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DNA methylation: past, present and future directions

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DNA methylation, or the covalent addition of a methyl group to cytosine within the context of the CpG dinucleotide, has profound effects on the mammalian genome. These effects include transcriptional repression via inhibition of transcription factor binding or the recruitment of methyl-binding proteins and their associated chromatin remodeling factors, X chromosome inactivation, imprinting and the suppression of parasitic DNA sequences. DNA methylation is also essential for proper embryonic development; however, its presence can add an additional burden to the genome. Normal methylation patterns are frequently disrupted in tumor cells with global hypomethylation accompanying region-specific hypermethylation. When these hypermethylation events occur within the promoter of a tumor suppressor gene they will silence the gene and provide the cell with a growth advantage in a manner akin to deletions or mutations. Recent work indicating that DNA methylation is an important player in both DNA repair and genome stability as well as the discovery of a new family of DNA methyltransferases makes now a very exciting period for the methylation field. This review will highlight the major findings in the methylation field over the past 20 years then summarize the most important and interesting future directions the field is likely to take in the next millennium.

Introduction

Mammalian cells possess the capacity to epigenetically modify their genomes via the covalent addition of a methyl group to the 5-position of the cytosine ring within the context of the CpG dinucleotide (1). Approximately 70% of the CpG residues in the mammalian genome are methylated; however, the distribution of CpG is non-random with the majority of the genome being CpG-poor (2). Certain regions of the genome, which are often clustered at the 5′-ends of genes, possess the expected CpG frequency and have been termed CpG islands (1). CpG islands are protected from methylation in normal cells by mechanisms which remain unclear. DNA methylation has been shown to be essential for normal development (3), X-chromosome inactivation (4), imprinting (5) and the suppression of parasitic DNA sequences (6); however, its presence in mammalian cells also adds additional burdens. DNA methylation can cause an increase in mutation rate (7) and heritably silence genes whose promoters are associated with CpG islands and which control cellular proliferation (8,9). When this occurs in the promoter region of a growth-regulatory gene it can act as one of the steps in the Knudson 'two-hit' model of tumor formation. Mechanisms, which remain unclear, prevent *de novo* methylation of CpG island-associated promoter regions in normal cells. Recent studies on the potential roles for DNA methylation in DNA repair (10,11), genomic instability (12) and the cloning and characterization of new enzymatic activities which both add (13) and remove 5-methylcytosine from DNA (14,15), make this an extremely interesting period for the DNA methylation field. This review will discuss the major advances in the methylation field over the past twenty years and then end with a discussion of the most current and exciting directions the field is likely to take in the next millennium.

5-Azacytidine, DNA methylation and gene expression

Studies carried out >20 years ago first revealed a connection between DNA methylation and gene expression. In these early studies the methylation inhibitor 5-azacytidine was used to treat an undifferentiated mouse embryo cell line (10T1/2) with the striking result being the formation of several differentiated cell phenotypes (such as muscle and fat cells) (16). Such changes in phenotype were stably inherited and DNA isolated from such variants was capable of inducing differentiation when transfected back into the undifferentiated parental line (17).

It was later shown that 5-azacytidine, and its deoxy version 5-aza-2′-deoxycytidine, trap the DNA methyltransferase (DNMT) enzyme in a covalent complex with the DNA, resulting in a progressive loss of DNA methylation with each round of cell division (18). While both compounds are relatively poor chemotherapeutic agents they have been extremely valuable in studying the role of DNA methylation in gene expression. Treatment of a variety of cell lines revealed that a large number of genes could be reactivated; however, there appeared to be some specificity to this effect. It was later realized that cell lines contained altered patterns of 5-methylcytosine distribution relative to primary cells, presumably an adaptation to tissue culture conditions, and it was mostly this 'abnormal' or culture-associated methylation which was preferentially removed, and the associated gene reactivated, by 5-azacytidine treatment (17,19).

Detailed studies of the effects of DNA methylation on promoter activity have revealed that DNA methylation is a potent suppressor of gene activity (20). Two mechanisms have been proposed for this repression. The first involves the direct inhibition of binding of sequence-specific transcription factors whose binding sites contain CpG sites such as c-Myc/Myn, AP-2, E2F and ATF/CREB-like proteins binding to cAMP responsive elements (21). This mechanism requires that a CpG dinucleotide be present within the binding site, a criterion clearly not fulfilled by all transcription factors. The second mechanism of repression is mediated by methyl-CpG binding proteins (for example MeCP1 and MeCP2 also known as the MBD family) which are not sequence-specific but are specific for methylated DNA. Such proteins may compete with tran-

Table 1. Examples of CpG island promoters of tumor suppressor genes which can become hypermethylated and silenced in human tumors

Tumor suppressor gene	Tumor type(s)	Reference
pRb	Retinoblastoma	(29)
VHL	Renal carcinoma	(30)
p16INK4a	Melanoma and many others	(73)
p15INK4b	Hematologic malignancies	(74)
hMLH1	Colorectal carcinoma	(31)
APC	Colorectal carcinoma	(75)
BRCA1	Breast cancer	(76)

scription factors for their binding sites in methylated DNA or reorganize DNA into tightly packed chromatin structures incompatible with transcription (22), as will be discussed later. Studies utilizing a variety of different systems have shown that repression of transcription from some promoters appears to rely more on the former mechanism (23) while others have indicated a more prominent role for the latter mechanism (24). The exact mode of transcriptional repression *in vivo* most likely results from a combination of these two mechanisms and is also dependent on the CpG density and regulatory element composition of the specific promoter.

