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DNA hypomethylation in cancer cells

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Abstract

DNA hypomethylation was the initial epigenetic abnormality recognized in human tumors. However, for several decades after its independent discovery by two laboratories in 1983, it was often ignored as an unwelcome complication, with almost all of the attention on the hypermethylation of promoters of genes that are silenced in cancers (e.g., tumor-suppressor genes). Because it was subsequently shown that global hypomethylation of DNA in cancer was most closely associated with repeated DNA elements, cancer linked-DNA hypomethylation continued to receive rather little attention. DNA hypomethylation in cancer can no longer be considered an oddity, because recent high-resolution genome-wide studies confirm that DNA hypomethylation is the almost constant companion to hypermethylation of the genome in cancer, just usually (but not always) in different sequences. Methylation changes at individual CpG dyads in cancer can have a high degree of dependence not only on the regional context, but also on neighboring sites. DNA demethylation during carcinogenesis may involve hemimethylated dyads as intermediates, followed by spreading of the loss of methylation on both strands. In this review, active demethylation of DNA and the relationship of cancer-associated DNA hypomethylation to cancer stem cells are discussed. Evidence is accumulating for the biological significance and clinical relevance of DNA hypomethylation in cancer, and for cancer-linked demethylation and *de novo* methylation being highly dynamic processes.

Keywords

cancer; DNA methylation; DNA repeats; genomic sequencing; hypermethylation; hypomethylation

DNA hypomethylation: a ubiquitous feature of carcinogenesis

The first-described epigenetic changes in human cancer were losses of DNA methylation (m^5C residues replaced by unmethylated C residues) reported in 1983. We observed this alteration of DNA methylation throughout the genome in various cancers versus a wide variety of normal tissues [1], and Feinberg and Vogelstein described hypomethylation of a few cancer-irrelevant gene regions in colon adenocarcinomas versus normal colonic epithelium [2,3]. It was apparent that metastases were even more susceptible to cancer-linked DNA

In memoriam: This article is dedicated to the memory of Charles Gehrke, PhD and Professor of Biochemistry, University of Missouri, Columbia. His expertise in analytical biochemistry and gracious willingness to collaborate with me, starting in 1979, when I was very much his junior, were critical to launching my career in the then nonexistent field of cancer epigenetics [1,84].

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hypomethylation than primary tumors. Many subsequent reports have confirmed the frequent overall genomic hypomethylation in cancers relative to control tissues (e.g., [4–9]).

The opposite methylation change, hypermethylation of promoters of tumor suppressor genes, homeobox genes and other sequences, is also one of the most constant features of the cancer genome [10–15]. In spite of the prevalent increases in DNA methylation, overall deficiencies in the m^5C content of DNA are found in almost every type of cancer, but not in all specimens (Figure 1). The following list contains just some of the types of tumors that have been shown to display overall losses in genomic m^5C:

- Ovarian epithelial carcinomas versus the corresponding cystadenomas or various normal postnatal somatic tissues [16,17];
- Prostate metastatic tumors versus normal prostate [4];
- Leukocytes from B-cell chronic lymphocytic leukemia versus normal leukocytes [18];
- Hepatocellular carcinomas versus matched nonhepatoma liver tissue [19];
- Cervical cancer and high-grade dysplastic cervical lesions versus normal cervical tissue or low-grade dysplasia of the cervix [20];
- Colon adenocarcinomas versus adjacent normal mucosa [21];
- Wilms' tumors versus various normal postnatal somatic tissues [22].

Even a murine model of prostate cancer based upon early and late T-antigen transgenes expressed in a prostate-specific manner displays both satellite DNA hypomethylation and gene locus-specific hypermethylation in the tumors (~2% of ~1200 analyzed loci) [23].

Just as there are cancer-type specific differences in DNA hypermethylation patterns [12], so some DNA sequences are more or less susceptible to DNA hypomethylation depending on the kind of cancer [24]. Some types of cancer, for example, testicular germ cell seminomas, display an especially large amount of genomic hypomethylation. However, in the case of testicular germ cell seminomas, it could be the result of the cell of origin of the tumor having unusually hypomethylated DNA [25,26]. Moreover, for some DNA sequences, cancer-linked DNA hypermethylation can be seen in some specimens and hypomethylation seen in others, as discussed below.

Contribution of hypomethylation of DNA repeats to overall genomic hypomethylation

Hypomethylation of highly repeated DNA sequences [5,17,27,28], which comprise approximately half of the genome, is largely responsible for the global DNA hypomethylation that is observed so frequently in cancers (Figure 1), as we first demonstrated for satellite DNAs [29–31]. Tandem centromeric satellite α, juxtacentromeric (centromere-adjacent) satellite 2, the interspersed Alu and long interspersed elements (LINE)-1 repeats are the most frequently studied DNA cancer-hypomethylated repeats [17,27–34]. Although hypomethylation of centromeric and pericentromeric tandem repeats in cancers can be significantly associated with each other, sometimes one of these types of repeats is hypomethylated and the other is not [17,35,36].

Cancer-linked hypomethylation of gene regions

Hypomethylation of transcription regulatory regions in cancer seems to be much less frequent than hypermethylation of CpG islands overlapping promoters [5,14,23,27,37]. Nonetheless,

some of the cancer-associated loss of DNA methylation encompasses gene regions, including transcription control sequences [38–45]. For example, the gene encoding the protease urokinase (*PLAU/uPA*) is overexpressed and hypomethylated in conjunction with tumor progression in breast cancers (grades 1, 2 and 3) and prostate cancers (benign prostate hyperplasia vs cancer) [46,47]. Decreased methylation of this gene that is implicated in breast cancer invasion was induced by incubation of a breast cancer cell line with 5-azadeoxycytidine and was associated with increased expression of this gene [48]. Among the genes displaying cancer-linked hypomethylation and transcriptional activation are cancer-testis genes, *S100A4*, mesothelin, claudin4, trefoil factor 2, maspin, *PGP9.5*, *POMC* and the heparinase gene [49–53]. Cancer-linked hypomethylation of gene promoters is often associated with decreases in overall genomic methylation [9,54] or satellite DNA hypomethylation [55].

DNA hypomethylation can be found early in carcinogenesis, but can also be associated with tumor progression

Regional hypermethylation can occur very early in tumorigenesis, but it can also be so strongly associated with tumor progression that it serves as a significant indicator of survival [56–58]. Similarly, hypomethylation of DNA sequences is often observed during the early stages of tumorigenesis or in abnormal non-neoplastic tissue, such as hyperplasia [21,29,38,59] (Figure 1). For example, age-related hypomethylation of certain DNA sequences appears to precede aneuploidy in a subset of gastrointestinal cancers [60].

