Therapeutic implications of DNA methylation

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Cancer growth and metastasis requires reprogramming of the expression of multiple genes. The epigenome, which is comprised of chromatin and the patterns of DNA methylation, sets up and maintains gene expression programs. As expected from the broad changes in gene expression in cancer, which are characterized by both silencing and activation of multiple genes, the epigenome of cancer cells is distinguished by aberration of DNA methylation patterns, which include both hypo- and hypermethylation and aberrant regulation of DNA methylation enzymes. In contrast to genetic alterations, which are fixed and are not amenable to therapeutic intervention, pharmacological agents could alter DNA methylation patterns. This raises the prospect that DNA methylation-targeted drugs will reverse cancer growth and metastasis. One of the main challenges however, is to understand the relative role of hypo- and hypermethylation in order to achieve a balance of epigenetic therapeutic agents with positive outcome and reduced adverse effects.

The epigenome directs the genome to execute multiple gene expression programs required for normal life. This exquisite programming is disrupted in cancer by either genetic mutations or epigenetic deregulation. The epigenome, as we understand it, includes chromatin which is comprised of protein structures that package the DNA in either accessible or inaccessible modes [1,2], and a pattern of chemical modifications of the DNA molecule which is part of the chemical structure of the genome, DNA methylation [3]. The building block of chromatin is the nucleosome, a structure composed of an octamer of histone proteins, H3–H4 tetramer flanked on either side with a H2A–H2B dimer [4]. The N-terminal tails of these histones are extensively modified by methylation [5,6], phosphorylation and acetylation [7,8]; and different histone variants also play a regulatory role. These modifications regulate accessibility of genes to the transcription machinery. Histone modifications are dynamic and are catalyzed by an equilibrium of enzymes such as histone acetyltransferases (HAT) which are targeted to specific genes by site-specific *trans*-activators and histone deacetylases (HDACs) that are recruited by transacting repressor complexes [9]. Histone modifications can act by attracting other proteins; for example, methylation at the K9 residue of H3-histone tails attracts the heterochromatin protein (HP)-1, which binds methylated histones and precipitates an inactive chromatin structure [10]. In addition to histone modifications, the positioning of nucleosomes on regulatory regions of genes is modulated by energy-dependent chromatin remodeling complexes [11].

There is vast literature associating different methods of chromatin modification with critical cellular processes, which is beyond the scope of this review. Different histone modifications are proposed to create a histone code, which dynamically programs and maintains gene expression profiles and is responsive to different developmental, physiological, environmental and pathological cues [1]. Therefore, it is clear why the enzymes regulating different histone modifications such as HDACs and histone methyltransferases, which involve global alteration of gene expression, are emerging as new therapeutic targets in cancer and other diseases.

A second level of epigenomic information is the DNA methylation pattern. Cytosine residues residing in the dinucleotide CGs are modified by a methyl moiety at the fifth position on the carbon ring [12]. Since not all CGs are methylated and the methylation pattern varies from cell type to cell type in a manner which correlates with the state of gene expression, it is clear that the pattern of distribution of methyl groups in different cell types carries information that either reflects or determines the unique cell-specific properties of the genome [3]. DNA methylation is an extremely stable modification and is part of the chemical structure of the genome, in contrast to chromatin, which is composed of proteins that are associated with DNA. The state of chromatin activity and DNA methylation patterns are tightly linked. It has been known for some time that open regions of chromatin are associated with hypomethylated DNA and close inactive regions of chromatin are associated with

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hypermethylated DNA [13]. DNA methylation marks silenced genes and bears the long-term memory of epigenomic programming [14,15]. Methylation of DNA adds long-term stability to states of expression, which are first defined by changes in chromatin structure (Figure 1).

Although it is clear that DNA methylation is extremely stable, it is also potentially dynamic and

plastic as will be discussed below. This tension between stability and plasticity is at the heart of the pathological changes impacting on the DNA methylation pattern during cancer progression, and on the potential therapeutic opportunities in modulation of the DNA methylation machinery.

Cancer progression requires silencing of genes, which suppress tumorigenesis and

Figure 1. A scheme of epigenomic states of a gene.

