combine to determine repressive versus active states of gene transcription. In turn, these modifications regulate gene expression through their interactions with chromatin-associated proteins, in marking regions of transcriptionally active euchromatin and regions of transcriptionally inactive heterochromatin [82]. Key to our current review of cancer-related epigenetic abnormalties is that the balance between many of the above marks can be altered in cancer and these alone may cause dysregulated states of gene transcription. Moreover, these modifications are very interactive with DNA methylation and, thus, as we will discuss, can potentially be key to what triggers initiation and maintenance of cancer-specific abnormalities such as promoter CpG island DNA hypermethylation. For example, histone marks such as acetylated histone H3, and especially di- or trimethylated histone H3 lysine 4 (H3K4me2, H3K4me3), are antagonistic in experimental models to imposition of DNA methylation [83-86].

On the other hand, histone marks for repression of transcription, such as deacetylated histone H3, trimethylated histone H3 lysine 9 (H3K9me3), and trimethylated histone H3 lysine 27 (H3K27me3) are correlated with DNA methylation in normal and abnormal settings [87, 88].

Many of the above possible correlations between histone marks and transcription are evident in cancer. For instance, epigenetically silenced genes, including those marked by promoter DNA methylation, are marked simultaneously, to variable extent, by the repressive marks, H3K9me3 and H3K27me3. In this setting, the active marks of H3K4me2, H3K4me3, and H3K9 acetylation are reduced [87, 89, 90]. Interestingly, when the DNA-hypermethylated genes are reactivated by 5-aza-2′-deoxycytidine, a DNA demethylating agent, or examined in a colon cancer cell line where the DNMT1 and 3b have been genetically disrupted and DNA-hypermethylated genes are demethylated and re-expressed, the repressive chromatin does not fully return to an active euchromatic state [89]. Rather, the gene promoters are left in a "semi-heterochromatic" state, in which the gene promoters have restored levels, to a variable degree, of the active H3K4me mark but retained some levels of the repressive mark, H3K27me3 [90]. This chromatin pattern closely resembles one that Bernstein *et al.* [91] have termed bivalent chromatin. In normal embryonic stem cells (ESCs), this bivalent promoter pattern marks a set of CpG island-containing genes which are held in a poised, low transcription state to prevent premature lineage commitment [91, 92].

The studies of polycomb group (PcG) proteins and complexes are shedding important light on how genes may succumb to abnormal silencing in cancer [93-95]. First, the link of PcG marking and bivalency has been advanced by the initial findings that some 50% of genes with promoter CpG island DNA hypermethylation in colon cancer, are among the ~10% of PcG-marked genes, most of which have the CpG islands in a bivalent state, in ESC and embryonic progenitor cells [88, 96, 97]. Second, several constituents of these PcG complexes have been shown to interact with DNMTs, and possibly to promote initiation and maintenance of cancer-specific silencing in adult cancer [98, 99]. In this regard, EZH2, the PcG protein in the polycomb repressive complex 2/3 (PRC 2/3) that catalyzes the trimethylation of histone H3 lysine 27 (H3K27me3) may be a key player [100, 101]. While EZH2-mediated gene silencing usually takes place in the absence of DNA methylation, a study by Vire *et al.* [99] suggests EZH2 can directly interact with DNMTs. Growing evidence suggests a strong link of EZH2 to oncogenesis and to cancer-specific gene silencing. Overexpression of EZH2 has been found in many cancers and the expression level is correlated with tumor progression and prognosis [102-106]. Depletion of EZH2 leads to growth arrest of cancer cells [102, 107]. Moreover, we have previously shown that EZH2 and H3K27me3 are retained to a variable degree at the promoter of genes, which are DNA hypermethylated and abnormally silenced in cancer [89]. However, knockdown of EZH2 does not fully reactivate genes that are densely DNA hypermethylated and silenced in adult cancer [108, 109], suggesting EZH2 may not be absolutely required for maintenance of DNA methylation.

