

Review

DNA methylation and the regulation of gene transcription

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Abstract. The regulation of gene transcription is not simply dependent on the presence or absence of DNA-binding transcription factors that turn genes on or off, but also involves processes determining the ability of transcription factors to gain access to and bind their target DNA. Methylation of DNA cytosine bases leads to the inaccessibility of DNA regulatory elements to their transcription factors by a number of mechanisms. Our understanding of DNA methylation has advanced rapidly in recent years with the identification of an increasingly large number of novel proteins involved in this process. These include methylcytosine-binding proteins as well as additional members of the DNA methyltransferase family. The cre-

ation of mice with targeted deletions in a number of genes involved in DNA methylation has further elucidated the functions of many of these proteins. The characterization of complexes that contain proteins known to be involved in DNA methylation has led to the identification of additional proteins, especially those involved in histone deacetylation, indicating that DNA methylation and histone deacetylation very likely act in a synergistic fashion to regulate gene transcription. Finally, the implication of DNA methylation in tumorigenesis and the realization that some congenital diseases are caused by deficiency of proteins involved in DNA methylation has confirmed the importance of this process in regulating gene expression.

Key words. DNA methylation; methylcytosine; gene regulation; methyltransferase; methylcytosine-binding protein; chromatin; histone deacetylase; genomic imprinting.

Methylcytosine, CG dinucleotides and CpG islands

First discovered over 50 years ago in calf thymus [1], methylation of DNA occurs mostly at the 5 position of cytosine in higher eukaryotic cells (fig. 1). Although approximately 4% of cytosines present in the genome are methylated, all of the methylcytosine residues are found in the context of 5'-cytosine guanine-3' (CG) dinucleotides, of which ~70–80% contain methylcytosine (for early reviews see [2, 3]). Of great functional significance is the fact that methylcytosines occur in a nonrandom distribution throughout the genome [4]. When DNA is analyzed for CG dinucleotide pairs, regions with higher proportions of CG

sequences than would be expected by chance are found to exist [5]. Such CG-rich regions, referred to as CpG islands, constitute between 1 and 2% of the total genome, but account for most of the nonmethylated CG dinucleotides. The remaining CG dinucleotides occur outside of CpG islands and are largely methylated. CpG islands have been identified in organisms with large genomes such as vertebrates and some higher plants [6]. A connection between CpG islands and gene structure first became apparent with the finding that regions of the genome accessible to nucleases, and therefore likely to be involved in active gene transcription, contained hypomethylated DNA [4]. Further studies on CG dinucleotide distribution found that their frequency was higher at the 5' end compared to the 3' end of 15 randomly selected genes [5].

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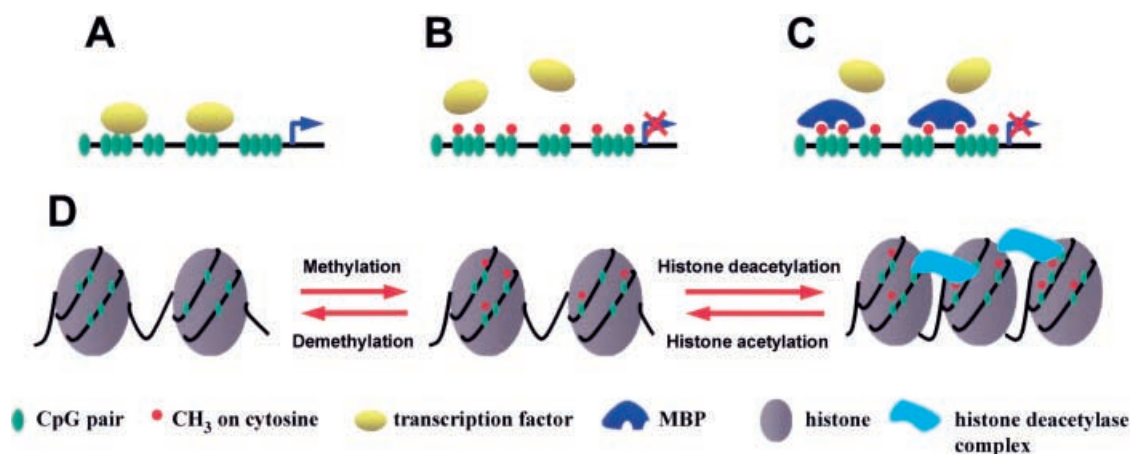


Figure 1. Mechanisms by which DNA methylation inhibits gene transcription. (A) Hypomethylation of CpG dinucleotides within a promoter region allows transcription factors to bind and transcription to occur. (B) Methylation of CpGs inhibits transcription factor binding and therefore gene transcription. (C) The presence of methylcytosine-binding proteins (MBPs) bound to CpGs within a promoter region also blocks access of transcription factors to their binding sites. (D) MBPs bound to CpGs in the promoter region form complexes with histone deacetylases and corepressors, leading to histone deacetylation, chromatin condensation and a transcriptionally inactive chromatin structure.

CpG islands were subsequently identified at the 5' end of a number of individual genes, including murine MHC class I and II genes, as well as dihydrofolate reductase and chicken $\alpha 2$ type I collagen [7]. Similar results were obtained when restriction fragments were generated with *HpaII* cleavage, an enzyme whose target site contains an unmethylated CG dinucleotide. These fragments were found to be relatively unmethylated and consisted of sequences with high C + G content (65%), compared with bulk DNA (40%) [8]. The increased frequency of CG pairs in CpG islands is reflected in the CG/GC ratio of approximately 1, a fivefold increase compared with 0.2 for bulk DNA [9]. The low CG/GC ratio in bulk DNA reflects the tendency for methylcytosine to undergo deamination to thymine, which is repaired less efficiently by thymine-DNA glycosylase than the corresponding deamination of unmethylated cytosine to uracil, which is corrected by uracil-DNA glycosylase [10].

An early indication of a relationship between CpG islands and the presence of genes came from restriction enzyme analysis using methylation-sensitive CG restriction enzymes [11]. Using the enzyme *SacII*, 3 out of 4 CpG islands were found to be associated with active gene expression. As chromatin structure can also act as a marker for active genes, CpG island chromatin was compared with that of bulk chromatin and found to have significantly reduced amounts of histone H1, higher levels of histone H3 and H4 acetylation, and regions within CpG islands free of nucleosomes [12]. All these features are characteristic of transcriptionally active chromatin and further support the notion that CpG islands colocalize with the promoter region of active genes. This was confirmed in a comprehensive analysis of CpG islands in the European Molecular Biology Laboratory (EMBL) data-

base [13]. Examination of 375 genes and 58 pseudogenes revealed 240 CpG islands, virtually all of which covered part of at least one exon. More than half the genes contained CpG islands, and all housekeeping genes and widely expressed genes were found to have a CpG island covering the transcription start site [13]. It should be noted, however, that CpG islands do not occur exclusively within the 5' region of genes but have also been identified downstream of transcription initiation sites within the transcribed region of a number of genes [14].