DNA methylation and cancer

It has been recognized for >15 years that methylation patterns in tumor cells are altered relative to those of normal cells. A global hypomethylation of the genome (25) is accompanied by region-specific hypermethylation (20). The regions which appear to be frequent targets of hypermethylation events are CpG islands (9). CpG islands are GpC and CpG-rich regions of ~1 kb that are usually associated with genes (26). CpG island methylation is rare in normal cells, playing a role in X-chromosome inactivation (4) and imprinting (5), increasing with age (27), and with *in vitro* cell culture (19). Numerous examples of CpG island promoter hypermethylation of tumor suppressor genes, accompanied by silencing and presumably a growth advantage for that cell, have been described and are summarized in Table I.

There has been some debate as to the direct role of DNA methylation in carcinogenesis. Selection of cells containing hypermethylated alleles of tumor suppressor genes such as those listed in Table I most likely represent a more generalized defect in the maintenance of methylation-free CpG islands (20) (Figure 1A). Several lines of evidence point to a direct causal role for DNA methylation in tumorigenesis. The first is that reduced DNA methylation suppresses the formation of intestinal polyps in $Apc^{Min/+}$ mice (28). Secondly, promoter-region methylation of the retinoblastoma (*pRB*) gene and the von Hippel Lindau (VHL) gene have been documented in familial cases of unilateral retinoblastoma (29) and renal cancer (30), respectively. Thirdly, studies of sporadic cases of colorectal carcinomas exhibiting microsatallite instability demonstrated a high frequency of promoter region hypermethylation of the mismatch repair (MMR) gene *hMLH1*. Treatment of colon cell lines containing a hypermethylated *hMLH1* gene with 5-aza-2′-deoxycytidine resulted in re-expression of *hMLH1* and restoration of MMR ability indicating that hypermethylation of the *hMLH1* CpG island was the primary inactivating event (31). Lastly, there are now several examples in which one copy of a tumor suppressor gene is wild-type but silenced by hypermethylation while the second copy is either mutated or lost, again supporting the role of DNA hypermethylation as one of the primary, inactivating events contributing to tumorigenesis (32).

5-Methylcytosine as an endogenous mutagen

CpG sites have been shown to act as hotspots for mutations and have been estimated to contribute to 30% of all point mutations in the germline (33). In addition, CpG sites in the coding regions of tumor suppressor genes are strong hotspots for acquired somatic mutations leading to cancer (7,34). For example, the CpG sites in the *p53* coding region are methylated in all human tissues studied (35) and contribute to as many as 50% of all inactivating mutations in colon cancer and 25% in cancers in general (34) (Figure 1B).

The increased mutability of 5-methylcytosine versus cytosine is thought to be influenced by three factors: differential repair efficiency, rate of spontaneous deamination, and rate of cell division. Deamination of cytosine forms uracil, which is readily recognized and repaired by the highly abundant and efficient uracil DNA glycosylase (UDG). However, deamination of 5-methylcytosine forms thymine, a naturally occurring DNA base which is significantly more difficult to detect and repair via the thymine DNA glycosylase (TDG) (36). The end result is an increased C→T transition rate at methylated CpG sites. Aberrations in the repair pathways themselves do not seem to be common in human tumors (37). The second factor, the rate of spontaneous deamination, appears to be relatively constant and is more than sufficient to account for all mutations observed in double-stranded DNA (38). The third factor influencing the enhanced mutability of 5-methylcytosine versus cytosine is the rate of cell division. In a model bacterial system, 5-methylcytosine was as stable as cytosine when the cells were not dividing (39). It has in fact been noted that CpG mutations are more common in human cancers where cell division is stimulated by hormones, or in damaged tissues undergoing repair (34).

The methylation machinery itself may contribute to the increased mutability of 5-methylcytosine. Mutations in prokaryotic DNA methylases which leave DNA binding functions intact but destroy catalytic function or result in a methylase unable to bind the methyl donor *S*-adenosyl-L-methionine (SAM), result in a mutator enzyme which increases 5-methylcytosine deamination (40). Such mutant DNA methylases can also actively block repair of deaminated 5-methylcytosine, presumably due to a steric hindrance mechanism (41). Similar naturally occurring catalytic site mutations in the human *DNMT1* have not been detected in human cancers, and human tumors appear to have levels of SAM sufficient for catalysis (42) so it remains unclear if these mechanisms contribute significantly to the enhanced mutability of 5-methylcytosine *in vivo*.

Interaction between DNA methylation and carcinogens

As mentioned in the previous section, CpG sites frequently act as mutational hotspots in the *p53* gene. The *p53* mutation spectrum for a given tumor type varies, however, and while transition mutations resulting from spontaneous deamination at CpG sites are frequent in colon carcinomas and other internal cancers, G→T transversion mutations predominate in lung cancer, and transition mutations at dipyrimidine sequences predominate in skin cancer, reflecting the specificity of the causative exogenous carcinogen (34,43). Elegant studies from