CBX7, another PcG protein, is a constituent of PRC1, and has also been shown to read the repressive histone marks, H3K9me3 and H3K27me3, and to participate in mediating gene silencing in the development of cancer [98, 110-112]. Similar to EZH2, in an experimental setting, CBX7 is able to recruit DNA methylation machinery to gene promoters and facilitate repression of genes, which are frequently silenced in adult cancer [98]. Given the close connection between chromatin-modifying complexes, gene silencing, and DNA methylation, PcG complexes and their constituents are actively being pursued for their link to abnormal gene silencing and how aberrant DNA methylation is initiated and maintained in cancer.

In addition to chromatin-modifying complexes outlined above, another major player, which participates in mammalian gene regulation is chromatin remodeling complexes. The SWI/SNF family is one such player, which mediates ATP-dependent chromatin remodeling processes and alters the position of nucleosomes along DNA. Malfunction of the SWI/SNF multisubunit complexes has been associated with cancer development [113, 114]. For example, BAF47 (encoded by *SMARCB1*), a subunit of the SWI/SNF complexes, was found to be inactivated in various types of cancer, including rhabdoid tumors [115], central nervous system tumors [116], and chronic myeloid leukemia [117]. Loss of heterozygosity of other components in the SWI/SNF complexes, BRM (encoded by *SMARCA2*) and BRG1 (encoded by *SMARCA4*), has been found in primary lung cancers [118, 119]. Recently, another member of the SWI/SNF complex, BAF250A (encoded by *ARID1A*) was shown to be frequently mutated in ovarian clear cell carcinoma [120]. Although the epigenetic changes caused by these mutations have yet to be defined, the frequent mutations, in many types of cancer, of genes encoding proteins involved in chromatin remodeling have indicated its important role in the pathogenesis of cancer.

Cancer epigenomics

Recent advances of high-throughput technology have enabled scientists to map the human cancer genome at

single-nucleotide resolution [121-127]. Similar to genomic studies, research in cancer epigenetics for gene discovery, has been steadily moving beyond candidate gene approaches to large-scale epigenomic designs for characterization of global epigenetic alterations in cancer, including global patterns of DNA methylation, histone modifications, and factor occupancy of their gene targets. The hope is that understanding the human cancer epigenome landscape will provide valuable insights into the molecular mechanisms involved in oncogenesis and offer implications in translational research [6, 128].

Indeed, the epigenetic alterations in cancer have been shown to be a global event. Several genome-wide methylation studies indicate that hundreds of genes can coordinately undergo CpG island promoter DNA hypermethylation and become silenced in adult cancers, including individual tumors from patients [31, 60, 129, 130]. In some instances, genes can be silenced simultaneously throughout long stretches of chromosomes and the spreading of silencing seems to affect neighboring unmethylated genes through repressive chromatin [131]. However, more often, DNA methylation appears to take place locally with no initial preference for nuclear position or chromatin architecture [132].

Epigenomic studies may provide an additional perspective to sites of cancer-specific abnormalities in DNA methylation-dependent gene regulation. A recent study by Irizarry *et al*. [133] suggests DNA methylation in the "shore" regions, hundreds of base pairs away from classic CpG islands and/or transcriptional start sites, can also contribute to gene regulation. The molecular mechanism of how this "shore" methylation regulates gene expression is not yet well understood. However, one possibility may be that "shore" methylation controls enhancer activities thereby modulating gene expression in cancer.

Characterization of genome-wide methylated cytosines has clinical implications as well. Almost any given type of cancer is a heterogeneous disease composed of distinct clinical and biological subtypes. Genomic and epigenomic profiling may help identify molecular signatures of existing and new subtypes, thereby helping to derive more accurate classifications to guide clinical management [57, 134]. For example, as part of The Cancer Genome Atlas project, global analysis of promoter DNA methylation patterns in 272 glioblastomas identified a distinct subset of tumors with a glioma-CpG island methylator phenotype, which is closely associated with presence of somatic mutations of the *IDH1* gene [57]. Patients with these tumors are younger and have better clinical outcome. Similarly, biologically distinct subtypes in acute myeloid leukemia were discovered using global methylation profiles [135].