Hypomethylation of DNA is generally more pronounced with tumor progression or the degree of malignancy [17,30,61,62], although this depends on the type of tumor and the individual specimens [35,59,63] (Figure 1). Among the examples of tumor progression-linked DNA hypomethylation is the transition from the chronic phase of chronic myeloid leukemia to blast crisis; this transition is significantly associated with hypomethylation of a CpG island in the LINE-1 repeat promoter, as determined by a quantitative methylation-specific PCR assay [64]. Overall, DNA hypomethylation is associated with the grade of cervical neoplasia [20], with multifocal versus unifocal hepatocellular carcinomas [65], and the disease stage, tumor size and histological grade for breast tumors [66]. For prostate adenocarcinomas, LINE-1 hypomethylation has a highly significant relationship with lymph node involvement [67]. For hepatocellular carcinomas, the degree of hypomethylation of either of two repetitive sequences was significantly correlated with postoperative recurrence of disease and was a better predictor than conventional factors [68]. Hypomethylation of both centromeric and juxtacentromeric satellite DNAs was significantly associated with tumor grade in ovarian carcinomas and was a highly significant marker of the risk for relapse and overall survival time [35]. Although more studies have revealed tumor progression-linked hypomethylation of DNA repeats, gene hypomethylation increasing with tumor progression has also been described [46,47].

Overview of relevance to cancer biology

Recently, there has been a surge in reports about cancer-associated DNA hypomethylation. These include genome-wide mapping of hypomethylation, investigating the roles of this hypomethylation in carcinogenesis, identifying and testing cancer markers, and designing and monitoring anticancer therapies. However, for a while, the importance of decreases in DNA methylation associated with carcinogenesis was overshadowed by the emphasis on cancer-linked hypermethylation of tumor-suppressor genes [69]. The biological significance of both decreases and increases in DNA methylation to cancer is underscored by their contribution to other human diseases, normal vertebrate development, bioengineering and by their susceptibility to environmental influences [33,70–80].

Some of the observed cancer-associated hypomethylation in gene regions contributes to the disease [5,53,81–83]. As described below, studies of cancer-linked DNA hypomethylation indicate that it is not simply a consequence of cancer-associated hypermethylation. Moreover, DNA methylation changes in cancers are much more dynamic than previously envisioned.

Some of the confounding factors in assessing cancer-associated changes in DNA methylation

Normal cell-type specificity, individual or age-related variations in DNA methylation & tumor purity

Cancer-linked DNA hypomethylation denotes less methylation relative to some control tissue (s), which should be specified. However, as we had demonstrated in collaboration with Charles Gehrke (formally Distinguished Emeritus Professor of Biochemistry, University of Missouri, Columbia, MO, USA, and recently deceased), there are tissue-specific differences, as well as species-specific differences, in the amounts and distribution of DNA methylation [84,85]. For example, normal human cerebellum DNA has 17% more m^5C than heart DNA. In addition, tissues are generally composed of a heterogeneous mixture of cell types, which can differ in their genomic methylation. At the gene level, normal tissue-specific changes in promoter methylation could obscure oncogenesis-mediated changes if the appropriate control tissue is not used, for example, with the tumor-suppressor gene, *SERPINB5* [86].

Even similar cell types may differ epigenetically because of derivation from different stem cell niches [87]. Moreover, a cancer may arise from a minor stem cell component or from independently transformed progenitor cells as part of a neoplastic field effect [88–90], and these might have an atypical methylation pattern (see below). Another factor confounding analysis of cancer-linked changes in DNA methylation is heterogeneity at the allelic level due to imprinting [91], or allelic differences that are independent of parent of origin [92].

Moreover, in quantitative studies of cancer-linked changes in DNA methylation, the percentage of cells from the tumor sample that are non-neoplastic may vary and affect the results if the tumor sample has not been microdissected. For some kinds of tumors, like Wilms' tumors and ovarian epithelial carcinomas, which consist mostly of neoplastic cells, this is not an important factor, but it is important for others, like breast adenocarcinomas.

In addition, there can be epigenetic variability among individuals. Increases or decreases in DNA methylation can occur with aging, and individual-specific differences in DNA methylation patterns have been found [93,94]. However, these are usually localized and subtle [85,95–97]. In some cancer studies, the variability of DNA methylation in non-neoplastic tissues is not adequately addressed by the proper choice of control tissue or by the use of a diverse set of control tissues. Nonetheless, numerous articles on cancer-linked changes in DNA methylation indicate that differences in DNA methylation in malignancies versus control tissues vastly exceed individual-specific differences in a given normal postnatal tissue [7-9, 16-22].

Methods of analysis

There are many new methods for analyzing DNA methylation [28,37,98-101]. The types of alterations in DNA methylation that can be observed are strongly affected by the method of DNA methylation analysis and the choice of sequences analyzed; for example, normally unmethylated or partially methylated CpG islands [102] (Box 1). For example, in order to visualize both increases and decreases in methylation of the same DNA subregion, it is optimal for the DNA sequence in the control tissue to have an intermediate extent of methylation or

some CpGs with highly conserved methylation and others with conservation of the unmethylated status [24].

Another confounding factor in DNA epigenetics is the normal heterogeneity of DNA methylation patterns from molecule to molecule owing to the lack of precise preservation of methylation patterns *in vivo*. It is this heterogeneity that helps make epigenetics a major contributing factor to tumorigenesis. Some of the heterogeneity in the patterning of methylation changes of individual sequences, including laterally along the DNA and across the two strands of a given CpG dyad, evades detection in studies where the average methylation content at each examined CpG is determined. Such heterogeneous changes can be observed when individual DNA molecules are analyzed by molecular cloning [103-106].

Box 1

A summary of some of the salient spatial–temporal features of cancer DNA epigenetics

At the level of several hundred base pairs: a high degree of site-to-site dependence

- A neighboring-sites model rather that an independent-sites or context-dependent model best describes methylation changes in regions that are not under strong cancer-selection pressure.
- Sometimes hypomethylated and hypermethylated CpG dyads are neighbors.
- Some CpG dyads persist as hemimethylated sites.

At the level of genes, gene clusters or other large regions: long-range coupling

- Selection pressures during tumorigenesis help shape DNA methylation patterns, for example, homogeneous hypermethylation of promoters of genes whose silencing facilitates cancer formation.
- There are regions of long-range epigenetic changes containing either:
  - –Clusters of hypermethylated CpG islands;
  - –Pockets of hypomethylation; or
  - –Long tandem repeats with overall hyper- or hypomethylation.

Snapshots of a specific time & place

- The detection of cancer-linked DNA methylation changes depends on: the method of analysis and choice of DNA sequences to be analyzed, the tumor specimen, tumor subregion, amount of ‘contaminating’ normal cells, the stage of carcinogenesis and the use of an appropriate normal tissue for comparison.
- Usually only DNA sequences with an intermediate level of methylation in normal tissues will reveal both hypo- and hyper-methylation.

Interplay between cancer-linked hypermethylation & hypomethylation

Cancer-linked hypo- & hypermethylation are generally independent, although most studied cancers display both changes in their genome

In a comprehensive study of satellite DNA hypomethylation, global DNA hypomethylation and hypermethylation of 55 gene loci in three types of ovarian epithelial tumors of different malignant potential and a variety of normal somatic tissues [17], we found that DNA hypomethylation and hypermethylation usually co-exist in the same tumor but in different

sequences. Moreover, progressive changes in both promoter hypermethylation and hypomethylation of DNA repeats or global DNA hypomethylation can be seen in comparisons of premalignant lesions and certain types of cancer, cultured cell or animal models of tumor progression [62,107,108]. Even for tandemly repeated DNA sequences, the same cancer specimen can display hypomethylation of one type of repeat (satellite DNA) and hypermethylation of another (*NBL2* and *D4Z4* in subtelomeric regions or in the short arms of the acrocentric chromosomes) [24,109,110].