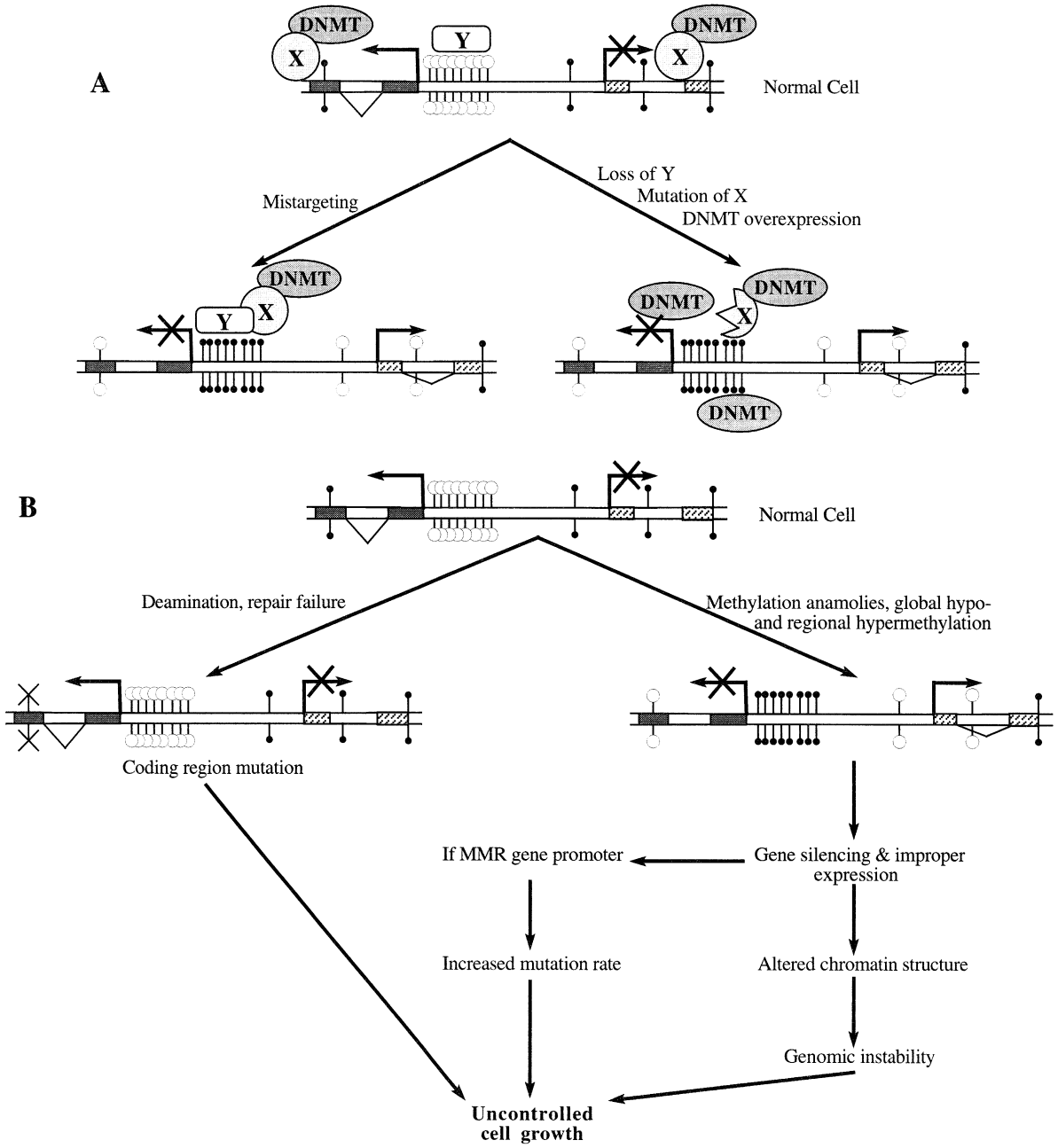

Fig. 1. (**A**) Schematic diagram showing how interaction of the various DNMTs (DNMT1, 3a or 3b in this case) with other cellular proteins may target methylation to the proper regions (shown by interaction with hypothetical protein X) and protect CpG islands from *de novo* methylation (shown by hypothetical protein Y blocking access of the DNMT to DNA). Aberrant methylation patterns in tumors may result from mistargeting of the DNMT or improper expression during the cell cycle (left) or through any one or a combination of loss of Y, mutation in X, and overexpression of one or more of the DNMTs (right). (**B**) Mechanisms by which CpG methylation may contribute to tumorigenesis. The presence of a methylated CpG within the coding region of a gene may predispose that region to mutation due to deamination and failure to repair resulting in a point mutation (left). Alternatively, alteration in the normal cellular methylation patterns, by mechanisms that remain unknown, result in gene silencing and altered chromatin structure (right). If *de novo* methylation occurs within the promoter region of a tumor suppressor gene or a gene involved in maintaining genome stability then that cell may gain a growth advantage. Black lollipops are methylated CpGs and open lollipops are unmethylated CpGs. An 'X' represents a point mutation or promoter silence. Filled boxes are exons and bent arrows are promoters.

several groups have shown that the occurrence of the latter two types of *p53* mutations were also enhanced by methylated cytosine (43).

The G→T transversion mutations in the *p53* gene, which occur with high frequency in smoking-related lung cancers, are thought to result from the bulky adduct-producing compounds [polycyclic aromatic hydrocarbons, for example benzo[*a*]pyrene (B[*a*]P)] present in tobacco smoke (43). Denissenko *et al*. (44) used a highly sensitive ligation-mediated PCR method to show that benzo[*a*]pyrene diolepoxide (B[*a*]PDE; the activated form of B[*a*]P) reacted preferentially (3–10 fold) with guanines flanked by 5-methylcytosine. This site preference of B[*a*]PDE for guanines adjacent to 5-methylcytosine was also observed for other bulky chemical carcinogens (43).

The majority of mutations observed in skin cancer are C→T transitions or CC→TT mutations at dipyrimidine sequences resulting from UV-light-induced cyclobutane pyrimidine dimer formation (45). Methylation of cytosine was shown to enhance

pyrimidine dimer formation resulting from exposure of cells to sunlight by 5–15-fold due to the higher energy absorption (λmax) of 5-methylcytosine versus cytosine in DNA (45). Thus the presence of 5-methylcytosine in mammalian DNA adds to the mutational burden via several different mechanisms.