Global alterations of histone modifications are of great importance in basic cancer research as well. A study by Fraga *et al.* [136] showed losses of acetylated histone H4 lysine 16 and trimethylated histone H4 lysine 20 were associated with tumorigenesis in a mouse model of multistage skin carcinogenesis. Similar findings were also observed in breast and liver cancer development [137, 138]. In addition to histone modifications, studies on various PcG proteins have provided a link between gene silencing and cancer development.

Epigenetics and cancer stem cell hypothesis

It has long been known that individual cancers harbor heterogeneous cell populations and recent work has emphasized their diverse tumorigenic potentials. Thus, the concept of the cancer stem cell hypothesis has arisen, which stresses that only certain subpopulation(s), known as cancer stem cells or cancer-initiating cells or tumor propagating cells, may sustain and perpetuate tumors [139, 140]. In 1994, John Dick and colleagues demonstrated that CD34+CD38− leukemic stem cells possessed differentiative and proliferative capacities, and were capable of initiating human leukemia in NOD/SCID mice [141]. This seminal study on leukemic stem cells provided a paradigm for later studies on cancer stem cells in solid tumors, such as glioblastomas [142], breast cancer [143], prostate cancer [144], hepatocellular carcinomas [145, 146], colon [147-149], pancreatic [150], and head and neck cancers [151], among others.

Over the past decade, a growing body of evidence has indicated a huge relevance of the cancer stem cell hypothesis to clinical cancer management. As demonstrated in multiple studies, the cancer-initiating cells are usually resistant to standard chemotherapy [152] or radiotherapy [153], leading to clinical recurrence and treatment failure. Furthermore, they may be capable of forming metastatic foci at distant sites. Thus, understanding the origins and molecular characteristics of these cells may pave the way for developing therapies that directly target and eliminate cancer-initiating cells, thereby helping to prevent tumor recurrence or distant metastasis.

Despite the existence of the above discussed tumor cell subpopulations, the cancer stem cell hypothesis, including its clinical relevance and the precise origins of these cells, continue to generate many controversies. Therefore, much effort has focused on how cancer stem cells are derived and what role epigenetic events play during this process. A key hypothesis is that tumors are initiated through abnormal expansion of clonal stem/progenitor cells, which evolve in the setting of the chronic cell renewal events attendant to high-risk states for

cancer, such as chronic inflammation. Here, in concert, genetic and epigenetic changes may help provide the survival advantage which allows these cell subpopulations to withstand the toxic environment of inflammation constituted by accumulating reactive oxygen species, and which then contributes to tumor initiation and progression [154, 155] (Figure 1).

Studies on epigenetic alterations both in stem cells and in cancer are providing important insights into the stem/progenitor cell origin of cancer [154]. There is compelling evidence, as we outlined earlier, showing that the PRC, which we have discussed as linked to abnormal gene silencing in cancer, target similar sets of CpG island-containing genes in ESC as in cancer [88, 96, 97]. A working hypothesis suggested vulnerability for these PcG-marked genes, which do not have promoter DNA methylation in ESC, to gain this change as stem/progenitor-like cells emerge during tumorigenesis [88, 97, 154] (Figure 1). This abnormal methylation may, then, help abnormally lock in activation of stem cell pathways and contribute to the self-renewing ability of cancer cell subpopulations. Many data suggest epigenetic-mediated silencing events, among others, may bestow such properties on cancer cells during oncogenesis [156]. This is well demonstrated by abnormal activation of the Wnt signaling pathway at early stage of colon cancer devel-