It had been proposed that there is cross-talk between demethylation and *de novo* methylation pathways during tumorigenesis, making one dependent on the other. For example, DNA demethylation might be used as a type of epigenetic repair in normal postnatal cells to attempt to compensate for physiologically inappropriate methylation of CpG islands overlapping promoters of tumor suppressor genes or polycomb-complex target genes. As for conventional DNA repair pathways, this epigenetic repair pathway might be only partially effective and might demethylate many more sequences than just the incorrectly methylated CpG islands. Alternatively, DNA hypomethylation might occur early in oncogenesis and be followed by hypermethylation [111] as a kind of increased, compensatory *de novo* methylation consequent to the genomic hypomethylation. This might be analogous to an *Arabopsis* antisense DNA methyltransferase (*DNMT*) gene, which caused gene hypermethylation despite an overall decrease in genomic cytosine methylation [112].

However, we demonstrated in the above-mentioned study of ovarian carcinomas [17] that DNA hypomethylation could not just be described as a consequence of DNA hypermethylation or *vice versa*. The degree of gene hypermethylation was associated with the degree of malignancy of ovarian epithelial tumors (benign, low-malignant potential or carcinomas). However, this association was independent of the association of satellite or global DNA hypomethylation with the degree of malignancy [17]. These results were consistent with previous less extensive analyses of other types of tumors [22,67] and with more recent studies [113]. In addition, in our analysis of ovarian tumors, no DNA hypomethylation variables were significantly associated with DNMT RNA levels after adjustment for multiple comparisons [17].

Nonetheless, both hyper- and hypo-methylation of DNA are linked to carcinogenesis and have some inter-relationship other than one being dependent on each other. They may have some step(s) in common in the poorly understood pathways that lead to these divergent epigenetic changes [105]. Indeed, cancer is generally characterized by the instability of the epigenome at the levels of DNA (methylation of cytosine) and chromatin (protein associations, modifications and chromatin conformation). Moreover, different types of cancer-linked epigenetic abnormalities can interact in various ways [90,114,115]. In addition, epigenetic abnormalities in cancer are occasionally associated with genetic changes, for example, methylation of the promoter of O^6-methylguanine DNA methyltransferase and increased G–A mutations in glioblastomas [116].

Tumor specificity of DNA hypomethylation

There is tumor-type specificity in cancer-associated DNA hypomethylation of a given sequence, just as there is for a gene region hypermethylation in cancer [24,117,118] (Figure 1). For example, we found that the extent of hypomethylation of juxtacentromeric satellite 2 DNA from chromosome 1 was often greater in ovarian carcinomas than in Wilms' tumors. Nonetheless, at least moderate levels of hypomethylation were frequent in both types of cancer [30,31,35,36,119].

Surprisingly, DNA sequences that display an intermediate level of methylation in normal tissues may exhibit opposite cancer-associated alterations depending on the tumor specimen or the tumor type [105]. For example, some ovarian epithelial carcinoma specimens exhibited

strong overall hypermethylation of the NBL2 (a repeat found mostly on acrocentric chromosomes) and others had strong hypermethylation of this tandem repeat [24]. Such opposing changes in DNA methylation could be observed because the intermediate level of methylation of NBL2 in all control somatic tissues [24] allowed the determination of a decrease in methylation in some cancers and an increase in others. We also found opposite cancer-linked changes in methylation at a subtelomeric tandem repeat, D4Z4 [109].

Importantly, among 17 ovarian carcinomas and 44 examined Wilms' tumors, there was a significant correlation ($p < 0.001$) between the direction and degree of methylation change at D4Z4 and the dissimilar NBL2 [109]. This suggests that diverse sequences on different chromosomes can be recognized by the same demethylation or *de novo* methylation mechanisms during carcinogenesis. However, many of these cancers displaying hypermethylation of D4Z4 and NBL2 repeats had hypomethylation of satellite DNA, a very different type of tandem repeat [24]. This is similar to the findings of Katargin *et al.* on D4Z4 and satellite 2 methylation in cervical cancer [118]. We recently demonstrated that even satellite 2 DNA, which is highly susceptible to decreases in methylation in cancer, can exhibit focal hypermethylation in some cancers [106].

DNA methylation changes probably overshoot their biological targets in cancer & often spread over large genomic regions

A high-resolution tiling-array analysis of two entire chromosomes demonstrated that cancer-linked hypermethylation favors CpG islands and hypomethylation favors repeated sequences, including tandem repeats, interspersed repeats and segmental duplications [14], as we had inferred previously [5]. Most increases and decreases in DNA methylation are not strictly targeted to specific loci. Rather, they are found in many regions of the genome that are probably irrelevant to cancer [2,24,28]. This overshooting of cancer-linked DNA methylation changes to many more sites than are biologically relevant may parallel overshooting of tissue-specific alterations in DNA methylation during development [73]. For example, although aberrant promoter methylation is mostly associated with effects on transcription in cancer, sequences in the body of genes can also become hypermethylated without noticeable effects on transcription [15,108].

Recently, it has been observed that large regions of chromosomes may be coordinately methylated or demethylated in cancer [37,45,120] (Box 1). These apparently coordinated methylation changes might be related to the type, frequency, spacing of DNA repeats or to the presence of clusters of interrelated gene. However, cancer-linked DNA hypermethylation does not necessarily spread from repeats to neighboring sequences. Within a subtelomeric segmental duplication in several cancers, we have observed hypermethylation of the bulk of a tandem repeat array but hypomethylation of border sequences at the beginning of the repeat array and nonrepeated sequences proximal to the array [109].

Dynamic nature of cancer-linked DNA methylation changes

That a given DNA sequence can be susceptible to both increases and decreases in methylation during carcinogenesis is relevant to the one statistically significant association that we found between DNA hypomethylation and gene hypermethylation. This was an inverse relationship between hypomethylation of the juxtacentromeric satellite 2 DNA and hypermethylation of *CDH13* in ovarian carcinomas and breast cancers [17,35]. This association might reflect waves of DNA demethylation during carcinogenesis that could affect some CpG-rich regions and reverse their cancer-linked *de novo* methylation [121,122].

The fluidity of epigenetic changes in cancer is also indicated by our above-mentioned findings that NBL2 and D4Z4 repeats could be extensively hypomethylated in some cancer specimens

and hypermethylated in others [24,109]. Moreover, for NBL2 repeats, we showed that hypomethylated CpG dyads could be next to hypermethylated dyads [105]. These results and the low, but appreciable, frequency of stable hemimethylated CpG dyads in normal tissues and cancers [104–106,123] suggest that DNA methylation after differentiation is maintained by more dynamic mechanisms than generally assumed, rather than just by maintenance methylation. Similarly, Pfeifer *et al.* concluded that normal human cells have a dynamic methylation system from a study of bisulfite-mediated genomic sequencing of cloned DNA molecules from an X-linked gene in cultured cells with or without treatment with a DNA inhibitor [124].