DNA methylation and repair

Several recent lines of evidence point toward a role for DNA methylation in DNA repair, particularly the MMR system. In the first study, it was demonstrated that *DNMT1* is targeted to newly replicated DNA via the replication-associated protein or proliferating cell nuclear antigen (PCNA) (10). PCNA, also known as the polymerase processivity factor or sliding clamp, is required for DNA replication (46) as well as DNA repair [both MMR (47) and nucleotide excision repair (48)]. The cell cycle regulatory protein $p21^{WAF1/CIP1}$ also interacts with PCNA and mediates the ability of p53 to arrest cell division in response to DNA damage (49). The studies of Chuang *et al*. (10) showed that the binding of $p21^{WAF1/CIP1}$ and *DNMT1* to PCNA were mutually exclusive and their levels were inversely related in both normal and transformed cells. Since p53 function is commonly lost in cancer (50) with a concomitant loss of $p21^{WAF1/CIP1}$, this may result in a disruption in the balance between the $p21^{WAF1/CIP1}$–*DNMT1* interaction with PCNA and could result in a mistargeting of methylation especially since damaged DNA is a good substrate for *DNMT1* (51).

A clear link between DNA methylation and MMR has been established through the demonstration that the *hMLH1* promoter is frequently hypermethylated and silenced in sporadic colorectal tumors (31). An additional connection between DNA methylation and MMR came from a paper by Lengauer *et al*. (11) which described an interesting phenomenon in which retroviral infection of MMR-deficient cell lines led to *de novo* methylation and transcriptional silencing of the proviral DNA, whereas infection of MMR-proficient cell lines did not lead to *de novo* methylation of proviral DNA and these lines were competent to express the retrovirally encoded reporter gene. This led to the proposal that there were two distinct pathways which may lead to the genomic instability observed in many tumors and which have been termed MMR^+/MET^- (methylation proficiency) and MMR^-/MET^+ (11). Other studies lend support to this idea by showing differences in the methylation patterns of several cellular genes in MMR-deficient versus MMR-proficient cell lines (52). While it is still unclear exactly what the relationship is between MMR and DNA methylation, the role of the PCNA–*DNMT1* interaction in repair, and the nature of the genes involved in causing the MET^- phenotype, these results all point toward a major role for DNA methylation in the DNA repair process.

DNA methylation and chromatin structure

A strong correlation between DNA hypermethylation, transcriptional silence and tightly compacted chromatin has been established in many different systems (53). Work over the past 5 years has led to a remarkable increase in the knowledge of the mechanisms of chromatin structure modulation and have revealed that chromatin is a dynamic structure which plays a large role in transcriptional regulation (54). At least a major portion of chromatin remodeling appears to be accomplished through acetylation and deacetylation of the histone octamer 'tails'. Many of the acetylation and deacetylation enzymes turn out to be known transcriptional enhancer and repressor proteins, respectively (54). Inactive regions of DNA which were demonstrated to be heavily methylated were also found to be enriched in hypoacetylated histones (55). A major advancement in understanding the link between DNA methylation, histone hypoacetylation, and gene silencing came from the recent studies of two laboratories. Both groups demonstrated that the methyl-binding protein MeCP2, known to be involved in transcriptional repression of methylated DNA (56), recruited a histone deacetylase, HDAC1, via the bridging protein Sin3 (57,58). This provided the first direct connection between two transcriptional silencing pathways. These findings also have implications for cancer where altered methylation patterns, both hypo- and hypermethylation events, may result in inappropriate gene expression or silencing by altered targeting of the histone deacetylase and also indicates that inhibitors of histone deacetylases, alone or in combination with DNA hypomethylating agents, may be useful in reactivating silenced tumor suppressor genes in cancer cells.

The methylation machinery

Until recently, only one DNMT, DNMT1, had been cloned from human and mouse cells. DNMT1 is a large enzyme (~200 kDa) composed of a C-terminal catalytic domain with homology to bacterial cytosine-5 methylases and a large N-terminal regulatory domain with several functions, including targeting to replication foci (59). Disruption of Dnmt1 in mice results in abnormal imprinting (5), embryonic lethality, greatly reduced levels of DNA methylation (3) and derepression of endogenous retroviruses known as IAPs (6). $Dnmt1^{-/-}$ embryonic stem (ES) cells, however, are viable and still possess the ability to *de novo* methylate viral DNA, which suggested the existence of an independently encoded *de novo* DNMT (60).

Targeting of DNMT1 to replication foci via the N-terminal domain is believed to allow for copying of methylation patterns from the parental to the newly synthesized daughter DNA strand. Several forms of DNMT1 have been detected which differ in their translation start sites and their preferences for hemimethylated versus unmethylated substrates (61). Forced overexpression of DNMT1 or cleavage between the N-terminal regulatory domain and C-terminal catalytic domain has been shown to result in increased *de novo* methylation activity (62,63) and cellular transformation (64). Furthermore, inhibition of DNMT1 activity through the use of antisense, knockout, pharmacologic means, or a combination of the latter two, inhibits tumor cell growth and induces differentiation (28,65).