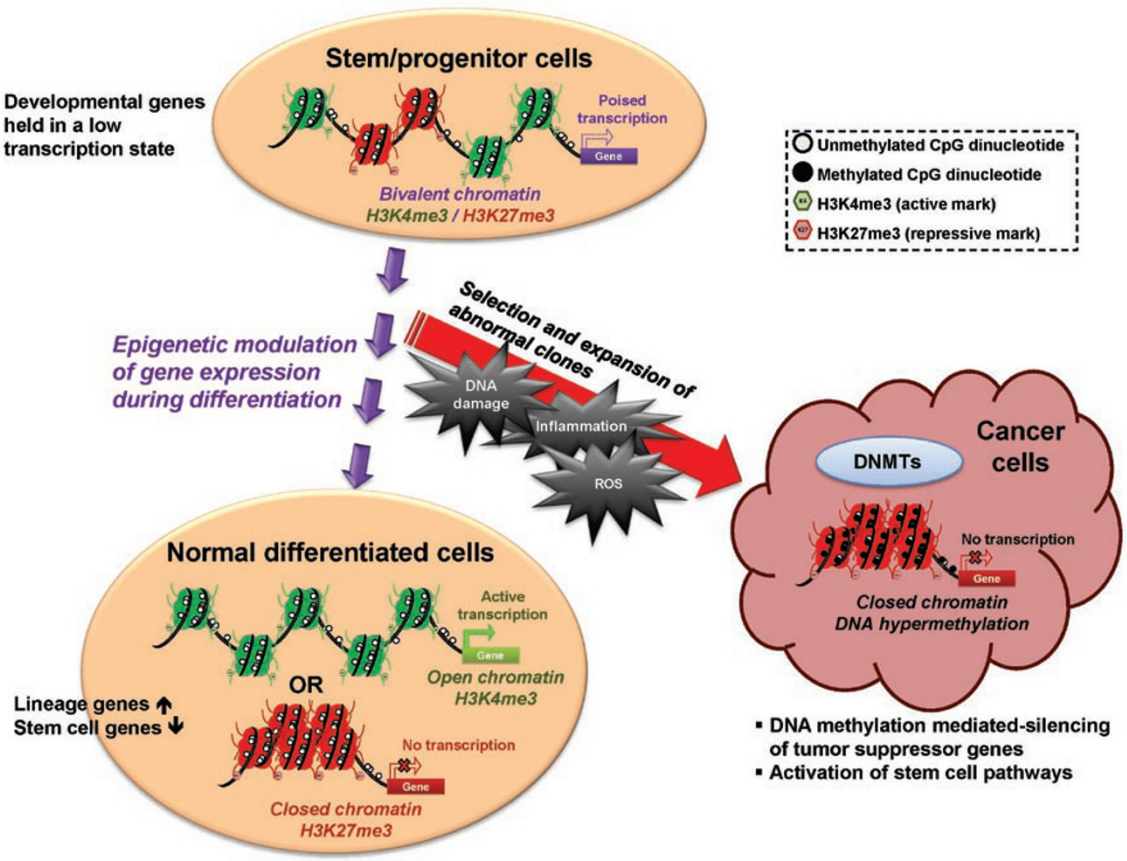

Figure 1 In normal stem/progenitor cells, the promoter regions of many CpG island-containing developmental genes are marked by both active (trimethylated histone H3 lysine 4; H3K4me3) and repressive marks (trimethylated histone H3 lysine 27; H3K27me3), termed "bivalent chromatin" by Bernstein *et al.* [91]. This chromatin pattern holds these genes in a low, poised transcription state to prevent premature lineage commitment. When the stem/progenitor cells respond to environmental cues and start to differentiate, a shift of the balance between the active and repressive epigenetic marks takes place with corresponding changes in chromatin architecture, leading to the silencing of stemness genes and upregulation of lineage-specific genes. However, repeated environmental stress such as chronic inflammation or accumulating reactive oxygen species (ROS) may promote clonal expansion of cells with genetic or epigenetic abnormalities, which then contribute to tumor initiation and progression. During this course of oncogenesis, the repressive marks in the promoter regions of tumor suppressor genes may recruit DNA methylation machinery to impose abnormal CpG island methylation on these genes leading to permanent gene silencing. At the same time, these epigenetic abnormalities may also contribute to activation of stem cell pathways, such as the Wnt pathway, and bestow self-renewing properties on cancer cells.

opment through epigenetic-mediated silencing of key genes, such as Wnt pathway antagonists, including the secreted frizzled-related gene family (*SFRP1*, *SFRP2*, *SFRP4*, and *SFRP5*) and SRY-box containing gene 17 (*SOX17*) [53, 157].