The idea that the control of DNA methylation even in normal postnatal tissues involves *de novo* methylation and demethylation is gaining support [40,125–129]. In cancer cells, methylation and demethylation mechanisms appear to be much less regulated, which leads to DNA methylation instability in the directions of both gains and losses of methylation. This nonuniform loss of control of DNA methylation is seen in the metastatic tumor heterogeneity of promoter hypomethylation (LINE-1 interspersed repeat promoter) in prostate cancers [130]. Moreover, bidirectional changes in DNA methylation imply that DNA hypermethylation might spontaneously reverse during the course of carcinogenesis or be reversed in response to treatment.

Usually, studies of abnormal DNA methylation in cancers are snapshots of a specific time and place (Box 1). These analyses only reveal the epigenetic status of the analyzed portions of the genome in the tumor when and where it was sampled, and do not detect fluxes in DNA methylation changes during tumor development or progression. Selective advantages to certain alterations in DNA methylation during carcinogenesis, such as repressive methylation of promoters of tumor suppressor genes, probably help shape more uniform methylation patterns in cancer. Non-gene DNA repeats, like the tandem repeats mentioned above, may give insights into the nature of cancer-linked DNA methylation changes better than gene regions, because they are probably not subject to strong selection pressures for transcriptional silencing or activation during carcinogenesis, as are tumor-suppressor genes, polycomb-complex target genes and oncogenes. Moreover, their intermediate stages of methylation changes may be more stable because they reside in condensed regions of the genome.

Evidence for cancer-linked hypomethylation being causally involved in carcinogenesis

Nutrition & DNA methylation

Experiments involving DNA methylation inhibitors *in vivo* and *in vitro* [131–133] or analyzing DNMT-deficient mice [134,135] indicate that the loss of DNA methylation is important for oncogenesis. Studies of tumorigenesis in rodents fed methyl-deficient diets [70,136,137] are in accordance with a causative role for DNA hypomethylation in cancer. Global DNA hypomethylation could be induced in rats by dietary methyl deficiency and, if prolonged, was irreversible upon resumption of a normal diet [138]. This DNA hypomethylation was associated with formation of initiated hepatic foci. Evidence has been presented that diet can affect DNA methylation and thereby expression of some genes [139,140]. Moreover, for certain human cancers, inadequate folate intake is implicated as a risk factor, especially in combination with the C667T polymorphism in *MTHFR* [141]. However, a recent study showed that dietary folate deficiency in $Ung^{-/-}$ mice caused a significant decrease in DNA methylation in epithelial cells without inducing tumor formation [142]. Nonetheless, most of these nutritional studies argue for causal roles of DNA hypomethylation in carcinogenesis.

Mouse models with *Dnmt* deficiency or other genetic sources of loss of DNA methylation

Many animal models have been used for studying the effects of changes in DNA methylation on cancer formation but, as indicated above, the type of model greatly influences the results. DNA hypomethylation in $Apc^{Min/+}$ mice induced by mutation (heterozygosity for a *Dnmt1* mutation) and/or 5-azadeoxycytidine treatment (drug-induced demethylation) decreases, rather than increases, the number of intestinal polyps [143]. Experimentally induced DNA hypomethylation was associated with decreased progression of microadenomas to macroscopic tumor growths [144]. However, transgenic mice containing a hypomorphic mutant *Dnmt1* allele and consequent DNA hypomethylation had increased formation of hepatocellular adenomas and carcinomas despite the decreased formation of macroscopic intestinal adenomas [135]. Transient decreases in Dnmt1 activity during murine development led to multiple tumors in association with the loss of imprinting [145]. A hypomorphic allele of *Dnmt1* in mice resulted in thymic lymphomas that displayed frequent insertion of a transposable element into the *Notch* oncogene [146]. Genetic knockout of the chromatin remodeling protein Lsh in mouse transplantation experiments led to upregulation of retroviral elements and oncogenes, hypomethylation of DNA (especially of repeated sequences), impairment of normal erythropoeisis, and erythroleukemia [83]. The variety of tumor outcomes from animals in which DNA methylation levels are experimentally altered is likely to be dependent on the type of tumor that is studied, the genetic background of the animal, chromosome-epigenetic variations and the specific DNA sequences affected by the methylation changes [12,24,135].

Gene hypomethylation contributing to tumor formation & tumor progression

Hypomethylation of gene regions, which has been implicated in various diseases [147], may foster the formation and progression of tumors in diverse ways. Some of the gene-regulatory regions that display cancer-linked hypomethylation have correlated increases in transcription [5,82]. Hypomethylation and overexpression of some imprinted genes, including the *IGF-II* and *H19* genes, are implicated in carcinogenesis [145,147,148]. Gene region hypomethylation could contribute to oncogenesis by affecting classical oncogenes, but evidence suggests a greater involvement in the activation of genes associated with tumor invasion or metastasis [48].

The synuclein-γ gene is among the cancer invasion and metastasis genes that seems to be a target of the oncogenic effects of DNA demethylation in a wide variety of cancers [81,149–151]. It is a marker of metastasis and can be mechanistically linked to tumor progression. Among the pathways by which its tumor progression- and demethylation-linked overexpression may influence tumor progression are its roles in neurofilament integrity, signaling pathways [301] and proliferation [81]. Another gene target of tumor progression-linked DNA methylation changes is drug-resistance genes. The increasing destabilization of DNA methylation patterns that characterizes much tumor progression may interfere with cancer treatment by increasing the drug resistance of cancer cells through promoter region hypomethylation for some genes and hypermethylation for others [152].

Important insight into DNA methylation changes and oncogenesis came from a study of global DNA hypomethylation and estrogen receptor gene hypermethylation observed in colorectal adenomatous polyps and adenocarcinomas, but not at the earlier hyperplastic polyp stage [153]. These methylation abnormalities were more reversible by treating patients with a nonsteroidal anti-inflammatory drug (celecoxib, Pfizer, Inc., NY, USA) at the adenomatous polyp stage than at the adenocarcinoma stage. This suggests an epigenetic progression that might involve chromatin changes subsequent to DNA methylation changes.

Hypomethylation of DNA repeats contributing to carcinogenesis & chromosomal rearrangements

The hypomethylation of interspersed repeats and tandem repeats might promote tumor formation or progression by fostering DNA rearrangements [31,134,154–158]. For example, there is an association between hypomethylation of satellite DNA at the juxtacentromeric heterochromatin of chromosomes 1 and 16 and chromatin decondensation and rearrangements in these regions in lymphoid cells. This is seen in cells from patients with the immunodeficiency, centromeric region instability, facial anomalies (ICF) syndrome [72,159, 160], and in certain human cell lines [161,162]. In cancer, pericentromeric rearrangements involving these regions are over-represented and often manifest as unbalanced translocations that can promote tumor formation by affecting the copy number of genes relevant to cancer [163,164]. Murine embryonic stem cells show an increase in chromosomal translocations upon knockout of *Dnmt1* [156]. Associations between DNA hypomethylation and chromosomal rearrangements in human cancer have been observed [157,165]. There is also cause-and-effect evidence for DNA hypomethylation predisposing to chromosomal rearrangements [154,155, 158]. The carcinogenesis-associated role of DNA hypomethylation in activation of transposable elements or endogenous viruses [146] is another example of its role in promoting cancer-predisposing or tumor progression-linked genomic rearrangements. However, the amount of cancer-linked DNA hypomethylation in individual cancer cells versus the frequency of chromosomal rearrangements in those cells suggest other pathways for DNA hypomethylation favoring tumorigenesis and tumor progression, in addition to the enhancement of rearrangements [36].