Since *de novo* methylation activity remains in Dnmt1 knockout ES cells and because the exact role of DNMT1 in tumor-specific methylation abnormalities remain unclear, a search for additional DNMTs was carried out by several groups. A second potential DNMT, Dnmt2, was isolated by two groups but has not been shown to possess methylation ability (66,67). Recently, another group of DNMTs, Dnmt3a and 3b, was isolated by database search. These enzymes were shown to be expressed at increased levels in undifferentiated ES cells and were downregulated in differentiating ES cells and adult murine tissues. Furthermore both Dnmt3a and 3b methylated hemimethylated and unmethylated DNA with equal efficiencies, making them potential candidates for the long sought *de novo* methyltransferases (13). Overexpression of both DNMT1 as well as the DNMT3 family has been reported in human tumors and may contribute to the global methylation

abnormalities seen in cancer cells although increased expression of the DNMTs is likely to be only partially responsible for the observed methylation abnormalities since not all tumors overexpress these enzymes (68,69) (Figure 1A).

The enzymatic removal of 5-methylcytosine from DNA has also been described but has been far less extensively studied. One mechanism identified involves a 5-methylcytosine DNA glycosylase activity (14). A more recent mechanism has been reported which appears to involve specific removal of the methyl group from 5-methylcytosine in the form of methanol by a single polypeptide which also contains a methyl-CpG binding motif (15). It is likely that there is an interplay between both methyltransferases (*de novo* and maintenance) and demethylase(s) when new methylation patterns are established during embryogenesis (70), and a dysregulation of these pathways is likely responsible for the global hypomethylation and CpG island hypermethylation observed in tumor cells.

Future directions

One of the greatest tasks for researchers in the DNA methylation field in the next millennium will be to use the new knowledge of the methylation machinery just described to determine how dysregulation of one or more of these systems contributes to cancer or can be used to specifically target tumor cells for destruction. While we and others have reported that DNMT1 and the recently discovered DNMT3 family of methyltransferases are overexpressed in tumors relative to adjacent normal tissue (68,69) there is clearly more to the story. The frequency and degree of DNMT1 overexpression in tumors remains a controversial issue (71). With the new enzymes in hand it will be essential to characterize the catalytic activity of the new DNMTs and compare this with the more well-characterized DNMT1 as well as determine the preferred substrate of each DNMT. For example a particular DNMT, or DNMT splice variant, may preferentially bind CpG-rich sequences, like CpG islands, and could thus be responsible for initiating the aberrant CpG island hypermethylation of such sequences in cancer. Furthermore, each DNMT may be targeted to a different region of the nucleus, or be regulated differently during the cell cycle, so for each DNMT it will be crucial to determine their respective cell-cycle profiles, their protein interaction partners, the subcellular localization of each DNMT, whether this targeting varies during the cell cycle, and if targeting is altered in transformed cells (Figure 1A).

A second major area of research which is likely to dominate the DNA methylation field in the future is determining its role in DNA repair and genome stability (Figure 1B). As discussed above there is evidence that hypermethylation of MMR genes contributes directly to the MMR phenotype and that the MMR status of a cell is inversely related to its ability to *de novo* methylate foreign DNA sequences (11,31). A recent report suggests that methylation may be involved in targeting MMR. A novel protein termed MED1 (also called MBD4) has been identified which associates with *hMLH1*, binds preferentially to methylated DNA via a methyl binding domain at its N-terminus, and possesses endonuclease activity at its C-terminus (72). Further investigation will be required to determine if the function of this novel protein is simply to preferentially target repair to deaminated 5-methylcytosine or if MED1 may represent the human homolog of the bacterial *MutH* gene, which allows for strand discrimination and targeting of the repair apparatus to the newly synthesized DNA strand.

The role of DNA methylation in genome stability (Figure 1B), which is only beginning to be explored, is also likely to be a major focus of future research. A recent study using murine embryonic stem cells deficient in Dnmt1 indicated that reduced DNA methylation led to increased genomic instability, as demonstrated by loss of the *hprt* locus, which may point to a role for DNA methylation in the suppression of mitotic recombination (12). The finding of Lengauer *et al*. (11) that MMR proficiency was inversely related to methylation proficiency is interesting in the context of genome stability since MMR^- (MET^+) cell lines are known to have a stable (near diploid) chromosome composition while MMR^+ (MET^-) lines have unstable genomes which exhibit aneuploidy, chromosomal losses, duplications and translocations. While the pathways and genes responsible for the MET phenotype are unknown, future work in this area is likely to greatly enhance our knowledge of the role of DNA methylation in tumorigenesis. Two other important areas that are likely to dominate the field in the future include characterization of the demethylation machinery and studies of the relationship between DNA methylation and chromatin structure. Such studies are likely to yield major contributions to understanding the role of DNA methylation in cancer and, since methylation changes are reversible, may also lead to the development of novel therapeutic strategies to reverse the transformed phenotype through the use of known or novel demethylating agents to correct aberrant methylation patterns and restore growth control in tumor cells.