In parallel to the fact that different subpopulations within a tumor possess distinct biological phenotypes, epigenetic-mediated silencing may not be a universal phenomenon in every sub-population. In a glioblastoma cell line model, the methylation status of the promoter of one marker used to identify stem-like cells, CD133 (prominin-1), is heterogeneous between CD133+ and CD133− subpopulations. In most cases, the presence of methylation in the CD133− cells correlated with absence or decreased expression of this surface glycoprotein [158]. This suggests aberrant DNA methylation in tumors can be dynamic and can be imposed during the transition between active and repressive state of gene transcription. Interestingly, however, promoter hypermethylation of other tumor suppressor genes, such as *SFRP1* and *SOX17*, which is often considered to drive oncogenesis at early stages, is already present in the stem-like CD133+ subpopulation and preserved in the CD133− subpopulation, possibly consistent with the stem/progenitor cell origin of epigenetic abnormalities in cancer [158].

Clinical applications

The universal occurrence of epigenetic alterations in cancer has broad potential for important clinical applications. Similar to genetic changes, epigenetic alterations are heritable and stable. Therefore, their potentials as molecular markers in cancer patients are being extensively explored for cancer risk evaluation, early detection, prognosis stratification, and treatment response prediction [4, 159]. On the other hand, unlike genetic mutations, epigenetic changes, including DNA methylation and histone modifications, are pharmacologically reversible, which makes them an attractive target in cancer therapeutics [160, 161].

Biomarker development

The use of monitoring sequences containing promoter CpG island DNA hypermethylation as a diagnostic tool in cancer is gaining widespread appreciation. The high prevalence and abundance of involved genes in cancer tissues, presence of the abnormality at early stages of oncogenesis, relative stability of the methylation marks, and ease of assaying the change in sites such as serum, sputum, stool, and so on with non- or minimally invasive procedures, make use of hypermethylated sequences an attractive biomarker approach [162].

The fact that CpG island promoter methylation of some genes may precede cancer development rationalizes its use to predict risks for cancer. A series of studies showed detection of a panel of DNA-hypermethylated genes in sputum can identify subjects with high risk for lung cancer development [163, 164]. Moreover, methylation markers can be useful for early detection of cancer. For instance, presence of *TFPI2* or *GATA4* methylation in stool DNA has reasonably high predictive value of colorectal cancer and can be used as a non-invasive screening tool coupled with conventional screening methods [165, 166].

Similarly, accumulating data indicate gene-specific methylation can be a useful clinical marker for patient prognosis stratification. One example is *RASSF1A*, for which inactivation by promoter methylation is associated with poor prognosis in patients with different types of cancer [167, 168]. Likewise, Brock *et al.* [169] showed that detection of *p16* and *CDH13* methylation simultaneously in DNA from tumors and mediastinal lymph nodes of patients with stage I non-small cell lung cancer who underwent curative resection is associated with early recurrence. This molecular re-staging strategy may, then, be powerful for predicting which patients with this disease may benefit from more than just surgery alone. These findings suggest that prognosis prediction markers may be used to guide clinical management. Sometimes, a panel of multiple genes may be required for such purposes. In a recent study by Shen *et al.* [170], a panel of 10 DNA hypermethylation genes was used to predict overall survival in patients with myelodysplastic syndrome. Notably, some attempts have been made to identify novel markers through genome-wide methylation profiling. With this approach, Figueroa *et al*. [135] were able to discover a panel of 15 genes predictive of overall survival in patients with acute myeloid leukemia.

In addition, DNA methylation patterns may be predictive of patients' response to chemotherapy and correlated with clinical outcome. One such example is for the gene encoding, O^6-methylguanine-DNA methyltransferase (*MGMT*), a DNA repair protein, which reverses the addition of alkyl groups to the guanine base of DNA. Promoter methylation-mediated silencing of *MGMT* in gliomas is a useful predictor for response to alkylating agents, such as carmustine (BCNU) or temozolomide [171-174]. Similarly, methylation of a mismatch repair gene, *hMLH1* in ovarian and colon cancer cell lines confers chemoresistance to many chemotherapeutic agents. Treatment with a DNA demethylating agent, 5-aza-2′-deoxycytidine, can reactivate *hMLH1* and reverse the chemoresistance [175, 176]. Likewise, epigenetic silencing of apoptotic peptidase activating factor 1 (*APAF-1*), a

proapoptotic gene, confers chemoresistance to melanoma and leukemia cells through mediating resistance to cytochrome *c*-dependent apoptosis [177, 178]. These findings demonstrate the potential for clinical use of DNA methylation markers in tailoring medical care to the need of individual patients.