A gene can be found in different epigenomic states resulting from differences in histone modification and DNA methylation. For the sake of simplicity only histone tail lysine 9 (K9) acetylation and methylation are shown. An active gene (on) is packaged around a nucleosome with K9 acetylated histone tails (Ac). Histone deacetylation is catalyzed by histone deacetylases (HDACs), which are recruited by repressor complexes to specific genes. Histone acetylation is catalyzed by histone acetyl transferases (HATs), which are recruited to specific sequences by transacting activators. Binding of a repressor(s) to a gene results in loss of acetylation, increased methylation of K9 and silencing of gene expression (off). This is a relatively unstable silencing and is easily reversible, as indicated by the arrows. Histone deacetylases recruit DNMTs to the gene, which cause its methylation (CH₃). Methylation attracts the methyl binding protein MeCP2, which recruits additional corepressors and HDACs. This state of gene silencing is highly stable. Any aberrant acetylation will be countered by the HDAC recruited by MeCP2 to the methylated sequence. This state of methylation could be reversed only if acetylation is induced either pharmacologically, by TSA, or by a strong transcription factor recruiting HATs to the gene and over-riding inhibition of binding by methylation. Histone acetylation would increase the accessibility of the gene to demethylases. This event is rare but still possible. Thus, DNA methylation is a stable mark of gene silencing. The positions at which different drugs modulate the epigenome are indicated. MG 98 is a DNMT1 antisense, which is currently in clinical trials [98] and MBD2-AS is an antisense oligonucleotide targeting MBD2 [84]. DNMT: DNA methyltransferase.

induction of genes that promote growth and metastasis. Genetic alterations such as inactivating mutations or activating mutations and rearrangements, could accomplish this switch in gene expression. However, it is also possible to alter the gene expression program using epigenomic changes. Since general enzymatic machineries control genome-wide epigenomic patterns of gene expression, a change in the DNA methylation machinery could result in variations in epigenomic programming and state of expression of numerous genes. Thus, epigenetic modulation seems to be an effective way for a cancer cell to control the state of expression of several genes concurrently. It is perhaps therefore not surprising that both hypermethylation of certain genes, which suppress tumorigenesis [16–18] and global hypomethylation [19,20], take place in cancers (Figure 2). Since the epigenome, in contrast to the genome, is reversible, it might be possible to overturn some of these epigenomic changes by targeting the DNA methylation machinery. Thus, the epigenome is a promising target for anticancer drugs. The critical question is to

learn how to target the required epigenetic changes to affect tumor inhibition and to steer clear of changes, which could result in promoting tumorigenesis and metastasis. In view of the fact that hypermethylation of tumor suppressor genes has been the focus recently, most attention in the field has been directed at DNA methylation inhibitors as potential activators of tumor suppressor genes. DNA methylation inhibitors are now being tested as potential anticancer agents [21,22]. However, if hypomethylation is also important for tumorigenesis, and might promote the activity of genes involved in metastasis as will be discussed below, then DNA methylation inhibition treatment might also result in the adverse effect of activation of pro-metastatic genes [22]. Thus, although interfering with the DNA methylation machinery is a potentially powerful approach to cancer therapy, it is imperative to gain a full understanding of the mechanisms involved in these two processes; hypermethylation and demethylation, to properly utilize agents targeting the DNA methylation machinery in anticancer therapy.

Figure 2. Hypermethylation and hypomethylation of critical genes in cancer.

Two classes of methylation changes are encountered in cancers. Many tumor suppressor genes are hypermethylated (CH₃) and silenced. The silencing of tumor suppressors leads to uncontrolled growth. In metastatic cancer however, prometastatic genes, which are normally methylated and are methylated in non-metastatic breast cancer, are demethylated and activated by demethylase. Inhibition of DNA methylation by DNA methylation inhibitors might lead to activation of these genes [71,80]. DNMT: DNA methyltransferase.

DNA methylation patterns: mechanisms of formation & maintenance

DNA methyltransferases

The transfer of the methyl group from the methyl donor *S*-adenosylmethionine (SAM) onto DNA is catalyzed by DNA methyltransferases (DNMTs) concurrently with DNA replication [23]. A number of DNMT genes and their splice variants are known in vertebrates. *DNMT1* was originally proposed to be responsible for maintaining the DNA methylation pattern during cell division including cancer cells [24], while *DNMT3A* and *DNMT3B* were suggested to be responsible for *de novo* methylation of specific sequences during early development [25–27] as well as for maintenance of the DNA methylation of specific repetitive sequences [28,29].