As noted above, most of the CG dinucleotides outside of CpG islands are methylated, and the large majority of these are found in transposable elements such as retrotransposons, L1 elements, Alu sequences and endogenous retroviruses, as well as juxtacentromeric satellite sequences. DNA methylation is believed to play a critical role in the suppression of parasitic and other repeat sequences (reviewed in [15–17]).

The DNA methyltransferases

DNA methyltransferase 1. Cloned in 1988, DNA methyltransferase 1 (Dnmt1) remained the only known mammalian DNA methyltransferase for 10 years [18]. Dnmt1, like bacterial methyltransferases, catalyzes the transfer of a methyl group from *S*-adenosylmethionine to the 5' position on cytosine (fig. 2). The protein, 1620 amino acids long, consists of a C-terminal domain containing the catalytic site, and is more similar to bacterial DNA C5-specific restriction methyltransferases than the other more recently identified mammalian DNA methyltransferases [18]. The N-terminal region consists of multiple domains, including a nuclear localization signal, a zinc-finger

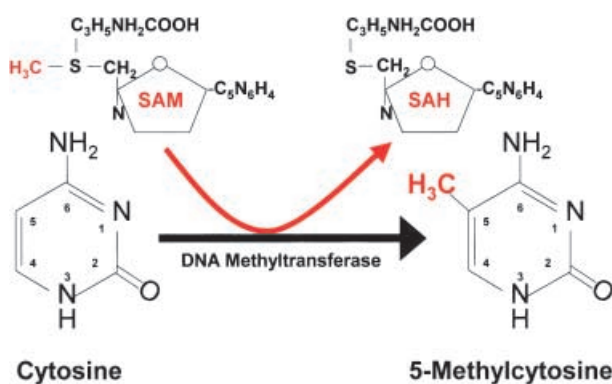


Figure 2. Formation of 5-methylcytosine. A DNA methyltransferase enzyme catalyzes the transfer of a methyl group (CH_3) from S-adenosylmethionine (SAM) to (deoxy)cytosine, producing 5-(deoxy)methylcytosine and S-adenosylhomocysteine (SAH).

DNA binding domain [18] and a domain for targeting Dnmt1 to the replication fork during S phase [19]. DNA binding may occur through a number of domains, including the zinc binding domain, the catalytic domain and the nuclear localization sequence containing the domain [20]. However, it has also been reported that the target recognition domain for hemimethylated CG is located within the N terminus between amino acids 122 and 417 [21].

Our understanding of the mechanism by which methyltransferase enzymes methylate cytosine has been greatly advanced by the crystallographic analysis of the bacterial methyltransferase *HhaI* [22], which showed that the target cytosine is everted from the DNA helix and inserted into the active site of the enzyme. All known DNA cytosine-5-transferases contain 10 sequence motifs, with 6 being very strongly conserved [23].

Dnmt1 is believed to primarily mediate maintenance methyltransferase activity during S phase because of its 5–30-fold preference for hemimethylated DNA as compared with nonmethylated DNA [24]. However, there is some evidence to suggest that Dnmt1 might also have de novo methyltransferase capabilities. First, an in vitro study of de novo methyltransferase activity found that all identifiable de novo activity in embryo lysates resided with Dnmt1 [24]. Second, forced overexpression of Dnmt1 led to de novo methylation in both myocytes [25] and fibroblasts [26]. Whether Dnmt1 mediates de novo methylation under normal biological conditions remains to be determined, although human immunodeficiency virus (HIV) infection was shown to result in upregulation of Dnmt1 expression and activity, resulting in de novo methylation of the interferon γ ($\text{IFN}\gamma$) promoter and suppression of the $\text{IFN}\gamma$ gene [27].

Differential usage of alternative exons at the 5' end of the gene gives rise to different isoforms in various tissues. In particular, the pattern of Dnmt1 expression in oocytes is quite different from that of other tissues. The oocyte-spe-

cific 5' exon is associated with a Dnmt1 protein that is truncated at the N terminus and sequestered in the cytoplasm during the later stages of oocyte growth [28]. Targeted deletion of the oocyte-specific promoter and first exon led to a loss of normal genomic imprinting [29]. By contrast, a spermatocyte-specific 5' exon was shown to interfere with translation and therefore synthesis of Dnmt1 protein [28], although an identical transcript has also been identified in differentiated skeletal muscle myotubes and shown to be translated despite the presence of a number of out-of-frame upstream ATGs [30].

Targeted homozygous Dnmt1 deletion leads to embryonic lethality prior to mid-gestation [31]. Although embryonic stem (ES) cells with the targeted mutation grow normally despite markedly demethylated genomes, they undergo apoptosis when induced to differentiate [31]. However, despite Dnmt1 deletion, the ES cells retain the capacity for de novo methylation. Dnmt1 also appears to be necessary for genomic imprinting [32] and X chromosome inactivation [33].

Three recent studies have confirmed or at least supported the tendency for apoptosis to occur in differentiated cells that are completely deficient in Dnmt1. First, Cre-mediated deletion of Dnmt1 in cultured fibroblasts lead to DNA hypomethylation followed by p53-dependent apoptosis [34]. Second, Cre-mediated deletion at the CD4+ CD8+ double-positive thymocyte stage resulted in a markedly reduced number of cell cycles by stimulated naive T cells, followed by growth arrest and cell death [35]. Finally, conditional Dnmt1 deletion in mitotic central nervous system (CNS) precursor cells in utero resulted in rapid elimination of mutated cells within 3 weeks of postnatal life [36].

Dnmt1 has now been shown to associate with many different proteins, including proliferating cell nuclear antigen (PCNA) [37], the transcription elongation factor E2F1, the retinoblastoma tumor suppressor protein Rb and the histone deacetylase HDAC1 [38]. It has also been shown to associate with a complex containing HDAC2, DMAP1, a recently identified DNMT1-binding corepressor protein, and TSG101, another corepressor [39].

De novo methylation and the methyltransferases Dnmt3a and Dnmt3b. The realization that additional DNA methyltransferases must exist arose from the observation that although Dnmt1 knockout embryos die prior to the eight-somite stage in association with widespread demethylation, Dnmt1-deficient embryonic stem cells are viable and maintain a low level of DNA methylation as well as cytosine methyltransferase activity. Furthermore, transfected proviral DNA that becomes integrated undergoes de novo methylation at the same rate as wild-type cells [40]. These findings suggested the existence of one or more de novo methyltransferases.