Notably, assay of histone modifications may also provide a potential molecular strategy to monitor clinical outcome in cancer patients. Several studies have shown that lower global levels of dimethylated histone H3 lysine 4 (H3K4me2) and acetylated histone H3 lysine 18 predict clinical recurrence in prostate, lung, kidney, breast and pancreatic cancer patients [179-182].

Epigenetic therapeutics in cancer

Targeting reversal of epigenetic alterations in cancer such as DNA methylation and histone modifications has emerged as an attractive strategy in cancer management owing to the reversible nature of these changes [160]. Many compounds have been discovered to target proteins that control DNA methylation, histone acetylation, and histone methylation. Some of them are already being used clinically with encouraging effects, which highlights the potential of epigenetic therapy and facilitates the development of novel drugs to target epigenetic mechanisms in cancer. Two clinically used compounds with DNA demethylating activities, azacitidine (Vidaza; Celgene, Summit, NJ, USA) and decitabine (Dacogen; SuperGen, Dublin, CA, USA), have been approved by the FDA for their promising efficacy in hematological malignancies, especially in the pre-leukemic disorder, myelodysplastic syndrome [183-187]. Both compounds are structurally similar to cytosine nucleosides and require incorporation into DNA to exert effects. They were synthesized in the 1960s as anti-metabolites and later found to have DNA demethylating activities through inhibition of DNMTs [188, 189]. In earlier years, the high toxicities observed in cancer patients treated with the drugs at high doses limited their widespread uses, especially in solid tumors [190]. Nevertheless, in the past decade, the drugs received renewed clinical interests and use of low dose regimens is yielding promising clinical efficacy with relatively mild side effects.

Many efforts have been made toward elucidating the actual mechanisms through which azacitidine and decitabine exert clinical efficacy. In addition to potential re-expression of tumor suppressor genes, which are silenced in association with DNA hypermethylation [4, 191], these drugs have multiple effects including cancer cell differentiation [192-195], DNA damage [196, 197], formation of covalent adducts between DNMTs and azanucleoside-substituted DNA [198, 199], immune modulatory effects through reactivation of cancer/germ-line antigens [200], inhibition of NFκB anti-apoptotic pathway [201], and so on. Notably, as some data suggested, these drugs might regulate gene expression in a DNA methylation-independent manner through breaking up complex protein interactions by inhibiting and removal of DNMTs from the nucleus [67, 69, 202]. It has also been speculated that global effects of the drugs, both DNA methylation-dependent and -independent, may reverse genome-wide epigenetic alterations in cancer through resetting multiple cellular pathways. Besides mechanisms of action, it would be equally important to study mechanisms of drug resistance for translational implications. Indeed, Qin *et al*. [203] found low deoxycytidine kinase, low nucleoside transporters (i.e., *hENT1*), and high cytosine deaminase are factors that confer resistance of cancer cell lines to decitabine. Moreover, in light of the cancer stem cell hypothesis and the epigenetic mechanisms involved, understanding whether the drugs have differential effects on different subpopulations may help guide future uses of these drugs in the clinic.