De novo & maintenance DNA methylation

It was known for some time that the DNA methylation patterns undergo dramatic changes during development and differentiation [30–32], and it was believed that the pattern of methylation is preserved through adulthood [12]. This was the common consensus, since it was thought that DNA methylation is irreversible and could only be removed by replication in the absence of DNA methyltransferase. Postmitotic nonreplicating cells would naturally preserve

their methylation pattern. In somatic dividing cells the maintenance enzyme DNMT1 was proposed to accurately replicate the DNA methylation pattern since it could only transfer a methyl group to a hemi-methylated site, which is formed when a methylated CG sequence is replicated [33]. However, based on recent data, the idea that DNA methylation is determined exclusively by the methylation pattern of the template must be revisited. In addition, the clear differentiation between *de novo* and maintenance DNMT is not fully supported by recent data. Recent experiments suggest that the putative *de novo* enzyme DNMT3b and the proposed ‘maintenance’ DNMT1 interact to maintain the DNA methylation pattern [29], they are found together in multiprotein complexes [34] and that knockdown of both activities is required to cause global loss of DNMT [35–38]. Thus, the author proposes that maintenance of DNA methylation pattern is not a passive process of photocopying of a methylation pattern of a parental strand, but is rather an

active process of repeated dynamic decisions to methylate specific CGs. The targeting of specific DNMTs to a gene is suggested to play a critical role in determining its state of methylation. Different splice variants of all DNMTs have been identified [39,40], and some variants are more abundant in certain tumors. The repertoire of different DNMTs and their splice variants associated with a gene possibly play an important role in shaping as well as maintaining its state of methylation. DNMTs act in the context of other DNMTs and other epigenomic components. Therefore, it is proposed here that the decision to either maintain or alter a DNA methylation site is not predetermined by the original pattern of methylation. Thus, there is no inherent reason for a site to remain methylated. A methylation pattern is maintained as long as the context of DNA methylation machinery around a gene remains the same. The context of DNA methylation enzymes around a gene is defined by other *trans*-acting factors interacting with the gene and the chromatin structure. This model suggests that DNA methylation is dynamic and is not an automatic mirror image of its history. This dynamic nature of DNA methylation is consistent with the *de novo* methylation events observed in cancer.

Figure 3. Inhibition of metastatic genes by demethylation inhibitors.

DNA demethylation agents cause activation of metastatic genes. The methylation status of metastatic genes such as *uPA*, is in steady state equilibrium, which is a balance of methylation catalyzed by DNMTs and demethylation catalyzed by demethylases. In metastatic cancer cells that express high demethylase activity, the balance is tilted towards demethylation. Inhibition of demethylase activity with inhibitors such as the methyl donor *S*-adenosylmethionine (SAM) or an antisense to MBD2 blocks demethylation and tilts the equilibrium towards the left, resulting in methylation, silencing of the genes and inhibition of metastasis [46]. In nonmetastatic cancer cells, genes required for metastasis are methylated and silenced resulting in inhibition of metastasis. Treatment of such tumors with the DNA methylation inhibitor 5-aza-CdR results in hypomethylation, activation of metastatic genes and induction of metastasis. DNMT: DNA methyltransferase. *uPA*; urokinase plasminogen activator.

Demethylation & demethylases

If DNA methylation is dynamically maintained throughout life it should also be reversible, otherwise it will gradually drift toward increased methylation. Due to the chemical stability of DNA methylation, it has been a long-standing accepted belief that the DNA methylation pattern is a fixed characteristic of genomes in mature cells and therefore should not play a role in the dynamic changes in gene expression programming throughout adulthood. However, the author and colleagues have proposed that DNA methylation could be enzymatically reversed by demethylase activity [41] and that the DNA methylation pattern is an equilibrium of methylation and demethylation (Figure 3) [22]. Therefore, a given pattern of methylation is a steady state, which potentially could be altered by different environmental, physiological and pathological signals such as those occurring in cancer. Bhattacharya and colleagues have proposed that the methylated DNA binding protein MBD2 is a demethylase [42]. This discovery has been a matter of significant controversy since Adrian Bird's group has shown that MBD2 associates with the chromatin remodeling