By screening the dbEST database with the bacterial restriction methyltransferase *M.XorI*, a second methyl-

transferase, Dnmt2, was identified [41]. However, although Dnmt2 contains all 10 of the sequence motifs characteristic of DNA (cytosine-5)-methyltransferases, it lacks the N-terminal regulatory domain of other eukaryotic methyltransferases and has no detectable methyltransferase activity *in vitro* [42]. Furthermore, embryonic stem cells deficient for Dnmt2 display normal methylation of endogenous viral sequences as well as *de novo* methylation of newly integrated retroviral DNA [42]. Given that it binds strongly to DNA through a specific target-recognizing motif, Dnmt2 may identify specific sequences within the genome for some as yet unknown biological function.

With additional EST database screening using full length bacterial type II cytosine-5 methyltransferase sequences as queries, two additional DNA methyltransferases, Dnmt3a and Dnmt3b, were discovered [43]. Both enzymes are capable of methylating unmethylated and hemimethylated DNA equally *in vitro* [43]. Structurally, both of the Dnmt3 proteins contain a catalytic domain at the C terminal, an adjacent cysteine-rich domain and a variable domain at the N terminal. The catalytic domain of both Dnmt3a and Dnmt3b contains all of the invariant amino acids in the five highly conserved domains I, IV, VI, IX and X [44]. The cysteine-rich domain is homologous to a similar domain in the X-linked ATRX gene of the SNF2/SW1 family that is known to mediate its association with the histone deacetylase HDAC1 [45].

Although Dnmt1 possesses *de novo* methylation activity *in vitro*, targeted deletion of either of the Dnmt3 genes has confirmed the essential requirement of Dnmt3a or Dnmt3b for *de novo* methylation *in vivo* [46]. Methylation of a recombinant retrovirus transfected into ES cells required either Dnmt3a or Dnmt3b, but not both, indicating redundancy of *de novo* methylation function. Although Dnmt3a knockout mice appeared normal at birth, most became runted and died at about 4 weeks of age. Dnmt3b embryos, however, died *in utero* from multiple developmental defects. Endogenous C-type retroviral DNA was normally methylated in Dnmt3a knockout mice and slightly undermethylated in Dnmt3b knockout mice. However, double-knockout mice demonstrated marked hypomethylation of these endogenous retroviral sequences. Examination of the role of Dnmt3a and Dnmt3b in the methylation of imprinted genes produced mixed results. Whereas the methylation status of the region 2 intronic CpG island of Igf2r and the 5' upstream region of H19 was unchanged in double-knockout ES cells, almost complete demethylation of DMR2, another region of the Igf2r gene, occurred in the same cells. Finally, a unique role for Dnmt3b in the methylation of juxtacentromeric minor satellite repeats was revealed in ES cells from the Dnmt3b knockout, which had substantially hypomethylated satellite repeats. This is consistent with the association of Dnmt3b mutations and the congenital disease ICF

(see below). Although the studies on *de novo* methylation of transfected retroviruses suggested redundancy of function of Dnmt3a and Dnmt3b, their unique patterns of tissue expression in the embryo suggest that they have separate functions.

The demonstration of physiological *de novo* methylation activity has largely been confined to the postimplantation embryo and primordial germ cells of midgestation embryos. With the exception of *de novo* methylation of the CD8 coreceptor gene [47], there is no evidence to date that *de novo* methylation plays a significant role in the normal regulation of gene expression apart from embryonic tissues. Similarly, little is known regarding the functions of the *de novo* methyltransferases in nonembryonic tissues. However, expression of Dnmt3a and 3b has been shown in various adult tissues [43, 48], and it is reasonable to suggest that there may be *de novo* methyltransferase activity occurring in these tissues. We have identified upregulation of both Dnmt3a and Dnmt3b during the early stages of T cell activation [authors' unpublished data]. Dnmt3a has recently been shown to associate with RP58, a DNA-binding protein with transcriptional repressor activity, and also with HDAC1 via its ATRX-homologous domain. Furthermore, Dnmt3a was shown to act as a corepressor with RP58, independent of its methyltransferase activity [45].

DNA demethylation. Very little is known about the process of genomic demethylation. That it occurs at all is clearly demonstrated in the developing embryo, in which a wave of demethylation following fertilization leads to an almost complete conversion of methylcytosines to cytosines [49, 50]. However, demethylation of DNA in adult tissue is poorly characterized and with few exceptions has not yet been clearly demonstrated in normal cells. The generally accepted view is that demethylation occurs during development when gene expression patterns in somatic cells are being laid down and genes become activated in a cell-type specific fashion. Excluding embryonic studies, most evidence for demethylation events comes from analysis of transfected genes [51]. In addition, a global loss of methylcytosines occur in many tumors as well as with ageing, as will be discussed later.

Demethylation of genomic DNA can occur in two ways. First, passive demethylation occurs only in the context of DNA replication. This is thought to be the primary mechanism of demethylation mediated by cytosine analogs such as 5-azacytidine. It has been suggested that certain DNA-binding factors may block cytosine methylation that normally occurs during S phase [52].

The second mechanism occurs independent of DNA synthesis, and involves the action of demethylases. The first evidence for demethylase activity occurred with the demonstration of demethylation activity in nuclear extracts of chicken embryos [53]. Demethylation was shown to occur through nucleotide excision repair of 5-

methyldeoxycytidine. Subsequently, the protein responsible for this activity was identified as 5-methylcytosine DNA glycosylase (5-MCDG) [54] and found to require RNA for its demethylating function [55]. Both enzyme and RNA appear to exist in a larger complex that also contains an RNA helicase and the G/T mismatch DNA glycosylase [56]. The RNA may play a role in recognition of methylated CG dinucleotides. When overexpressed in human embryonic kidney cells, 5-MCDG has recently been shown to specifically demethylate a stably integrated ecdysone-retinoic acid responsive enhancer-promoter [57]. Whether the function of 5-MCDG is primarily that of a demethylase rather than a DNA repair enzyme remains to be elucidated.

One of the methylcytosine binding proteins, MBD4, has also been shown to act as a demethylase with similar 5-methylcytosine glycosylase activity [58]. Although another methylcytosine-binding protein, MBD2, has been shown to have demethylase activity, catalyzing the direct reaction of the 5-methylcytosine moiety with water to form cytosine and methanol [59], this has not been confirmed by others [60, 61] (see below for further discussion of MBD2 and MBD4).