Another class of epigenetic-modifying agents used clinically is histone deacetylase (HDAC) inhibitors [204-208]. In cancer cells, HDAC enzymes, among many other functions, can modulate chromatin configurations and mediate cancer-related gene silencing as components of repressive protein complexes containing DNMTs. Thus, inhibition of HDAC enzymes may reverse abnormal gene silencing in cancer. Many HDAC inhibitors have been shown to have potent anti-tumor effects and entered clinical trials. Two such inhibitors, vorinostat (also known as, suberoylanilide hydroxamic acid; SAHA) and romidepsin (also known as depsipeptide or FK228) have been approved by the FDA for treating cutaneous T-cell lymphoma [206-208]. In addition to anti-tumor effects, other potential uses of HDAC inhibitors and other epigenetic-modifying agents in clinical oncology are being explored. A study by Sharma *et al*. [209] indicates drug resistance may derive via epigenetic mechanisms and can be reversed by various HDAC inhibitors. This suggests a novel use of epigenetic therapy in overcoming tolerance or resistance to standard chemotherapy in the clinical setting of cancer management, where drug resistance has always been a major concern.

Given that DNA methylation-mediated aberrant gene silencing in cancer involves transcriptional repressive complexes containing both DNMTs and HDAC, targeting both enzymes with combination therapy of a DNMT inhibitor and an HDAC inhibitor appears to be an inviting approach in cancer management (Figure 2). Indeed, sequential application of an HDAC inhibitor following a DNA demethylating agent has shown synergistic effects

in gene re-expression *in vitro* and enhanced anti-tumor effects clinically [210, 211]. Moreover, epigenetic-modifying agents may couple with other standard chemotherapeutic agents to boost clinical efficacy with lower doses of either drug. Emerging data indicate azacitidine and decitabine may modify multiple cellular pathways through gene reactivation, and sensitize cancer cells to other drugs that target similar pathways.

Despite the promising clinical efficacy of azacitidine or decitabine at low doses in hematological malignancies, when given alone [183, 184, 187, 212] or in combination with other drugs [211, 213], whether the drugs used at similar dosing schedules exert comparable anticancer activities on solid tumors is actively under investigation. In an ongoing lung cancer clinical trial at our institution, a low dose regimen for azacitidine and an HDAC inhibitor, entinostat (also known as SNDX-275 or MS-275) achieves robust and durable response in some patients with metastatic disease who failed several lines of previous chemotherapy. Clinical trials in other tumor types including breast and colon cancers are underway. Importantly, in addition to clinical efficacy, several areas warrant extensive research in the context of clinical trials to maximize patient benefits, such as optimization of dosing schedule and sequences, and searching for ways to identify those patients who would potentially benefit from epigenetic therapy.

Future directions

It is apparent that, over the past 20 years, our view of tumor biology has changed with a major addition being awareness that epigenetic abnormalities complement genetic alterations to drive all stages of cancer evolution. While the research in cancer epigenetics has already contributed to our understanding of fundamental steps