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References

1. Bird,A. (1992) The essentials of DNA methylation. *Cell*, **70**, 5–8.
2. Cooper,D.N. and Krawczak,M. (1989) Cytosine methylation and the fate of CpG dinucleotides in vertebrate genomes. *Hum. Genet.*, **83**, 181–188.
3. Li,E., Bestor,T.H. and Jaenisch,R. (1992) Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell*, **69**, 915–926.
4. Panning,B. and Jaenisch,R. (1998) RNA and the epigenetic regulation of X chromosome inactivation. *Cell*, **93**, 305–308.
5. Li,E., Beard,C. and Jaenisch,R. (1993) Role for DNA methylation in genomic imprinting. *Nature*, **366**, 362–365.
6. Walsh,C.P., Chaillet,J.R. and Bestor,T.H. (1998) Transcription of IAP endogenous retroviruses is constrained by cytosine methylation. *Nature Genet.*, **20**, 116–117.
7. Rideout,W.M.I., Coetzee,G.A., Olumi,A.F. and Jones,P.A. (1990) 5-Methylcytosine as an endogenous mutagen in the human LDL receptor and *p53* genes. *Science*, **249**, 1288–1290.
8. Jones,P.A. (1996) DNA methylation errors and cancer. *Cancer Res.*, **56**, 2463–2467.
9. Baylin,S.B., Herman,J.G., Herman,J.R., Vertino,P.M. and Issa,J.-P. (1998) Alterations in DNA methylation: a fundamental aspect of neoplasia. *Adv. Cancer Res.*, **72**, 141–196.
10. Chuang,L.S.-H., Ian,H.-I., Koh,T.-W., Ng,H.-H., Xu,G. and Li,B.F.L. (1997) Human DNA–(cytosine-5) methyltransferase–PCNA complex is a target for $p21^{Waf1.}$ *Science*, **277**, 1996–2000.
11. Lengauer,C., Kinzler,K.W. and Vogelstein,B. (1997) DNA methylation and genetic instability in colorectal cancer cells. *Proc. Natl Acad. Sci. USA*, **94**, 2545–2550.
12. Chen,R.Z., Pettersson,U., Beard,C., Jackson-Grusby,L. and Jaenisch,R. (1998) DNA hypomethylation leads to elevated mutation rates. *Nature*, **395**, 89–93.
13. Okano,M., Xie,S. and Li,E. (1998) Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. *Nature Genet.*, **19**, 219–220.
14. Fremant,M., Seigmann,M., Gaulis,S., Matthies,R., Hess,D. and Jost,J.-P. (1997) Demethylation of DNA by purified chick embryo 5-methylcytosine-