Relationship between DNA methylation and gene expression

Tissue-specific gene methylation. A role for DNA methylation in gene regulation was first proposed in the 1970s [62, 63], and supporting evidence has accumulated over the intervening years. Most of the early data was correlative. Methylation of the rabbit β globin gene [64] and subsequently other genes was noted to occur in a tissue-specific pattern, consistent with a role of methylation in tissue-specific gene regulation. Further studies on the tissue-specific expression of the chicken β globin [65] and ovalbumin [66] genes as well as the human β globin locus [67] demonstrated a positive correlation between hypomethylation and gene expression in different tissues. Cytosine analogs. Evidence has also come from the use of DNA methyltransferase inhibitors such as the cytosine analogs 5-azacytidine and 5-aza-2'-deoxycytidine. The earliest experiments involved treatment of mouse embryonic cells with 5-azacytidine, which gave rise to differentiated phenotypes, including muscle cells, adipocytes and chondrocytes [68]. These changes were subsequently shown to be associated with DNA hypomethylation [69]. DNA substituted with 5-azacytidine covalently binds DNA methyltransferase enzymes, leading to loss of activity and DNA hypomethylation [70]. However, the cytotoxicity of these cytosine analogs may be independent of their ability to cause DNA hypomethylation, as mice partially deficient in DNA methyltransferase activity are actually more resistant to the cytotoxic effects of 5-aza-

2'-deoxycytidine [71]. Since the original experiments with mouse embryonic cells, there have been numerous reports of the induction of new cellular phenotypes in response to treatment with cytosine analogs (reviewed in [72]), although the known inhibitory effects of these agents on cell division may have contributed to the observed phenotypic changes [73, 74]. Induction of specific gene expression by 5-azacytidine treatment was first reported for the HGPRT gene located on the inactive X chromosome [75]. This report also led to an understanding of the fundamental role that DNA methylation plays in X chromosome inactivation. Upregulation of perhaps many hundreds if not thousands of genes secondary to cytosine analog treatment have since been reported (early experiments are reviewed in [72]).

Genomic imprinting and X chromosome inactivation. In vivo evidence for the involvement of DNA methylation in gene regulation is found in the biological phenomena of genomic imprinting and X chromosome inactivation. In both, gene silencing is strongly associated with hypermethylation of CpG islands within the promoter region of silenced genes. Genomic imprinting is a process whereby only one of the two parental alleles is expressed, whereas the other gene is imprinted or silenced by DNA methylation (reviewed in [76–80]). Imprinted genes are germline derived and inherited from either the maternal or paternal gamete. About 45 imprinted genes have been identified in the mouse, and ~88% have CpG islands [81]. By generating uniparental mouse embryonic fibroblasts and treating with DNA methylation inhibitors, it was possible to induce expression of some but not all imprinted genes [82]. However, the mechanism of imprinting is quite complex and, in addition to CpG island methylation, involves insulator or boundary elements, repressor proteins such as CTCF and antisense transcripts (reviewed in [80, 83–86]). Nevertheless, DNA methylation appears to play a critical role in the regulation of these processes as well. The functional significance of genomic imprinting is unclear, but, as hypothesized in the 'conflict theory', may be a genetic mechanism to balance the in utero growth requirements of the developing fetus versus the health of the mother [87–90].

X chromosome inactivation is a related methylation-dependent phenomenon and consists of the transcriptional silencing of one of the two X chromosomes in mammalian females. This is to ensure equivalent levels of gene expression from the sex chromosomes in both males and females, i.e. gene dosage compensation (reviewed in [91–94]).

Transfection of differentially methylated genes (patch methylation). Although it has been possible to pharmacologically demethylate CG dinucleotides in DNA of cultured cells, pharmacological methylation of specific unmethylated CGs in vivo has not been feasible to date. One experimental approach used to overcome this problem is

the methylation of genes in vitro using bacterial methyltransferases and their transfection back into cells. An early example is the in vitro methylation of the hamster adenine phosphoribosyltransferase (*aprt*) gene at all CCGG sites with *HpaII* methylase and its subsequent transfection into murine L cells. Compared with cells transfected with the nonmethylated gene, methylation inhibited *aprt* expression [95]. Similarly, in vitro methylation of the human γ globin gene in its 5' region but not elsewhere inhibited its expression following transfection into mouse L cells [96]. Many similar experiments have now been performed, demonstrating a suppressive effect of DNA methylation on transfected genes. Recently, a more physiological approach was achieved by creating a transgenic mouse containing a 120-bp island element (IE) sequence that consisted of two Sp1 sites from the hamster *aprt* CpG island [97]. This sequence was able to protect itself and also CG dinucleotides up to 150 bp away from de novo methylation. By creating a loxP-flanked version of this sequence, it was possible to generate Cre-mediated deletion at either the preimplantation stage before the normal wave of de novo methylation had taken place, or later in the adult mouse. With the IE placed within a reporter gene, it was possible to show that Cre-mediated deletion prior to the wave of de novo methylation lead to persistent methylation and suppressed expression of the reporter construct, whereas deletion in the adult mouse did not lead to further methylation [97].

Mechanisms by which DNA methylation regulates gene expression

Direct inhibition of transcription factor binding. DNA methylation may suppress gene transcription in two general ways (fig. 1). First, methylated CG dinucleotides may directly interfere with the binding of transcription factors to their recognition sequences, presumably by protruding into the major DNA groove. The movement of RNA polymerase along the gene does not seem to be affected by methylation, because coding regions downstream of the promoter usually contain CG dinucleotides that are mostly methylated. This suggests that for methylated CG dinucleotides to directly inhibit transcription, they must lie in or near promoter sites or other cis-acting regulatory elements. This has been demonstrated for the binding of AP-2 to its recognition sequence within the promoter site of the proencephalin gene. Methylation of a CCGG site within the AP-2 binding sequence inhibited binding of AP-2 and is thought to explain the inhibition of expression of a proenkephalin-CAT fusion gene methylated at CCGG sites [98]. Similar inhibition has been demonstrated for the binding of ATF/CREB to the cyclic AMP (cAMP)-responsive element [99] and c-Myc

to its regulatory element [100]. However, although Sp1 binding has also been shown to be inhibited by DNA methylation [101], the presence of methylated CG within its recognition sequence did not prevent its binding to a synthetic oligonucleotide containing this methylated recognition sequence [102]. In addition to this direct interference by methylated cytosines with the binding of transcription factors to DNA, methylcytosine binding proteins can also prevent the binding of transcription factors to their promoter sequences [103].