Indirect effects of hypomethylation of DNA repeats on gene expression

We have proposed another pathway for hypomethylation of DNA repeats contributing to carcinogenesis that involves regulating, in *trans*, the expression of genes by altering the sequestration of transcription control proteins at DNA repeats. Given the highly reiterated nature of satellite DNA (Alu repeats and LINE-1 elements), these sequences have high potential to act as holding or delivery docks for transcription control proteins. These four categories of DNA repeats are very frequently subject to cancer-linked DNA hypomethylation [5,27,28]. Because DNA methylation affects the DNA binding of many proteins [166–169], transcription control proteins could be sequestered by DNA repeats in a methylation-responsive fashion. This would provide a novel, possibly tissue-specific, pathway by which hypomethylation of DNA repeats could impact gene expression and carcinogenesis.

Examples of mammalian transcription regulators that bind selectively to centromeric or juxtacentromeric heterochromatin by DNA–protein and/or protein–protein interactions are accumulating: Ikaros; ATRX; heat shock factor 1; C/EBPα, C/EBPβ and C/EBPδ; DNMT1, DNMT3A and DNMT3B (which can repress transcription independently of their methylating activities); the MeCP proteins MBD1, MBD2 and MeCP2; HDAC1 and 2; and HP1-associated proteins like TIF1 β [170–178]. Heat shock factor 1 localizes to 9qh and the centromeric regions of chromosomes 12 and 15 upon heat shock [172], with induction of transcription of satellite 3 at chromosome 9 [179]. The transcription is associated with the co-localization of a transcription factor to this heterochromatic region. Additional pathways for transcription-regulatory changes in methylation of nongene DNA repeats may be through alterations in subnuclear compartmentalization of chromatin [27,180–183] or altering transcription of noncoding RNAs [184–187].

Cancer stem cell hypothesis is compatible with demethylation during carcinogenesis

Can the term ‘demethylation’ be applied to the origin of the pervasive hypomethylation of DNA in cancer? In the context of DNA epigenetics, demethylation means the exchange of C residues for m^5C residues without implying the mechanism. Instead of DNA demethylation during carcinogenesis, cancer-associated hypomethylation might have been due to selection for a pre-existing hypomethylated state of many DNA sequences in cancer stem cells that give rise to tumors. Studies of stem cells and early embryonic cells clearly indicate that most cancer-linked hypomethylation of DNA repeats, the predominant sequence subject to this hypomethylation, can be described as carcinogenesis-associated demethylation (see below). Moreover, as mentioned above, the frequent finding that increased tumor progression is associated with increased DNA hypomethylation indicates that demethylation must be occurring during tumor progression. Some of the other arguments for DNA demethylation during carcinogenesis follow.

While there is renewed interest in the concept of cancer stem cells, including epigenetic evidence for such cells [188,189], the identification of cancer-initiating cells as normal stem cells is still controversial [190]. Even if normal stem cells are the source of many cancers, it is clear that epigenetic changes in DNA and chromatin are part of the early stages of carcinogenesis [190]. Although DNA methylation changes have been observed in a recent genome-wide comparison of DNA methylation in mouse embryonal stem (ES) cells and ES-derived neural precursor cells, there was only approximately 2% demethylation (which is much less than the levels of global demethylation of the genome routinely observed in cancer [5]) and *de novo* methylation of CpGs [191].

Importantly, studies of satellite DNA methylation in normal differentiation precursor cells do not support the hypothesis that satellite DNA hypomethylation in cancer is a pre-existing condition in normal stem cells giving rise to tumors. Wild-type mouse embryonic stem cells have normal high levels of satellite DNA methylation [192]. Furthermore, by the early preimplantation stage, murine embryoblasts have remethylated satellite DNA sequences following extensive demethylation in the earliest stages of embryogenesis [193,194]. In addition, our comparison of satellite DNA methylation in Wilms' tumors to that in nephrogenic rests, embryonic kidney precursors of Wilms' tumors, indicated neither satellite DNA hypomethylation nor the promoter hypermethylation in the nephrogenic rests [22]. Similarly, Fanelli *et al.* found that satellite 2 DNA from glioblastoma-derived brain tumor stem cells was hypomethylated to a similar extent when compared with either normal human astrocytes or normal neural progenitor cells [89]. These findings are consistent with cancer stem cell populations from tumors having undergone DNA demethylation during their generation. Therefore, altered DNA methylation in cancer does not simply reflect the methylation status of the original cancer progenitor cell(s).

What is known & not known about the mechanism of DNA demethylation

Many studies suggest that there is active DNA demethylation in mammalian cells [40,125, 195] rather than just passive demethylation owing to a failure of maintenance methylation **(**Figure 2**)**. Active demethylation probably first occurs on one strand at a given dyad [40, 123]. In murine and bovine zygotes even before DNA replication, there is active demethylation of paternally inherited DNA, which includes demethylation of DNA repeats [196–199]. However, the mechanism is still not clear.

The best evidence for active demethylation comes from studies of *Arabidopsis*, which demonstrate that a DNA glycosylase hydrolyzes the m^5C-deoxyribose bond and then the

enzyme's lyase activity nicks the backbone at the resulting apyrimidinic site. This is followed by completion of base excision repair with incorporation at the formerly methylated C site of an unmethylated C residue [200]. There has been evidence for various pathways for active demethylation in vertebrates as follows:

- An m^5C DNA glycosylase [195] for m^5C removal as the free base by base-excision repair [195];
- A demethylase that catalyzes the release of the 5-methyl group from m^5C [201];
- A growth arrest protein, GADD45A [125], that may function in nucleotide excision repair to release m^5C as a nucleotide [125,126];
- DNMT3A or DNMT3B for catalysis of the oxidative deamination of the C4 position of m^5C to give a T residue replaced with an unmethylated C residue by mismatch repair [126,128].

There is controversy about each of these pathways [126,202]. Moreover, the existence of active demethylation in mammalian cells has recently been questioned [203].