complex NurD and plays a role in the silencing of methylated genes, and they were unable to show demethylase activity for this protein [43]. Since then, evidence has been provided that MBD2 participates in active demethylation in human cells [44] by showing that antisense knockdown of this protein inhibits active demethylation [45] and results in hypermethylation of genes which are otherwise hypomethylated in cancer cells [46]. The fact that a protein, which is proposed to be a demethylase and therefore is predicted to be an activator of gene expression, also participates in gene suppression by association with a repressor complex such as NurD, seems to be paradoxical [43]. It is possible, however, to resolve this contradiction by arguing that MBD2 plays divergent roles when it is found in different promoter contexts by associating with different multiprotein machineries. In accordance with this hypothesis, Detich and colleagues showed that MBD2 demethylates in a promoter selective manner [44]. It is proposed, therefore, that MBD2 could associate with either a chromatin remodeling complex to silence genes, or serve as a demethylase associating with a different machinery.

Even if it is agreed that MBD2 bears demethylase activities, it is clear that other demethylases must exist since an MBD2^{-/-} mouse still exhibits normal active demethylation of the paternal genome after fertilization [47].

Direction of the DNA methylation equilibrium is determined by the chromatin structure of a gene

An important question is what determines the direction of the DNA methylation equilibrium towards either methylation or demethylation? It is well established that there is a tight correlation between the state of methylation of regulatory regions of genes and their state of activity [3,12,48]. Methylated regulatory regions of genes are associated with inactive deacetylated chromatin while unmethylated DNA is associated with hyperacetylated open chromatin, which is transcriptionally active [13,48]. The relationship between DNA methylation and chromatin structure was originally believed to be unidirectional; DNA methylation determines chromatin structure. A molecular explanation for this relationship has been provided by the discovery of methylated DNA-binding proteins such as MeCP2 that bind methylated DNA and recruit histone deacetylases and histone methyltransferases to regulatory regions of genes, resulting in chromatin inactivation [49,50]. However, recent data suggests

that there is a bilateral relationship between DNA methylation and chromatin structure. Inactivation of chromatin precedes and leads to DNA methylation of certain genes [51], and histone acetylation and chromatin activation bring about demethylation [52,53]. Szyf and colleagues therefore proposed that the direction of the DNA methylation equilibrium was determined by the chromatin structure of a gene. They have recently provided evidence that the DNA methylation is in a dynamic and reversible state in post mitotic tissues such as the brain long after birth, and that it could be reversed by active demethylation in response to pharmacological modulation of histone acetylation. The author and colleagues have also shown that high frequency of maternal licking and grooming could bring about demethylation of the glucocorticoid receptor in the hippocampus of the offspring early after birth at a point in time when very little DNA replication takes place. The difference in DNA methylation between high and low frequency licked offspring was maintained to adulthood. Strikingly, however, the methylation pattern could be reversed in adult rats by treatment with the histone deacetylase inhibitor trichostatin A (TSA), showing that active demethylation is induced by active chromatin *in vivo* [15] as it is in cell culture [52,53]. The fact that HDAC inhibitors could trigger active demethylation is of special importance in cancer therapy since HDAC inhibitors are in clinical trials as potential anticancer targets [54,55]. It is generally assumed that HDAC inhibitors only affect the state of acetylation and not DNA methylation [56]. Hypomethylation of tumor and metastasis promoting genes might be an undesirable consequence of HDAC therapy.

Although it is argued here that DNA methylation is dynamic and potentially reversible under certain conditions, it is evident that at the same time, DNA methylation is an extremely stable chemical modification of the cytosine ring, and therefore serves to stabilize silencing of genes. Therefore, it was proposed that only a persistent signal of chromatin activation would result in demethylation, whereas transient activation of chromatin would not be reflected in a change in DNA methylation [57]. Thus, DNA methylation guards the epigenome from noise but at the same time is amenable to change if the proper signals are triggered. In addition, because of the stability of the DNA methylation signal it stands to reason that it plays an important role in the stable silencing of heterochromatic regions and is recruited to these

regions by the histone methyltransferase Suv39h and the heterochromatin resident protein HP-1 [58], which are highly enriched in histone H3 lysine 9 methylation and HP-1 proteins [5].