Methylcytosine-binding proteins, histone deacetylase complexes and indirect inhibition of transcription factor binding. Second, regions of methylated DNA adjacent to the promoter sites of genes may recruit methylcytosine-binding proteins (see table 1) that in turn associate with large protein complexes containing corepressors and histone deacetylases. The binding of these complexes to DNA leads to a change in the chromatin structure from an active to an inactive form. The best-characterized methylcytosine-binding protein is MeCP2, identified by its ability to bind to DNA containing as little as a single methylated CG dinucleotide [104]. MeCP2 contains a methyl-CG binding region within an 85-amino acid domain near the N terminus [105] as well as a transcriptional-repression domain between amino acids 221 and 249 [106]. MeCP2 is concentrated in juxtacentromeric heterochromatin that contains a large fraction of all genomic methylcytosine [104, 107]. Murine MeCP2 associates with the Sin3A histone deacetylase complex, consisting of at least seven proteins including the transcriptional repressor Sin3A and histone deacetylases HDAC1 and HDAC2 [108], thus linking DNA methylation with histone deacetylation. Histone deacetylation suppresses gene expression by removing acetyl groups from histones, which in turn leads to more compact chromatin that is transcriptionally inactive (reviewed in [109]). A similar association occurs between homologous proteins in *Xenopus laevis* oocytes [110]. However, transcriptional repression by MeCP2 may also occur independent of histone deacetylases [103], suggesting that MeCP2 may directly compete with transcription factors, thereby preventing gene expression. Because of the abundance of methylated CG dinucleotides within the genome, MeCP2 may act as a global transcriptional repressor [106].

Two additional histone deacetylase complexes have been identified. These include MeCP1 [60, 111], which contains the methyl-CpG binding domain protein 2 (MBD2), the histone deacetylases HDAC1 and HDAC2, and the retinoblastoma protein-binding proteins RbAp46 and RbAp48 (also present in the Sin3A histone deacetylase complex). The latter were previously shown to copurify with histone deacetylases and may mediate core-histone binding of the histone deacetylase complex. The second histone deacetylase complex is the Mi-2 (or NuRD) complex that contains at least seven proteins, including Mi-2,

Table 1. DNA Methyltransferases and Methylcytosine-Binding Proteins.

Protein	Function	Binding Site	Deficiency/Disease States
1. DNA Methyltransferases			
Dnmt1	Maintenance of DNA methylation pattern during S phase. De novo methylation in vitro. Methylation independent inhibition of gene transcription in vitro	DNA replication foci	Embryo-lethal in muring knockout. SLE associated with reduced levels.
Dnmt2	Unknown. No detectable DNA methyltransferase activity in vitro	unknown	Normal phenotype of knockout ES cells. No known human deficiency.
Dnmt3a	De novo methylation in vivo (lost in Dnmt3a/3b double-knockout embryo) and in vitro. Methylation-independent inhibition of gene transcription in vitro	pericentromeric DNA and elsewhere in the nucleus	Death at about 4 weeks in murine knockout. No known human deficiency. Possible involvement in promoter hypermethylation in tumors.
Dnmt3b	De novo methylation in vivo (lost in Dnmt3a/3b double-knockout embryo). Methylation of pericentromeric DNA satellite repeats. Methylation-independent inhibition of gene transcription in vitro	pericentromeric DNA and elsewhere in the nucleus	Embryo-lethal in murine knockout. ICF syndrome associated with mutations in DNMT3b in humans. Possible involvement in promoter hypermethylation in tumors.
2. Methylcytosine-Binding-Proteins			
MeCP2	Suppression of gene expression in vitro and in vivo, both alone and in conjunction with Sin3A histone deacetylase complexes. May act as global suppressor of gene expression	methylated DNA; Pericentromeric satellite DNA especially rich in MeCP2; may require as many as 10 CpGs for binding	Neurological abnormalities in various murine knockout models. Rett syndrome and X-linked mental retardation associated with mutations in MeCP2 in human
MBD1	Suppression of gene expression. Probably associated with as yet unidentified histone deacetylases.	methylated DNA, especially pericentromeric satellite DNA; may require only a single CpG dinucleotide for binding	No published murine knockout. No known human deficiency.
MBD2	Suppression of gene expression. Associated with MeCP1, Sin3A and Mi2/NuRD histone deacetylase complexes. One report of demethylase activity, but not reproducible by others.	Methylated DNA, especially pericentromeric satellite DNA	Abnormal maternal behavior in murine knockout. No known human deficiency.
MBD3	Suppression of gene expression. Associated with Mi2/NuRD histone deacetylase complex.	nuclear foci, but apparently not methylated DNA	Embryo-lethal in murine knockout. No known human deficiency.
MBD4	G/T mismatch and 5-methylcytosine DNA glycosylase activity. Possibly involved in DNA demethylation.	methylated DNA, especially pericentromeric satellite DNA	No published murine knockout. No known human deficiency. Possible tumor suppressor gene, mutated in some colorectal carcinomas.

a protein believed to have chromatin remodeling activity, HDAC1, HDAC2, RbAp46, RbAp48 and also the methylcytosine-binding protein MBD3 [112]. Interestingly, MBD2 has been found in association with both Sin3A [113] and also the Mi2/NuRD complex [112], and is likely to play a role in gene repression mediated by both Sin3A and the Mi2/NuRD histone deacetylase complexes.

Dnmt1 has also been identified in a complex that contains both Mbd2 and Mbd3, binds to hemimethylated DNA and is located at the replication focus during late S phase [114]. The genes for both Mbd2 and Mbd3 have very recently undergone targeted deletion [115]. Mbd3 knockout mice die during early embryogenesis, whereas Mbd2 knockout mice appear to be largely normal except for defective maternal behavior.

In addition to MBD2 and MBD3, the MBD family of methylcytosine-binding proteins includes two further members, MBD1 and MBD4 [116, 117]. MBD1 contains a transcriptional repression domain, binds to methylcytosine-containing DNA and suppresses gene transcription at a distance from its binding site. Its ability to suppress transcription is overcome by the histone deacetylase inhibitor trichostatin A, indicating the involvement of deacetylases in gene suppression mediated by MBD1. However, MBD1 does not appear to associate with HDAC1 as do MeCP2, MBD2 and MBD3, suggesting it may differ in its mode of action [118]. Unlike the other MBD family members, MBD4 does not suppress gene transcription but functions as a G/T mismatch glycosylase activity as well as having 5-methylcytosine DNA glycosylase activity [119].