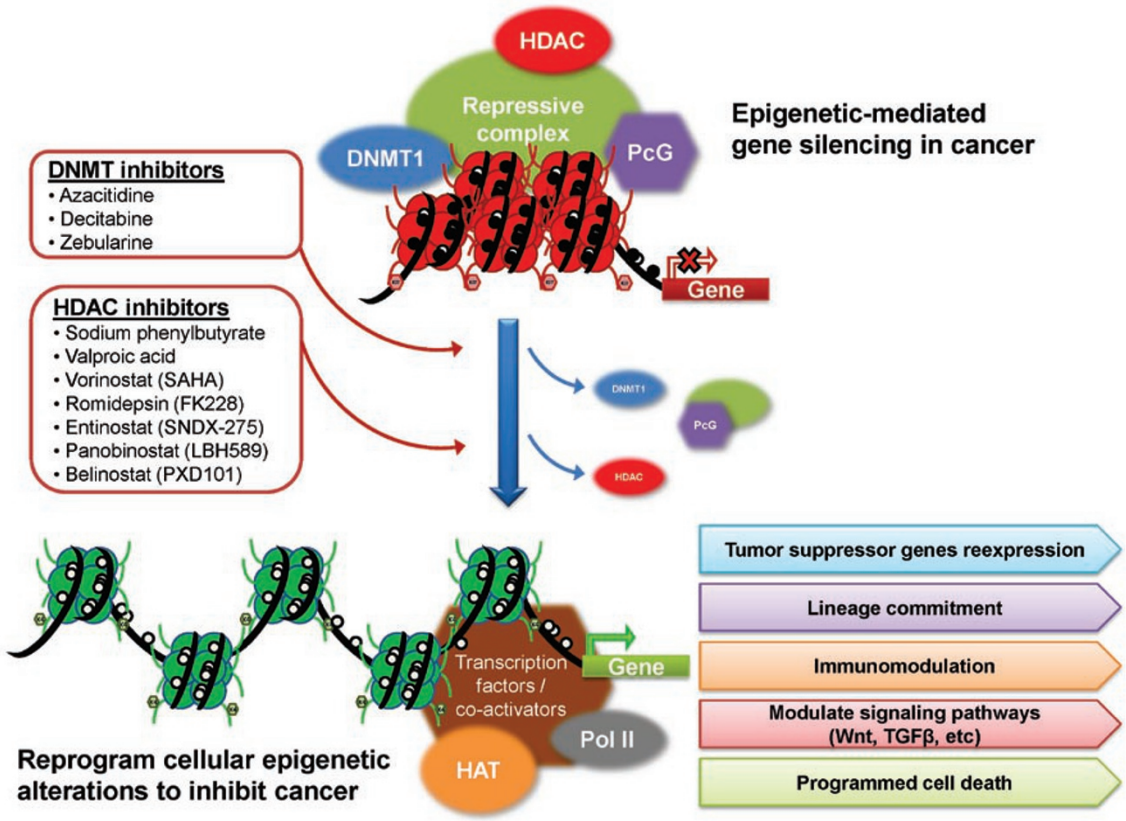

Figure 2 DNA methylation-mediated aberrant gene silencing in cancer involves transcriptional repressive complexes in the gene promoter region and interactions between DNA methylation machinery, chromatin modifiers (such as histone deacetylase, HDAC) and polycomb (PcG) proteins. Pharmacological inhibition of individual components in the repressive complex with DNMT inhibitors and HDAC inhibitors, either alone or in combination, may result in DNA demethylation and complex disintegration leading to reactivation of critical genes and reversal of genome-wide epigenetic alterations in cancer through resetting multiple cellular processes, including lineage commitment, immunomodulation, major cell signaling pathways, programmed cell death, and others. HAT: histone acetylase. Pol II: RNA polymerase II.

in cancer formation, to our knowledge about control of normal and abnormal gene regulation by the chromatin landscape, and to the growing potential for use of information gained for translational purposes, major challenges remain. We are far from having full understanding of the molecular mechanisms that are responsible for the initiation and maintenance of the epigenetic abnormalities that help drive tumorigenesis. We must, then for example, pursue the possibilities for molecular progression of abnormal gene silencing during tumor progression as contributed by PcG mediation of transcriptional repression. What drives this initiation and progression and how do cancer risk states play a role? What are the targeting mechanisms for PcG and its inter-actors in this progression? How precisely do they tie together the concept of cancer stem-like cells to events for derivation and maintenance of stem and progenitor cells in normal developmental and adult cell renewal settings? In this regard, especially, the molecular ties between PcG and targeting of DNA methylation in normal and neoplastic settings need much further clarification. Most broadly speaking, the full epigenomes of all cancer types, and their subpopulations, need to be mapped and compared accurately with the normal cell compartments from which they arise. This effort must take into account the interplay between genetic abnormalities in cancer and how these depend upon the epigenetic landscape for their oncogenic potential. Also, the precise biological ramifications of the growing number of recognized cancer mutations in genes encoding for proteins involved in regulation of chromatin and DNA methylation must be delineated [113, 114, 120]. Finally, we have much work ahead to exploit all of the above knowledge for translational purposes. We must continue the development of epigenetic biomarkers, which can enhance our capabilities to assess cancer risk, to make earlier cancer diagnoses, and to chart cancer prognosis and predict therapeutic responsiveness of different cancer subtypes. The potential for reversing epigenetic abnormalities for the purposes of cancer prevention and treatment is real but is probably in its very early stages in terms of delineating the best molecular targets, and developing or learning to use the drugs and agents that will be required. The future is a bright one and should hold bountiful rewards for both basic and translational cancer research.

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