DNA glycosylase requires both protein and RNA. *Nucleic Acids Res.*, **25**, 2375–2380.
15. Bhattacharya,S.K., Ramchandani,S., Cervoni,N. and Szyf,M. (1999) A mammalian protein with specific demethylase activity for mCpG DNA. *Nature*, **397**, 579–583.
16. Taylor,S.M. and Jones,P.A. (1979) Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine. *Cell*, **17**, 771–779.
17. Jones,P.A. (1985) Altering gene expression with 5-azacytidine. *Cell*, **40**, 485–486.
18. Juttermann,R., Li,E. and Jaenisch,R. (1994) Toxicity of 5-aza-2′-deoxycytidine to mammalian cells is mediated primarily by covalent trapping of DNA methyltransferase rather than DNA demethylation. *Proc. Natl Acad. Sci. USA*, **91**, 11797–11801.
19. Antequera,F., Boyes,J. and Bird,A. (1990) High levels of de novo methylation and altered chromatin structure at CpG islands in cell lines. *Cell*, **62**, 503–514.
20. Jones,P.A. and Laird,P.W. (1999) Cancer epigenetics comes of age. *Nature Genet.*, **21**, 163–166.
21. Tate,P.H. and Bird,A.P. (1993) Effects of DNA methylation on DNA-binding proteins and gene expression. *Curr. Opin. Genet. Dev.*, **3**, 226–231.
22. Boyes,J. and Bird,A. (1992) Repression of genes by DNA methylation depends on CpG density and promoter strength: Evidence for involvement of a methyl-CpG binding protein. *EMBO J.*, **11**, 327–333.
23. Robertson,K.D. and Ambinder,R.F. (1997) Mapping promoter regions that are hypersensitive to methylation-mediated inhibition of transcription: Application of the methylation cassette assay to the Epstein–Barr virus major latency promoter. *J. Virol.*, **71**, 6445–6454.
24. Kass,S.U., Goddard,J.P. and Adams,R.L.P. (1993) Inactive chromatin spreads from a focus of methylation. *Mol. Cell. Biol.*, **13**, 7372–7379.
25. Goelz,S.E., Vogelstein,B., Hamilton,S.R. and Feinberg,A.P. (1985) Hypomethylation of DNA from benign and malignant human colon neoplasms. *Science*, **228**, 187–190.
26. Gardiner-Garden,M. and Frommer,M. (1987) CpG islands in veterbrate genomes. *J. Mol. Biol.*, **196**, 261–282.
27. Ahuja,N., Li,Q., Mohan,A.L., Baylin,S.B. and Issa,J.-P.J. (1998) Aging and DNA methylation in colorectal mucosa and cancer. *Cancer Res.*, **58**, 5489–5494.
28. Laird,P.W., Jackson-Grusby,L., Fazell,A., Dickinson,S.L., Jung,W.E., Li,E., Weinberg,R.A. and Jaenisch,R. (1995) Suppression of intestinal neoplasia by DNA hypomethylation. *Cell*, **81**, 197–205.
29. Stirzaker,C., Millar,D.S., Paul,C.L., Warnecke,P.M., Harrison,J., Vincent,P.C., Frommer,M. and Clark,S.J. (1997) Extensive DNA methylation spanning the Rb promoter in retinoblastoma tumors. *Cancer Res.*, **57**, 2229–2237.
30. Herman,J.G., Latif,F., Weng,Y., Lerman,M.I., Zbar,B., Liu,S., Samid,D., Duan,D.-S.R., Gnarra,J.R., Linehan,W.M. and Baylin,S.B. (1994) Silencing of the VHL tumor-suppressor gene by DNA methylation in renal carcinoma. *Proc. Natl Acad. Sci. USA*, **91**, 9700–9704.
31. Herman,J.G., Umar,A., Polyak,K., Graff,J.R., Ahuja,N., Issa,J.-P.J., Markowitz,S., Willson,J.K.V., Hamilton,S.R., Kinzler,K.W., Kane,M.F., Kolodner,R.D., Vogelstein,B., Kunkel,T.A. and Baylin,S.B. (1998) Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal cancer. *Proc. Natl Acad. Sci. USA*, **98**, 6870–6875.
32. Myohanen,S.K., Baylin,S.B. and Herman,J.G. (1998) Hypermethylation can selectively silence individual *p16INK4a* alleles in neoplasia. *Cancer Res.*, **58**, 591–593.
33. Cooper,D.N. and Youssoufian,H. (1988) The CpG dinucleotide and human genetic disease. *Hum. Genet.*, **78**, 151–155.
34. Greenblatt,M.S., Bennett,W.P., Hollstein,M. and Harris,C.C. (1994) Mutations in the p53 tumor suppressor gene: Clues to cancer etiology and molecular pathogenesis. *Cancer Res.*, **54**, 4855–4878.
35. Magewu,A.N. and Jones,P.A. (1994) Ubiquitous and tenacious methylation of the CpG site in codon 248 of the *p53* gene may explain its frequent appearance as a mutational hot spot in human cancer. *Mol. Cell. Biol.*, **14**, 4225–4232.
36. Jiricny,J. (1996) Mismatch repair and cancer, In *Cancer Surveys: Genetic Instability in Cancer*. Imperial Cancer Research Fund, Vol. 28, pp. 47–68.
37. Schmutte,C., Baffa,R., Veronese,L.M., Murakumo,Y. and Fishel,R. (1997) Human thymine–DNA glycosylase maps at chromosome 12q22–q24.1: A region of high loss of heterozygosity in gastric cancer. *Cancer Res.*, **57**, 3010–3015.
38. Shen,J.-C., Rideout,W.M. and Jones,P.A. (1994) The rate of hydrolytic deamination of 5-methylcytosine in double-stranded DNA. *Nucleic Acids Res.*, **22**, 972–976.
39. Lieb,M. and Rehmat,S. (1997) 5-Methylcytosine is not a mutagen hot spot in nondividing *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **94**, 940–945.
40. Shen,J.-C., Rideout,W.M. and Jones,P.A. (1992) High frequency mutagenesis by a DNA methyltransferase. *Cell*, **71**, 1073–1080.
41. Shen,J.-C., Zingg,J.-M., Yang,A.S., Schmutte,C. and Jones,P.A. (1995) A mutant HpaII methyltransferase functions as a mutator enzyme. *Nucleic Acids Res.*, **23**, 4275–4282.
42. Schmutte,C., Yang,A.S., Nguyen,T.T., Beart,R.W. and Jones,P.A. (1996) Mechanisms for the involvement of DNA methylation in colon carcinogenesis. *Cancer Res.*, **56**, 2375–2381.
43. Pfeifer,G.P., Tang,M.-S. and Denissenko,M.F. (2000) Mutation hotspots and DNA methylation. In Vogt,P. and Jones,P.A. (eds) *Current Topics in Microbiology and Immunology.* Springer-Verlag, Heidelberg, in press.
44. Denissenko,M.F., Pao,A., Tang,M. and Pfeifer,G.P. (1996) Preferential formation of benzo[*a*]pyrene adducts at lung cancer mutational hotspots in p53. *Science*, **274**, 430–432.
45. Tommasi,S., Denissenko,M.F. and Pfeifer,G.P. (1997) Sunlight induces pyrimidine dimers preferentially at 5-methylcytosine bases. *Cancer Res.*, **57**, 4727–4730.
46. Krishna,T.S.R., Kong,X.-P., Gary,S., Burgers,P.M. and Kuriyan,J. (1994) Crystal structure of the eukaryotic DNA polymerase processivity factor PCNA. *Cell*, **79**, 1233–1243.
47. Umar,A., Buermeyer,A.B., Simon,J.A., Thomas,D.C., Clark,A.B., Liskay,R.M. and Kunkel,T.A. (1996) Requirement for PCNA in DNA mismatch repair at a step preceding DNA resynthesis. *Cell*, **87**, 65–73.
48. Nichols,A.F. and Sancar,A. (1992) Purification of PCNA as a nucleotide excision repair protein. *Nucleic Acids Res.*, **20**, 2441–2446.
49. Waga,S., Hannon,G.J., Beach,D. and Stillman,B. (1994) The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. *Nature*, **369**, 574–578.
50. Vogelstein,B. and Kinzler,K.W. (1992) p53 function and dysfunction. *Cell*, **70**, 523–526.
51. Smith,S.S., Laayoun,A., Lingeman,R.G., Baker,D.J. and Riley,J. (1994) Hypermethylation of telomere-like foldbacks at codon 12 of the human c-Ha *ras* gene and the trinucleotide repeat of the *FMR-1* gene of fragile X. *J. Mol. Biol.*, **243**, 143–151.
52. Ahuja,N., Mohan,A.L., Li,Q., Stolker,J.M., Herman,J.G., Hamilton,S.R., Baylin,S.B. and Issa,J.-P.J. (1997) Association between CpG island methylation and microsatellite instability in colorectal cancer. *Cancer Res.*, **57**, 3370–3374.
53. Kass,S.U., Pruss,D. and Wolffe,A.P. (1997) How does DNA methylation repress transcription? *Trends Genet.*, **12**, 444–449.
54. Wolffe,A.P. and Pruss,D. (1996) Targeting chromatin disruption: Transcriptional regulators that acetylate histones. *Cell*, **84**, 817–819.
55. Jeppesen,P. and Turner,B.M. (1993) The inactive X chromosome in female mammals is distinguished by a lack of histone H4 acetylation, a cytogenetic marker for gene expression. *Cell*, **74**, 281–289.
56. Nan,X., Campoy,F.J. and Bird,A. (1997) MeCP2 is a transcriptional repressor with abundant binding sites in genomic chromatin. *Cell*, **88**, 471–481.
57. Jones,P.L., Veenstra,G.J.C., Wade,P.A., Vermaak,D., Kass,S.U., Landsberger,N., Strouboulis,J. and Wolffe,A.P. (1998) Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nature Genet.*, **19**, 187–191.
58. Nan,X., Ng,H.-H., Johnson,C.A., Laherty,C.D., Turner,B.M., Eisenman,R.N. and Bird,A. (1998) Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature*, **393**, 386–389.
59. Leonhardt,H., Page,A.W., Weier,H. and Bestor,T.H. (1992) A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei. *Cell*, **71**, 865–873.
60. Lei,H., Oh,S.P., Okano,M., Juttermann,R., Goss,K.A., Jaenisch,R. and Li,E. (1996) De novo DNA cytosine methyltransferase activities in mouse embryonic stem cells. *Development*, **122**, 3195–3205.
61. Pradhan,S., Talbot,D., Sha,M., Benner,J., Hornstra,L., Li,E., Yaenisch,R. and Roberts,R.J. (1997) Baculovirus-mediated expression and characterization of the full-length murine DNA methyltransferase. *Nucleic Acids Res.*, **25**, 4666–4673.
62. Vertino,P.M., Yen,R.-W.C., Gao,J. and Baylin,S.B. (1996) De novo methylation of CpG island sequences in human fibroblasts overexpressing DNA (cytosine-5)-methyltransferase. *Mol. Cell. Biol.*, **16**, 4555–4565.
63. Bestor,T.H. (1992) Activation of mammalian DNA methyltransferase by cleavage of a Zn binding regulatory domain. *EMBO J.*, **11**, 2611–2617.
64. Wu,J., Issa,J.P., Herman,J., Bassett,D.E., Nelkin,B.D. and Baylin,S.B. (1993) Expression of an exogenous eukaryotic DNA methyltransferase gene induces transformation of NIH 3T3 cells. *Proc. Natl Acad. Sci. USA*, **90**, 8891–8895.
65. MacLeod,A.R. and Szyf,M. (1995) Expression of antisense to DNA