DNA methylation and the regulation of immune system genes

INF γ and other cytokine genes. Increasing evidence has emerged in recent years for a prominent role of epigenetic regulation, including DNA methylation, of immune system genes. Much effort has focused on the regulation of cytokine gene expression in the context of T-helper-cell subset generation and in particular the INF γ gene (reviewed in [120]). An early indication of the importance of DNA methylation in INF γ regulation was the finding that CG dinucleotides close to or within the gene were relatively hypomethylated in T cells relative to other tissues and cell lines [121]. Further, levels of INF γ expression correlated inversely with the degree of DNA methylation in a number of T cell lines [121]. Expression of INF γ was subsequently confirmed to correlate with the presence or absence of methylation of a CG dinucleotide within a TATA proximal regulatory element of the INF γ promoter region, with almost complete methylation of this site occurring in nonexpressing Th2 clones but partial or complete hypomethylation in Th1 clones, which express this gene. In addition, treatment of human Th2 clones with 5-azacytidine induced INF γ production [122]. Methylation analysis of three CG sites within the 5'-flanking region and first intron of the INF γ gene revealed that thymocytes, neonatal T cells and naive adult T cells had an almost completely methylated INF γ gene. This was in contrast to the relatively hypomethylated state of the INF γ gene of T cell lines that expressed INF γ at high levels [123]. A regulatory element within the INF γ promoter determining tissue-specific expression contains a CG dinucleotide that is selectively methylated in Th2 cells and other cell types that do not express INF γ [124]. In addition, methylation of this CG dinucleotide reduced binding of the transcription factors CREB, ATF-2 and Jun [124]. After TCR ligation, human naive CD4 T cells secreted INF γ and coincidentally partially demethylated a CG dinucleotide with the TATA proximal regulatory element [125]. Interestingly, both the upregulation of INF γ secretion and the associated CG demethylation were inhibited by prostaglandin E and interleukin (IL)-4. Increased INF γ gene methylation and the associated decreased expression have also been observed in CD4 T cells from tumor-bearing mice in response to treatment with phosphatidyl serine [126].

Analogous to T-cell-activation-related decreases in INF γ promoter methylation, demethylation of CG dinucleotides within the IL-4 promoter have also been shown to occur concomitantly with T cell activation under Th2- but not Th1-polarizing conditions [127]. Comparison of D5 and D10, Th1 and Th2 clones respectively, also revealed differential demethylation of the IL-4 locus in D10 cells associated with IL-4 secretion [127]. Treating naive T cells with 5-aza-2-deoxycytidine under nonpolarizing

conditions led to significantly increased numbers of IL-4-positive cells (as well as increased INF γ -positive cells) in a cell-cycle-dependent fashion [128]. Both the IL-4 and IL-5 loci were found to be differentially demethylated in a Th2 T cell clone as compared with a Th1 clone. Detailed kinetic analysis demonstrated IL-4 locus demethylation between days 4 and 7 following Th2 priming [128]. In fact, coordinate chromatin remodeling appears to occur within the whole IL-4/IL-5/IL-13 gene cluster with Th2 polarization of naive T cells, although the role of DNA methylation in this process remains to be determined [127, 129].

Finally, demethylation of CG sites within the IL-3 promoter occurs in activated CD8⁺ T cell clones, with most of the demethylation occurring at a specific CG dinucleotide 164 bp upstream of the transcriptional start site [130].

CD4 and CD8 coreceptor genes. Regulation of CD4 and CD8 coreceptor gene expression has also been associated with CG methylation. Early work from our lab demonstrated the induction of CD4 expression in CD8⁺ T cells treated with the DNA methyltransferase inhibitor 5-azacytidine [131]. Analysis of CD8 gene methylation status in thymocytes revealed progressive demethylation during the transition from CD4⁻CD8⁻ single-positive to CD4⁺CD8⁺ double-positive thymocytes [132]. Similar demethylation was observed in the CD4 gene locus during thymocyte maturation from CD4⁻CD8⁻ double-negative cells through CD4⁺CD8⁺ double-positive, CD4⁺ single-positive thymocytes and finally CD4⁺ peripheral T cells [133]. CD8 gene expression in a CD4⁺CD8⁺ T cell derivative of a CD4⁺CD8⁻ T cell line was associated with demethylation at a number of sites within the CD8 gene [134]. Examination of the CD8 β gene 5' regulatory region has also identified CG dinucleotides that are differentially demethylated in CD8 β -expressing thymocytes and peripheral T cells [135]. Recently, it was noted that continued CD8 expression is required for survival of peripheral CD8⁺ T cells. In the absence of engagement of both the T cell receptor and CD8 coreceptor, CD8 α downregulation occurs by de novo methylation of the CD8 α gene and subsequent apoptosis through a Fas-Fas ligand interaction [47]. An earlier study also reported the occurrence of de novo methylation in association with CD8 α downregulation in a T cell hybridoma [136].

Major histocompatibility complex genes. A role for DNA methylation in the regulation of MHC gene expression is more controversial, with evidence both for and against. In the case of the class II trans-activator (CIITA) gene, there is good evidence that CG methylation within its INF γ -inducible promoter IV is important in suppressing INF γ -inducible CIITA expression and therefore also the expression of major histocompatibility (MHC) class II genes in normal trophoblastic cells, which downregulate MHC expression in general to avoid immune recognition [137,

138]. As CIITA is also involved in the regulation of MHC class I genes, it is possible that methylation-induced suppression of CIITA expression may also lead to MHC class I gene downregulation in some tissues. As for the MHC class II genes themselves, there is some evidence that, at least in the case of the HLA-DR α gene, extensive methylation did not prevent transcription [139], and analysis of various melanoma cell lines treated with IFN γ [140] as well as other mononuclear cell lines [141] demonstrated no correlation between human leukocyte antigen (HLA)-DR gene expression and levels of methylation. In fact, early analysis of melanoma and other cell lines unexpectedly found a positive correlation between HLA-DR α gene methylation levels and gene expression [142, 143]. Most MHC class I genes are associated with hypomethylated CpG islands [144].

Although methylation of murine class I genes has been described, this has also been in the context of increased rather than reduced expression of the methylated gene [145, 146]. Similarly, whereas MHC class I expression is suppressed in trophoblastic tissue, there is no evidence that any of the MHC class I genes are methylated [147]. Further research on the issue of DNA methylation and MHC gene regulation would help clarify these issues, particularly as much of the research is not of recent origin.

T- and B-cell-receptor gene rearrangement. Another area of epigenetic immune gene regulation that has recently attracted considerable attention is the control of recombination events during the somatic rearrangement of T- and B-cell-receptor gene segments (reviewed in [148]). An early indication for a possible role of DNA methylation in antigen receptor regulation was the finding that the J chain gene was heavily methylated in B cell lines not expressing the J chain, whereas cell lines expressing the protein contained hypomethylated J chain genes [149]. Further work on B cells found that the degree of heavy-chain gene methylation correlated inversely with level of expression. In addition, all immunoglobulin loci were found to become increasingly demethylated with increasing B cell maturity [150].