Our analyses of symmetrical and asymmetrical methylation tandem DNA repeats in normal tissues and cancers give evidence in support of active demethylation *in vivo* in normal cells, where it may counteract aberrant *de novo* methylation, and in tumor cells, where it would create at least some of the cancer-linked hypomethylation. We used bisulfite-based hairpin genomic sequencing, which distinguishes CpG dyads that are symmetrically methylated, symmetrically unmethylated or hemimethylated **(**Figure 2**)** on individual DNA fragments whose strands are ligated together during the analysis [104]. In diverse normal somatic tissues, we found most CpG dyads highly methylated and a few mostly unmethylated in a 0.2-kb subregion of the 1.4-kb NBL2 repeat unit [105]. Compared with these somatic tissues, ovarian carcinomas and Wilms' tumors displayed hypomethylated CpG dyads next to hypermethylated CpG dyads in this subregion, suggestive of dynamic changes in DNA methylation. Approximately 2–6% of the CpG dyads were hemimethylated (Figures 1 & 2) in control tissues and cancers. These percentages are high enough to suggest that they are fairly stable structures. The dense packing of heterochromatic tandem repeats may prolong the lifetime of hemimethylated intermediates in cancer-linked DNA demethylation, thereby providing longer-lived intermediates in demethylation.

Much of the cancer-associated hypomethylation seen in NBL2 (1.4-kb repeat unit) and in satellite 2 DNA (1.3-kb higher-order repeat unit) was highly focal [105,106]. This was especially apparent in the runs of hemimethylated dyads overrepresented in the ovarian carcinomas and Wilms' tumors. Maintenance methylation is quite processive [204–206]. Therefore, if cancer-linked hypomethylation were owing to the inhibition of maintenance methylation, almost all of the hypomethylation in the examined 0.2-kb subregions would have been expected to occur in long runs, contrary to our findings [105,106].

Most importantly, some of the hemimethylated dyads in normal tissues and tumors in NBL2 and satellite 2 [105,106] were present in runs of the opposite orientation (Figure 2). While a local failure of maintenance methylation on the nascent strand could generate a hemimethylated dyad from a symmetrically methylated dyad, a simple failure of maintenance methylation cannot explain two nearby hemimethylated dyads with their m^5C residues on opposite strands (Figure 2). We conclude that there was at least some active demethylation in the normal tissues and in the cancers at these repeats.

In addition, some of the hairpin sequencing DNA clones from the tandem repeats gave evidence for nonrandom clustering of identically unmethylated or hemimethylated sites [105,106]. This suggests localized spreading of DNA demethylation. DNA hypermethylation appears to be

able to spread in *cis* [120,207]. There has been less analysis of the possible spreading of demethylation, which might involve demethylation on one strand at a single CpG dyad [40], followed by multiple, symmetrical demethylation events. The hypothesized spreading of active demethylation might occur by a hopping or sliding mechanism over small distances along a DNA molecule in analogy to how uracil DNA glycosylase (UNG) appears to catalyze the formation of abasic sites over regions less than 100 bp [208]. The spreading of UNG-mediated removal of uracil from DNA can even involve bases on opposite strands [208], as might be occurring during cancer-linked demethylation (Figure 2). Consistent with the UNG study and with evidence for involvement of base-excision repair in DNA demethylation [128], the longest runs of consecutive hemimethylated dyads that we have observed were five CpG dyads extending over about 60 bp in the cancers. The initial cancer-associated demethylation of clustered CpGs might subsequently seed more extensive spreading of demethylation to give rise to the fraction of cancer DNA clones that were derived from completely demethylated DNA sequences.

In a statistical modeling study based upon our hairpin sequencing data from NBL2 and satellite 2 in ovarian carcinomas, we considered three stochastic models for the spreading of methylation patterns [209]. The CpG dyad sites might independently assume their methylation status (independent sites model; Box 1). Alternatively, they might be subject to a ‘snowball effect’ of initial *de novo* or demethylation leading to fairly homogeneous methylation changes with the positions of such changes being random within the region (context-dependent model). The third model (neighboring sites model) depends upon stochastic growth or shrinkage of regions of epigenetic change, with these changes highly dependent on neighboring sites on both sides of an individual dyad. Our results on these tandem repeats do not fit the first model and best fit the third one. Our data supporting the neighboring sites model is consistent with demonstrated preferences of mammalian DNMTs for specific sequences [210].

Niehrs recently summarized many other studies that offer evidence for active demethylation in animal and plant cells and persuasively argues for maintenance of DNA methylation patterns involving a dynamic equilibrium of active demethylation and *de novo* methylation in addition to maintenance methylation [126]. He proposes a two-step model for active, strand-specific demethylation of DNA that would involve two different repair pathways in each step to convert m^5C residues to T residues at an m^5CpG dyad and product inhibition after the first step. This would allow repair of the first strand before repair begins on the second strand, and thereby avoid the introduction of deleterious double-strand breaks. The model fits well with our data on strand asymmetry of hemimethylated sites at the NBL2 repeat in cancers [105]. I further propose that the apparently persistent hemimethylation that we observed at some CpG sites could be due to delays in completing the first rate-limiting repair-demethylation step, for example, due to inappropriate dephosphorylation of a 5′-phosphate end at the site of repair.

Whatever the exact mechanism by which DNA demethylation occurs, it is likely to have diverse proximate causes. There could be decreases in DNMT isoforms, their subcellular distribution or their recruitment to DNA, although the often simultaneous development of cancer-linked demethylation and *de novo* methylation has to be explained. Among the newly discovered triggers for changes in DNMT3A and DNMT3B levels is microRNA-29b [211]. With respect to modulating chromatin interactions of Dnmts/DNMTs, it has been proposed that the SNP2 chromatin remodeling factor Lsh/LSH interacts with Dnmt3b/DNMT3B to recruit it to certain DNA sequences [83]. Deletion of Lsh causes widespread genomic demethylation, especially in DNA repeats [212] and erythroleukemia [83], as mentioned above. DNMT3B, which has many isoforms [213–215], is especially associated with the methylation of various tandem DNA repeats, including NBL2, satellite 2 and D4Z4 [71,216]. Certain isoforms of DNMT3B might be altered during carcinogenesis such that hypomethylation of a subset of DNA sequences results. In addition, DNMTs have many specific protein-binding partners, which

could influence their activity during carcinogenesis. For example, there is evidence that DNMT1 binds to PARP-1 after PARP-1 automodifies itself by poly(ADP-ribosyl)ation, and that this results in inhibition of DNA methylation [217]. In addition, viral and bacterial infections can alter DNA epigenetics, including inducing global DNA hypomethylation by various pathways [218].

How can cancer-linked hypomethylation affect the detection & treatment of cancer?

Diagnosis & prognosis

The association of global or focal DNA hypomethylation with the early stages of carcinogenesis or with tumor progression [1,7,8,20,[35],[52],[55],[61],[62],[67],[68],[105],149,151,219–221] provides cancer markers that should be very useful in the clinic. In addition, knowledge of which metastasis genes are activated by hypomethylation will be necessary for development of personalized treatment programs that are molecularly based. Translation of research findings to the clinical setting will be facilitated by the recent developments in high-throughput and high-resolution methods for DNA methylation analysis [28,37,98].

Cancer demethylation treatment

DNA methylation inhibitors, including, 5-aza-2′-deoxycytidine (decitabine), 5-azacytidine, 5,6-dihydro-5-azacytidine, zebularine and RNA interference (RNAi) and antisense inhibitors of DNMT1 are being used as cancer chemotherapeutic agents or in clinical trials [222–224]. For solid tumors, usually little or no clinical efficacy, and often no disease stabilization, is seen, and many toxic effects are observed [225–227]. 5-azacytidine and decitabine have had the most success as cancer chemotherapeutic agents for patients with myelodysplastic syndromes (MDS), acute myeloid leukemia and chronic myelogenous leukemia [228–232]. Clinical efficacy for this type of therapy applied to MDS patients has been increased by using optimized treatment schedules that take into account the mechanism of action of demethylating agents [230]. Combination therapies involving DNA demethylating agents have been tried for various cancers and are being refined with differing success rates [231,233–235].