methyltransferase mRNA induces DNA demethylation and inhibits tumorigenesis. *J. Biol. Chem.*, **270**, 8037–8043.
66. Yoder,J.A. and Bestor,T.H. (1998) A candidate mammalian DNA methyltransferase related to pmt1 from fission yeast. *Hum. Mol. Genet.*, **7**, 279–284.
67. Okano,M., Xie,S. and Li,E. (1998) Dnmt2 is not required for *de novo* and maintenance methylation of viral DNA in embryonic stem cells. *Nucleic Acids Res.*, **26**, 2536–2540.
68. Issa,J.-P., Vertino,P.M., Wu,J., Sazawal,S., Celano,P., Nelkin,B.D., Hamilton,S.R. and Baylin,S.B. (1993) Increased DNA-methyltransferase activity during colon cancer progression. *J. Natl Cancer Inst.*, **85**, 1235–1240.
69. Robertson,K.D., Uzvolgyi,E., Liang,G., Talmadge,C., Sumegi,J., Gonzales,F.A. and Jones,P.A. (1999) The human DNA methyltransferases (DNMTs) 1, 3a and 3b: Coordinate mRNA expression in normal tissues and overexpression in tumors. *Nucleic Acids Res.*, **27**, 2291–2298.
70. Kafri,T., Gao X. and Razin,A. (1993) Mechanistic aspects of genome-wide demethylation in the pre-implantation embryo. *Proc. Natl Acad. Sci. USA*, **90**, 10558–10562.
71. Lee,P.J., Washer,L.L., Law,D.J., Boland,C.R., Horon,I.L. and Feinberg,A.P. (1996) Limited up-regulation of DNA methyltransferase in human colon cancer reflecting increased cell proliferation. *Proc. Natl Acad. Sci. USA*, **93**, 10366–10370.
72. Bellacosa,A., Cicchillitti,L., Schepis,F., Riccio,A., Yeung,A.T., Matsumoto,Y., Golemis,E.A., Genuardi,M. and Neri,G. (1999) MED1, a novel human methyl-CpG-binding endonuclease, interacts with DNA mismatch repair protein MLH1. *Proc. Natl Acad. Sci. USA*, **96**, 3969–3974.
73. Ruas,M. and Peters,G. (1998) The p16INK4a/CDKN2A tumor suppressor and its relatives. *Biochim. Biophys. Acta*, **1378**, F115–F177.
74. Herman,J.G., Jen,J., Merlo,A. and Baylin,S.B. (1996) Hypermethylation associated inactivation indicates a tumor suppressor role for p15INK4B. *Cancer Res.*, **54**, 722–727.
75. Hiltunen,M.O., Alhonen,L., Koistinaho,J., Myohanen,S., Paakkonen,M., Marin,S., Kosma,V.M. and Janne,J. (1997) Hypermethylation of the APC (adenomatous polyposis coli) gene promoter region in human colorectal carcinoma. *Int. J. Cancer*, **70**, 644–648.
76. Dobrovic,A. and Simpfendorfer,D. (1997) Methylation of the *BRCA1* gene in sporadic breast cancer. *Cancer Res.*, **57**, 3347–3350.

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