Subsequent studies on the T cell receptor (TCR) demonstrated that the rearranged TCR- β -chain gene was highly unmethylated in mature peripheral blood T cells compared with nonexpressing B cells and monocytes [151]. Further research by the same group demonstrated that the TCR- β -chain region was hypomethylated in either CD4⁺ or CD8⁺ single-positive murine thymocytes as compared with the less mature CD4⁻CD8⁻ subset, whereas the same region was heavily methylated in macrophages [152]. Analysis of TCR methylation changes using methylation-sensitive enzymes has confirmed demethylation of the TCR- β locus during CD4⁻CD8⁻ to CD4⁺CD8⁺ thymocyte differentiation [153]. In addition, studies on the locus control region (LCR) for the TCR-

α/δ locus using germline deletions of two DNase I-hypersensitivity site clusters within the LCR suggest that these elements may mediate directed demethylation of a localized region within the LCR itself [154].

Deletion of the TCR- β enhancer leads to persistence of methylation within D β -J β clusters which normally become demethylated in developing thymocytes [155]. Finally, another mouse model containing deletion of the germline transcriptional promoter PD β 1 also demonstrates DNA hypermethylation in the D β 1-J β 1 region and reduced D β 1 rearrangement compared with normal mice [156]. In this case, methylation of a specific CG dinucleotide within one of the recombination signal sequences completely blocked the cleavage which would normally occur during VDJ recombination.

DNA methylation and disease states

Inherited disorders of methylation. With the increasing numbers of proteins known to be involved in the methylation process, it is not surprising that a number of genetic disorders have now been linked to congenital deficiencies of some of these proteins. Characteristic developmental abnormalities are a prominent feature of most of these disorders.

The ICF syndrome (immunodeficiency, centromere instability and facial anomalies) is a rare autosomal recessive disorder characterized by facial abnormalities, mental retardation and immunoglobulin deficiencies [157]. Lymphocytes from ICF patients demonstrate cytogenetic abnormalities, primarily localized to the juxtacentromeric regions of chromosomes 1, 9 and 16. Somewhat similar changes are seen when normal cells are treated with the demethylating agent 5-azacytidine [158]. These chromosomal regions are rich in tracts of classical satellites 2 and 3, which are usually heavily methylated, but are markedly hypomethylated in the ICF syndrome [159]. This disorder is now known to be associated with mutations in Dnmt3b [46, 160] and is consistent with Dnmt3b's known role in methylating juxtacentromeric satellite repeats [46]. None of the mutations leads to complete absence of the enzyme, a condition that is lethal in embryonic dnmt3b-deficient mice [46].

Another inherited condition, Rett syndrome, is one of the most common causes of female mental retardation [161]. Affected girls usually have no phenotype until 6–18 months of age, when they gradually lose speech and purposeful hand use and develop microcephaly, seizures, ataxia, episodes of hyperventilation and stereotypic hand movements. Mutations in the methylcytosine-binding protein MeCP2 are now known to occur in individuals with Rett syndrome [162]. The strongly neurodevelopmental nature of this disorder is consistent with the normally high levels of MeCP2 expression found in the

brain. Recently, MeCP2 knockout mice have been generated by targeted deletion [163, 164]. These mice develop severe neurological symptoms by about 6 weeks old, and heterozygous females also demonstrate abnormal behavior after several months. It is postulated that MeCP2 is involved in maintaining the stability of brain function rather than brain development [163].

A third condition associated with dysregulation of methylation is the ATR-X syndrome, characterized by severe, X-linked mental retardation, facial dysmorphism, urogenital abnormalities and alpha-thalassemia [165]. ATRX is a member of the SNF2 family, whose members all have similar adenosine triphosphate (ATP)ase and helicase domains. ATRX is localized to pericentromeric heterochromatin during interphase and mitosis. Mutations in the ATRX gene have been shown to be associated with alterations in the methylation patterns of a number of highly repeated sequences, including hypomethylation of ribosomal DNA arrays, hypermethylation of the Y-specific repeat DYZ2 and other subtle changes in subtelomeric repeats [166].

Defects in the imprinting process occurs in a number of developmental disorders, including Prader-Willi syndrome; the related disorder, Angelman's syndrome; and Beckwith-Wiedemann syndrome [167]. Prader-Willi syndrome is characterized by mild-to-moderate mental retardation associated with neonatal hypotonia, hypogonadism, short stature secondary to reduced growth hormone secretion, facial dysmorphism and hyperphagia [168]. The condition results from lack of transcripts expressed from the paternal copy of the imprinted chromosomal region 15q11-q13 [169]. Angelman syndrome is a similar disorder in which a deletion has occurred in the same region of chromosome 15 but on the maternal chromosome [169]. Beckwith-Wiedemann syndrome is an overgrowth condition associated with various abnormalities of the chromosome 11p15 region, a region that is known to be subject to genomic imprinting. One result is the overexpression of insulin-like growth factor 2 (IGF2) that may in part be responsible for the overgrowth [170, 171].

Finally, a number disorders arise from the expansion of trinucleotide repeats within genes. Fragile X syndrome, a common form of inherited mental retardation, is one such disorder and is associated with marked expansion of a CCG triplet within the 5'-untranslated region of the FMR1 gene on the X chromosome [172]. Such expansion occurs in germ cells and gives rise to 'anticipation' in succeeding families. The silencing of the FMR1 gene appears to occur via methylation of the expanded trinucleotide repeat as well as a CpG island within the promoter region of the gene itself, with associated histone deacetylation leading to absence of FMR1 protein [173]. Methylation and autoimmune disease. There have been few studies into the role of abnormal DNA methylation in

diseases apart from cancer. Systemic lupus erythematosus (SLE) is an autoimmune disease that primarily effects women and is characterized by increased antibody production and abnormalities in T cell function. The hypothesis that T cell DNA hypomethylation might be a factor in the etiopathogenesis of SLE originally arose from the observation in our lab that CD4+ T cells treated with 5-azacytidine become autoreactive and respond to autologous macrophages without the need for antigen [174]. In addition, 5-azacytidine-treated CD4+ T cells induce autologous B cell differentiation in the absence of any requirement for exogenous antigen [175]. The autoreactivity is due in part to the overexpression of the adhesion molecule LFA-1 (CD11a/CD18), since causing LFA-1 overexpression by transfection causes an identical autoreactivity [176]. Because of the known relationship between hydralazine and procainamide and the development of SLE, these drugs were investigated for their effects on DNA methylation, and found to inhibit DNA methylation as well as induce LFA-1 overexpression and autoreactivity in CD4+ T cells [177].