In addition, it has been proposed that the specificity of cancer-testis genes for some cancers could be the basis of a cancer vaccine [236,237]. Treatment of cancer cell lines with decitabine demonstrated that cancer-testis genes are especially sensitive to activation by this agent [238], which suggested that combination therapy with this drug followed by treatment with a vaccine for cancer-testis genes might be efficacious [239]. However, heterogeneous expression of the antigenic products of these genes by cancer cells in some tumors might limit the usefulness of this approach [240].

Mechanism of action of cancer demethylating chemotherapeutic agents

A total of five out of seven patients with MDS who were treated with decitabine displayed decreases of up to 70% in global methylation of bone marrow cells [241]. Major decreases in DNA methylation owing to therapy with these DNA demethylating drugs can be largely reversed after treatment [232,241,242]. Several studies have demonstrated that *p15* promoter hypermethylation was reversed by decitabine alone or in combination with the histone deacetylase inhibitor sodium phenybutyrate [234,243,244]. However, the effectiveness of DNA methylation inhibitors as cancer chemotherapeutic agents is partly owing to cytotoxic effects unrelated to their DNA-demethylating activity [245–248]. Decitabine and 5-azacytidine are DNA replication inhibitors as well as inhibitors of DNMT activity upon incorporation into DNA [246,249].

Caution is warranted

There is insufficient long-term survival data on cancer patients treated with DNA methylation inhibitors to evaluate the possible long-term risks associated with these treatments. In considering modalities for therapy for various types of patients, the pro-oncogenic potential of DNA hypomethylation should not be overlooked [250]. This includes associations of DNA hypomethylation with tumor progression, demethylation of promoters of genes that may contribute toward oncogenesis and increased chromosomal rearrangements, as well as experiments implicating DNA hypomethylation as a causal agent in carcinogenesis or tumor progression. Some other cancer therapies also have proven oncogenic potential. Nonetheless, the close association of DNA hypomethylation with oncogenesis and the finding that DNA hypomethylation often becomes more pronounced upon tumor progression, should be factored into clinical decisions, especially if other effective therapies are available for the type of cancer in question.

Conclusion

There is a hierarchy of patterns of DNA epigenetic changes in cancer, starting from the local level of individual CpG dyads to subregions of several hundred base pairs, several thousand base pairs and large regions of up to 1 million base pairs. These changes are the result of aberrant *de novo* methylation and demethylation, which probably includes active demethylation through a hemimethylated intermediate. Both demethylation and *de novo* methylation occur early in tumorigenesis, but often increase with tumor progression. Given the evidence causally linking DNA hypomethylation to cancer and the near ubiquity of cancer-associated DNA hypomethylation, it is likely that this loss of methylation plays important roles in tumor formation and progression which underscore the need for caution in design of DNA demethylation strategies to counteract hypermethylation-dependent silencing of tumor suppressor and homeobox genes.

Future perspective

Over the next 5–10 years, there will probably be many clinical tests in use that are based upon DNA hypomethylation markers for cancer detection and prognosis. In addition, in the era of personalized cancer medicine that is soon to be commonplace, DNA hypomethylation markers and oncogene targets will be examined for their methylation status to characterize cancers and design the best treatment for a given tumor and individual. Eventually, treatments will be developed for reversing cancer-causing gene-promoter hypermethylation that will be targeted to specific gene regions. This will replace the current blunt-force approaches to genome-wide demethylation that may be causing tumor progression as a late side effect. Lastly, the mechanisms for cancer-associated *de novo* methylation and demethylation will be elucidated with convincing detail, and lead to new kinds of epigenetic-based medicine.

Executive summary

Overview

- DNA hypomethylation is a ubiquitous feature of carcinogenesis.
- Most cancer-linked hypomethylation is in repeated DNA sequences, but gene regions show some too.
- DNA hypomethylation can be found early in carcinogenesis, but also is often associated with tumor progression.
- There are confounding factors to consider in assessing cancer-associated changes in DNA methylation.

- DNA hypomethylation patterns can be tumor-specific just as DNA hypermethylation patterns are.

Interrelationships of cancer-linked hypo- & hyper-methylation

- Cancer-linked hypo- and hyper-methylation are generally independent although most studied cancers display both changes in their genome.
- Some DNA sequences display cancer-linked hyper- and hypo-methylation.
- Cancer-linked DNA methylation changes can be dynamic.

DNA hypomethylation: mechanisms

- The cancer stem cell hypothesis is compatible with demethylation during carcinogenesis.
- Hypomethylation of DNA repeats can promote carcinogenesis by increasing DNA recombination and by direct and indirect effects on gene expression.
- There is evidence for active demethylation in normal tissues and cancers, although the mechanism is still unclear.
- The patterns of methylation in CpG dyads in repeated sequences favor a ‘neighboring sites’ model for DNA methylation changes.

Clinical applications of research on DNA hypomethylation in cancer

- Diagnosis and prognosis: Repeated DNA sequences that are frequently hypomethylated in cancer can provide markers for cancer diagnosis and prognosis, and are even more sensitive markers than unique sequences that are subject to cancer-linked DNA hypermethylation.
- Treatment: DNA demethylation therapy alone or in combination with other therapies is used for certain leukemias and myelodysplastic syndromes.
- Cautions: Treatment that involves demethylating DNA may inadvertently foster metastasis; methods should be developed for targeting the demethylation to promoters of tumor suppressor genes.