Regional hypermethylation in cancer & its therapeutic implications

Hypermethylation of tumor suppressor genes in cancer

A large list of genes, including classical tumor suppressor genes which were defined by germline mutations as well as other genes that were shown to suppress tumor cell growth in culture, were found to be epigenetically silenced in cancer [59–61]. Most of the epigenetically silenced genes in tumors are also hypermethylated in CG sequences located in the 5' regulatory regions. The profile of methylated genes in cancers might constitute a molecular signature, which could be used to classify and stage tumors [62–64].

DNA methylation & DNMT inhibitors as anticancer agents

Since DNA methylation can be reversed in dividing cells by inhibiting DNMTs during cell division, it was postulated that such inhibitors would cause demethylation and activation of critical tumor suppressor genes and thus block cancer cell growth. DNMTs were therefore proposed as candidate anticancer targets [21]. Nucleoside analogs of cytidine such as 5-aza-deoxy-cytidine (5-aza-CdR) [65] and zebularine [66], which are incorporated into DNA, trap DNMTs during movement of the replication fork and cause passive demethylation of nascent DNA, also bring about demethylation and activation of tumor suppressor genes [67]. Since nucleoside analogs exhibit cellular toxicities, attempts are being made to identify non-nucleoside DNMT inhibitors. Nevertheless, it should be mentioned that some nucleoside analogs such as zebularine might be less toxic than others such as 5-aza-CdR [66]. There have been a number of clinical trials with either 5-aza-CdR alone or in combination with histone deacetylase inhibitors. The basic assumption of the combination therapy is that HDAC inhibitors and 5-aza-CdR are acting independently on two different epigenetic modifications; histone acetylation and DNA methylation [56]. However, as discussed above our data suggests that the histone deacetylase inhibitor TSA could bring about demethylation of DNA as well as histone acetylation, and that these two processes are tightly related [15,52,53]. It is still unclear whether 5-aza-CdR will become an effective agent in anticancer therapy.

Somewhat encouraging effects were shown in treatment of leukemias but not solid tumors. The efficacy of the combination therapy with HDAC inhibitors is not yet known [68].

To bypass the problem of nucleoside-analog-associated toxicity as well as toxicity associated with the trapping of a large complex of proteins onto DNA as is the case with 5-aza-CdR [69], Ramchandani and colleagues have developed sequence-specific DNMT1 antisense oligonucleotides [70], which were shown to inhibit tumorigenesis in murine models, and a DNMT1 antisense MG98 is now in clinical trials [68].

DNA methylation inhibitors activate some prometastatic genes

The current strategy of looking for DNMT inhibitors which cause DNA methylation inhibition focuses on the methylated tumor suppressor genes. However, one needs to consider the possibility that hypomethylation induced by either nucleoside analogs of cytidine or HDAC inhibitors will lead to activation of prometastatic genes. For example, it has been shown that 5-aza-CdR, induced in a nonmetastatic human breast cancer cell line, the expression of the urokinase plasminogen activator (*uPA*) gene encoding a protease highly implicated in breast cancer metastasis [46,71], and similarly 5-aza-CdR was shown to induce the protease heparanase implicated in metastasis [72].

Methylation-independent transformation activities of DNMT1

Data from the author's laboratory suggests that knockdown of *DNMT1* by antisense inhibitors results in induction of tumor suppressors by a methylation-independent pathway [73], inhibition of DNA replication [74] and launching of an intra-S epigenomic damage checkpoint [75]. This treatment results in very limited DNA demethylation. The fact that antisense knock down of *DNMT1* causes very limited demethylation could be explained by recent data suggesting that knock down of both *DNMT3b* and *DNMT1* is required to bring about significant demethylation [36], as well as by the arrest of replication by *DNMT1* knock down [74] which prohibits passive demethylation. A whole-genome microarray comparison of the changes in the transcriptome caused by either inhibition of DNMT by a traditional nucleoside analog 5-aza-CdR or DNMT1 antisense, revealed that while 5-aza-CdR caused activation of methylated genes such as melanoma-associated antigen gene (MAGE)