These observations led to the question whether DNA hypomethylation might be associated with human SLE and other autoimmune diseases. Supporting evidence arose from studies of SLE and rheumatoid arthritis patients which demonstrated hypomethylated DNA in T cells from these patients as compared with normal controls [178]. These findings were confirmed by another group [179]. Further evidence supporting an etiologic role for T cell DNA hypomethylation in autoimmunity came from studies in which stimulated murine CD4+ T cells were treated with DNA methylation inhibitors, including 5-azacytidine, procainamide and hydralazine, then injected into syngeneic mice. The recipients developed a lupus-like disease with anti-DNA antibodies, as well as a liver disease resembling primary biliary cirrhosis [180]. More recent work has shown that the same region flanking the CD11a promoter is demethylated in the drug treated T cells as in T cells from patients with active lupus [B.C. Richardson et al., unpublished results]. The DNA hypomethylation in lupus T cells appears to result from decreased extracellular signal-related kinase (ERK) pathway signaling, leading to decreased Dnmt1 and Dnmt3a expression [B.C. Richardson et al., unpublished results].

Methylation and cancer. Both global hypomethylation and regional hypermethylation have been recognized as characteristic of many tumors [181]. Hypomethylation has been suggested to lead to activation of protooncogenes such as H-RAS and C-MYC, as well as members of the MAGE gene family (reviewed in [182]). However, the last few years has seen a remarkable explosion in research concerning the association of CpG island hypermethylation with tumorigenesis. This interest has arisen from the observation that CpG islands within the promot-

ers of many genes are found to be hypermethylated and suppressed in a variety of tumors [183, 184].

Although the association between CpG island hypermethylation and cancer has not been clearly shown to be causal, examination of affected genes is highly suggestive of a such a relationship. Causality predicts that hypermethylation of CpG islands within the 5' region of genes with various growth suppressing functions leads to suppressed gene expression with resultant dysregulated cell growth. Genes with hypermethylated CpG islands so far include tumor suppressor genes such as the RB1 gene in retinoblastomas and other tumors [185, 186] and the von Hippel Lindau (VHL) gene in renal carcinoma [187]; genes involved in apoptosis such as the death-associated protein (DAP) in lung cancer [188]; and DNA-repair genes such as MLH1 in colorectal tumors [189, 190].

Methylation changes for a specific gene occur quite frequently for a given cancer type [184]. Both CpG island hypermethylation and mutation changes in a specific gene can be found in a particular tumor type, further supporting the argument for a causal role of DNA methylation in tumorigenesis [191]. As discussed below, methylation changes may occur in normal tissue and precede the development of cancer [192, 193].

Interestingly and very relevant for diagnostics, abnormal promoter methylation occurs in the same location within an affected gene for different individuals and regardless of the tumor type [194]. The fact that the phenomenon of CpG island hypermethylation is relatively common in a wide variety of tumors suggests that for some reason, a directed methylation event targeted to CpG islands is occurring, whose normal biological equivalent is unclear at this time.

Methylation and ageing. Age-related changes in the levels of DNA methylation within promoter regions have the potential to mediate abnormal gene expression. Such changes might contribute to tissue senescence as well as the increased incidence of malignancy that occurs with age [195]. Early studies demonstrated that global levels of DNA methylation tend to decrease with age [196–199], although there are exceptions [200]. Such changes may represent demethylation primarily of repetitive DNA sequences [197, 201, 202]. Age-related demethylation of endogenous retroviruses has also been demonstrated and may be associated with the increase in expression of these sequences that occurs with age [203]. Our group examined total methylcytosine content of human T cells and found that although an increase accompanies the differentiation of thymocytes into mature T cells, total methylcytosine content decreases with age [204].

Individual genes undergo tissue-specific methylation changes with age, such that demethylation may occur in one tissue and not another [205]. For example, the *c-Myc* gene demethylated with age in murine spleen while dis-

playing increased methylation at some CG dinucleotides in murine liver [206], although the methylation changes did not consistently correlate with transcript levels [207]. Other genes have also been found to display age-related changes in methylation [205]; however, promoter involvement and changes in gene expression have not been clearly defined.

Because of their potential to influence the level of gene expression, CpG islands within the promoters of genes which are normally hypomethylated have recently been analyzed for evidence of age-associated increases in methylation (reviewed in [208, 209]). This work arose from the observation that gene inactivation in many tumors occurs as a result of hypermethylation of CG islands within their promoters, especially those of tumor suppressor genes (reviewed in [210]). The estrogen receptor (ER) gene was first identified as being associated with age-related CpG island hypemethylation in a subpopulation of normal human colonic mucosa cells [192]. Further studies by the same group demonstrated diminished or absent ER expression in virtually all colorectal tumors examined, and introduction of an exogenous unmethylated ER gene caused marked growth suppression, suggesting that ER CpG island hypermethylation may be an earlier event in the development of colorectal tumors [192]. Further analysis of CpG island methylation in colonic tissue revealed that 19/30 (63%) of CpG islands examined had undergone progressive methylation in an age-dependent fashion [193]. Aside from the increased risk of tumor development that is associated with ageing, it remains to be seen whether hypermethylation of CpG islands is a factor in other aspects of age-related gene dysfunction.

Our group has studied the effects of a heterozygous deletion mutation of *Dnmt1* (*Dnmt1*^{+/-}) with respect to immune senescence and autoimmunity [211]. As we had already shown that inhibiting T cell DNA methylation leads to autoimmunity, we hypothesized that *Dnmt1*^{+/-} mice would develop immune senescence and autoimmune disease more rapidly than their normal littermates. Surprisingly, *Dnmt1*^{+/-} mice showed a reduced degree of age-related lymphocytic infiltration in the liver and salivary glands, as well as lower titers of anti-DNA antibodies, compared with their normal littermates. Analysis of T cells from *Dnmt1*^{+/-} mice revealed that the CD44 high-memory subset developed more slowly, IL-2 secretion was maintained in old age and T cell proliferative responses increased rather than decreased with age. Analysis of lymphocyte DNA total methylcytosine content demonstrated that although DNA from young *Dnmt1*^{+/-} mice was hypomethylated, the methylcytosine content paradoxically increased with age compared with normal littermates, whose lymphocyte methylcytosine content declined with age. Interestingly, when transcript levels for methylcytosine-binding proteins were analyzed in

brain tissue, we found that although MeCP2 showed an age-related decline in normal littermates, levels in *Dnmt1*^{+/-} mice remained unchanged.

Conclusion

Over the past few years there has been enormous progress in our understanding of the mechanisms of DNA methylation and the role that this process plays in normal cellular function as well as in certain congenital diseases, tumor development, aging and autoimmune disease. It is likely that the next few years will lead to further insights into this process, with the expectation that such knowledge will allow the development of therapeutic modalities able to manipulate methylcytosine levels in DNA or interfere with the normal downstream effects of differentially methylated DNA. Certainly, the newly identified DNA methyltransferases and methylcytosine-binding proteins will be targets for pharmacological or genetic inhibition, especially in the development of anticancer agents. However, further research will be required to understand how abnormalities of the methylation machinery lead to disease states and what components of this machinery will be appropriate targets for therapeutic intervention.

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