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•• of considerable interest

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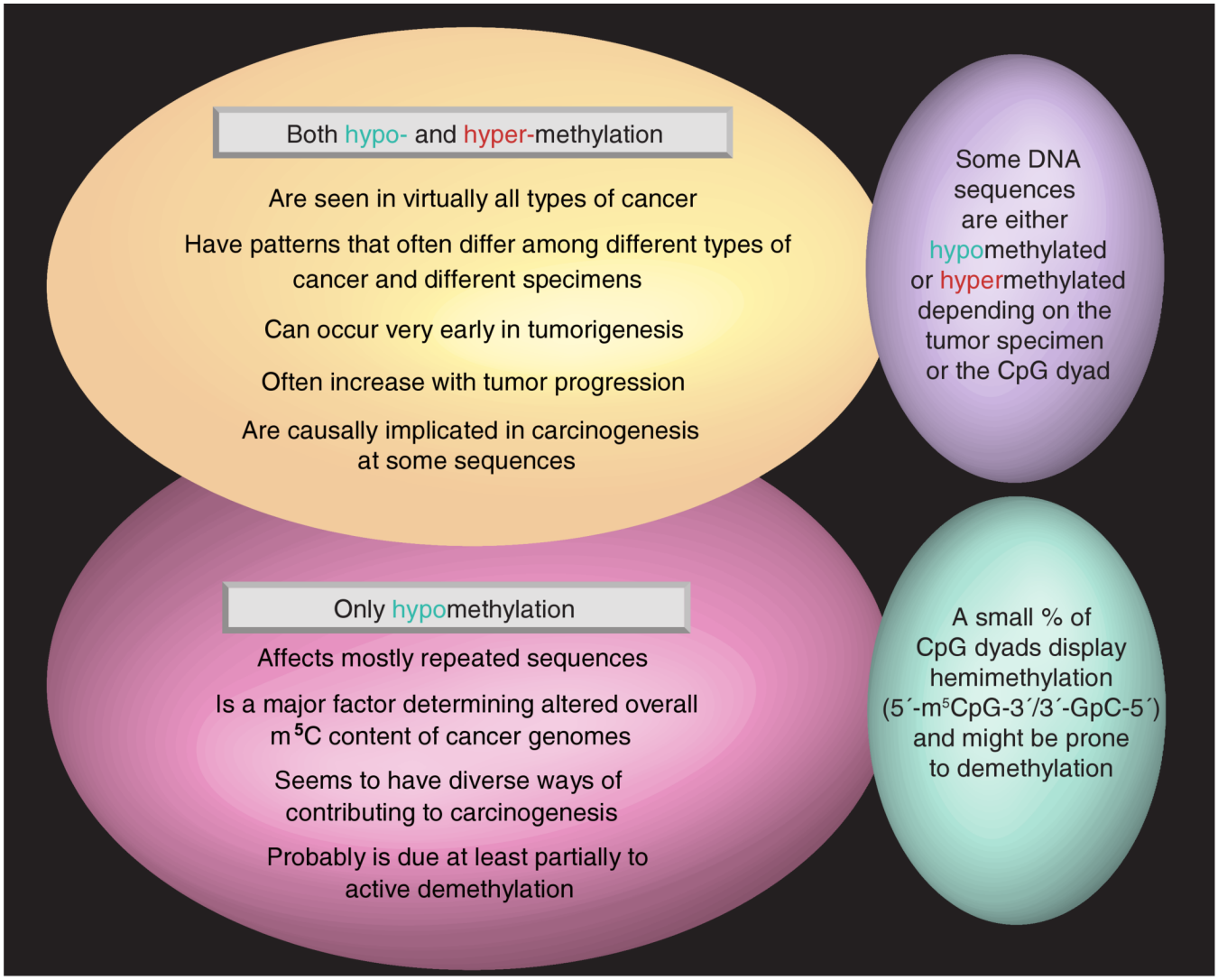

Figure 1. Similarilities and differences in cancer-associated hypo- and hyper-methylation of DNA

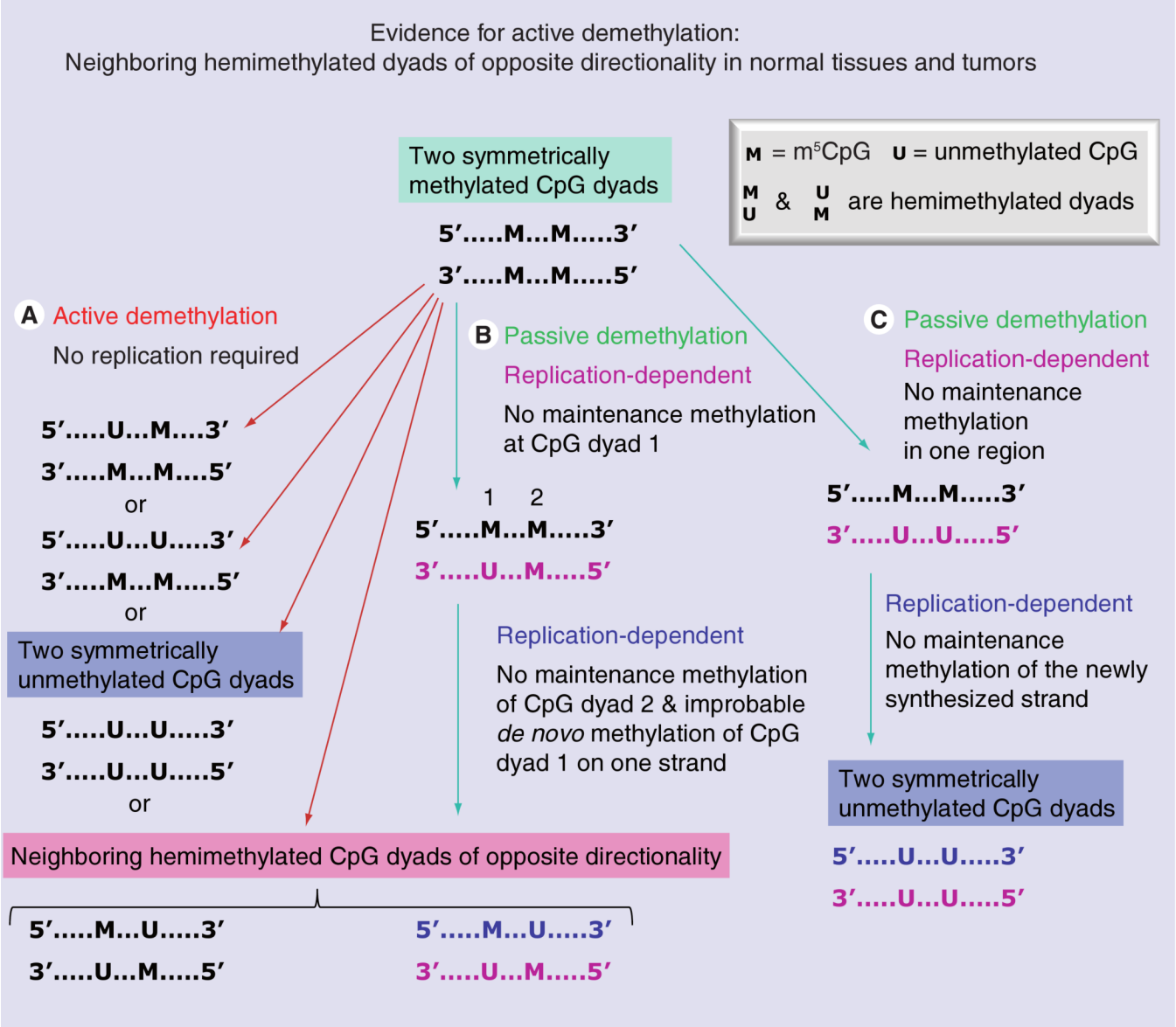

Figure 2. A comparison of active and passive pathways for DNA demethylation and, in particular, for generating neighboring hemimethylated CpG dyads with opposite polarity

This figure demonstrates that it is unlikely that inhibition of passive demethylation is responsible for producing such DNA methylation patterns, which were observed in both cancers and normal tissues. Although neighboring, oppositely-oriented hemimethylated dyads (pink highlighting) were seen only infrequently, they were found in three different hairpin sequencing studies of DNA methylation and in normal tissues as well as cancer samples [104–106]. We conclude that active demethylation is responsible for at least some of the demethylation *in vivo*.