family members, DNMT1 antisense activated predominantly ubiquitously expressed genes presumed to be unmethylated [76]. These data have two implications. First, that it is possible to bring about arrest of tumor growth by blocking the DNA methylation-independent activities of DNMT1. Second, that we need to target the DNA methylation-independent activities of DNMT1 rather than its catalytic activities. This might be a possible route for taking advantage of DNMT as an anticancer target, while avoiding the adverse effects of demethylating and activating prometastatic genes [57]. Paradoxically, we might have to look for inhibitors of DNMT1 which do not inhibit demethylation, and focus on the protein–protein interactions of DNMT1 leading to its methylation-independent effects [77].

Global hypomethylation in cancer & its therapeutic implications

Causal role of hypomethylation in cancer

An important question which has received very limited attention in recent years is whether pharmacological inhibition of the demethylation observed in cancer would be of therapeutic relevance. Global hypomethylation was implicated in chromosomal instability in cancer. This was supported by genetic evidence from hypomorphic mice with low *DNMT1* expression [78], and the human ICF syndrome where a defect in DNMT3B resulted in hypomethylation and instability of the centromere-adjacent heterochromatic region (qh) of chromosomes 1, 16 and sometimes 9 [79]. Perhaps the most interesting and clinically relevant impact of hypomethylation is the well-documented regulation of prometastatic genes such as *uPA* [46,71,80], *s100A4* [81,82] and heparanase [72] by methylation, and their activation by hypomethylation.

Based on this data, the author and colleagues suggested that inhibition of hypomethylation should be an attractive therapeutic approach for inhibiting metastasis [83]. If demethylation plays a role in regulating multiple metastatic genes, then inhibition of demethylation should lead to concurrent suppression of metastatic genes. Although there is much mystery surrounding the enzymatic machinery involved in DNA demethylation, the author and colleagues have recently illustrated that inhibition of either MBD2, the candidate demethylase with sequence specific antisense oligonucleotides [84], or treatment with SAM, the methyl donor of the DNA methylation reaction, could lead to hypermethylation, inhibition of metastatic

protein protease and inhibition of cell invasion *in vitro* and *in vivo* [46]. The author and colleagues have previously shown that SAM inhibits demethylase activity in HEK 293 cells and MBD2 demethylase activity *in vitro* [85]. SAM is especially interesting since its levels could be modulated by the methyl-enriching components of diets. There are animal [86–88] and some epidemiological studies correlating methyl-poor diets [89–93], and polymorphisms in methionine-synthesizing enzyme methylene tetrahydrofolate reductase (MTHFR) with some cancers [94,95]. In addition, SAM was shown to be chemoprotective against rat hepatocarcinogenesis [96] and this protection was demonstrated to involve DNA methylation since it was completely abolished by coadministration of 5-aza-CdR [97]. SAM is extremely unstable however, and can not be used clinically without modifications. However, stable analogs are available, and the author's data suggests that stable analogs of SAM could be tested as antimetastatic agents. In addition, if increased SAM levels can prevent metastasis, then there might be some therapeutic and prophylactic potential in methyl-enriching diets.

Since it is proposed here that *DNMT1* and hypomethylation play distinct roles in tumor progression, whereas *DNMT1* deregulated expression is critical for uncontrolled growth, and hypomethylation is required for invasion and metastasis, DNMT1 and DNA hypomethylation inhibitors might be used at different stages of the disease. DNMT1 inhibitors might be used to block tumor growth early in therapy and then replaced with DNA hypomethylation inhibitors in later stages when metastasis is a serious concern.

Conclusion

In summary, both global hypomethylation and regional hypermethylation confer a selective advantage upon cancer cells by targeting different sets of genes with opposing roles in cellular transformation. Regional hypermethylation targets the silencing of genes that suppress tumorigenesis, while global hypomethylation probably targets activation of genes, which are required for different stages of the transformation process. Our challenge is to learn how to selectively target these two opposite roles of DNA methylation in tumorigenesis. Although hypermethylation has been the focus of recent attention, inhibition of hypomethylation and its potential in inhibition of metastasis should be considered.

Executive summary**The epigenome**

- The epigenome directs the genome to execute gene expression programs.
- The epigenome includes chromatin and DNA methylation.
- Cytosines residing in the dinucleotide CGs are modified by a methyl moiety at the fifth position on the carbon ring.
- The pattern of DNA methylation in different cell types reflects their unique cell specific properties.
- DNA methylation is an extremely stable modification and is part of the chemical structure of the genome, in contrast to chromatin, which is composed of proteins, which are associated with DNA.
- DNA methylation patterns and chromatin structure are tightly linked.
- DNA methylation marks silenced genes and bears the long-term memory of epigenomic programming.

DNA methylation patterns: mechanisms of formation & maintenance

- Transfer of the methyl group from methyl donor S-adenosylmethionine (SAM) onto DNA is catalyzed by DNA methyltransferases (DNMTs).
- A combination of DNMTs are involved in maintenance and generation of DNA methylation patterns.
- The DNA methylation pattern is a steady state balance of DNA methylation and demethylation reactions.
- There is a bilateral relationship between DNA methylation and chromatin structure.
- Methylated DNA binding proteins (MBD) bind methylated DNA and recruit histone deacetylases to methylated genes precipitating an inactive chromatin configuration.
- Inactive chromatin structure also leads to DNA methylation; histone deacetylases and methyl transferases recruit DNMTs to silenced chromatin resulting in DNA methylation.
- Active chromatin attracts demethylases leading to demethylation.
- MBD2 was proposed to be a DNA demethylase but the demethylase activity of MBD2 is still controversial.
- HDAC inhibitors can cause demethylation indirectly by activating chromatin and increasing accessibility to demethylases.

Regional hypermethylation in cancer & its therapeutic implications

- A number of tumor suppressor genes are silenced by DNA methylation in cancer.
- Inhibitors of DNMT bring about passive demethylation and activation of tumor suppressor genes and possibly inhibition of tumorigenesis.
- Nucleoside analog inhibitors of DNMT such as 5-aza-CdR and zebularine and DNMT antisense oligonucleotides are currently being tested in clinical trials as anticancer agents.
- Inhibition of DNA methylation can bring about the adverse effect of activation of prometastatic genes.
- DNMTs play a role in tumorigenesis, which is DNA methylation independent.
- Targeting the DNA methylation-independent functions of DNMT might be an alternative approach to DNA methylation therapy.

Global hypomethylation in cancer & its therapeutic implications

- Hypomethylation might also play a causal role in tumorigenesis.
- Demethylase inhibitors are proposed as candidate anti-metastatic agents.
- The methyl donor SAM inhibits demethylation of the pro-metastatic gene *uPA* and blocks metastasis.
- SAM levels might be modulated by diet.
- MBD2 knockdown causes hypermethylation of *uPA* and inhibition of metastasis.
- DNA methylation therapy might involve agents which block methylation-independent functions of DNMT1 to block cell growth as well as inhibitors of demethylation to block metastasis.

Future perspective

Although there is no question that it is possible to modulate tumorigenesis and metastasis by targeting different proteins of the DNA methylation machinery, it is also clear that the involvement of DNA methylation in tumorigenesis is complex. We need to learn more about the following unresolved issues. First, we need to understand how DNMTs are targeted to specific CG islands and identify which DNMT isoforms play a role in silencing tumor suppressor genes. Second, we have to identify the transacting factors that precipitate the inactive state of chromatin associated with tumor suppressor genes which precedes hypermethylation. We then need to understand how inactive chromatin attracts DNMTs. Third, we need to increase our understanding of the methylation independent functions of DNMT.

By targeting drugs to these functions, we might be able to avoid the adverse effects associated with DNA methylation inhibitors such as activation of prometastatic genes by demethylation. Fourth, we need to define the demethylation machinery in order to identify the specific machinery responsible for hypomethylation in cancer cells. One promising target, MBD2, was identified, and its potential in inhibiting metastasis needs to be pursued. It is also imperative to study the potential of methyl-enriching diets and SAM analogs in inhibiting and protecting from metastasis.

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