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The Controversial Denouement of Vertebrate DNA Methylation Research

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Abstract—The study of the biological role of DNA methylation in vertebrates has involved considerable controversy. Research in this area has proceeded well despite the complexity of the subject and the difficulties in establishing biological roles, some of which are summarized in this review. Now there is justifiably much more interest in DNA methylation than previously, and many more laboratories are engaged in this research. The results of numerous studies indicate that some tissue-specific differences in vertebrate DNA methylation help maintain patterns of gene expression or are involved in fine-tuning or establishing expression patterns. Therefore, vertebrate DNA methylation cannot just be assigned a role in silencing transposable elements and foreign DNA sequences, as has been suggested. DNA methylation is clearly implicated in modulating X chromosome inactivation and in establishing genetic imprinting. Also, hypermethylation of CpG-rich promoters of tumor suppressor genes in cancer has a critical role in downregulating expression of these genes and thus participating in carcinogenesis. The complex nature of DNA methylation patterns extends to carcinogenesis because global DNA hypomethylation is found in the same cancers displaying hypermethylation elsewhere in the genome. A wide variety of cancers display both DNA hypomethylation and hypermethylation, and either of these types of changes can be significantly associated with tumor progression. These findings and the independence of cancer-linked DNA hypomethylation from cancer-linked hypermethylation strongly implicate DNA hypomethylation, as well as hypermethylation, in promoting carcinogenesis. Furthermore, various DNA demethylation methodologies have been shown to increase the formation of certain types of cancers in animals, and paradoxically, DNA hypermethylation can cause carcinogenesis in other model systems. Therefore, there is a need for caution in the current use of demethylating agents as anti-cancer drugs. Nonetheless, DNA demethylation therapy clearly may be very useful in cases where better alternatives do not exist.

Key words: DNA methylation, cancer, vertebrates

Despite the ubiquitous nature of vertebrate DNA methylation, the history of research in this field has involved considerable controversy about its functionality. The first descriptions of 5-methylcytosine (m^5C) in eukaryotic DNA were by Hotchkiss in 1948 [1] and Wyatt in 1951 [2]. Some of the early research on the species-specific and cancer-specific distribution of DNA methylation in vertebrate tissues was pioneered by Boris Vanyushin and colleagues [3-6]. Our laboratory subsequently confirmed the tissue-specificity of genomic m^5C levels in animals [7] and demonstrated such tissue-specificity also for human specimens [8]. Given the idea that cancer represents a special kind of derangement of differentiation, we subsequently looked for and found cancer-specific differences in global DNA methylation in human tissues [9], as described below.

In 1975, critical reviews of vertebrate DNA methylation by Holliday and Pugh [10] and Riggs [11] advanced our understanding of vertebrate DNA methylation with their hypotheses about maintenance vs. *de novo* methylation and the involvement of this methylation in differentiation and X chromosome inactivation. In the late 70's and early 80's, there was an initial flurry of activity to look for associations of differential promoter or gene methylation with tissue-specific repression or stages of virus infection [12-16]. Many such associations were found although many other tissue-specific differences in DNA methylation did not correlate with expression. Even more genes, especially constitutively expressed ones, were found to always have little or no methylation in their promoter regions in a largely tissue-independent manner. This complexity and the laborious methods for determining exact patterns of methylation discouraged many investigators from continuing research in this area. For a while, the result was insufficient attention to DNA methylation. The main exception was a small nucleus of

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scientists from geographically disperse countries who concentrated their research on the fifth letter in the vertebrate genomic alphabet.

Another of the impediments to research on vertebrate DNA methylation was the oversimplified notion emphasized by Walter Gilbert in a 1985 conference [17] that methylation of the vertebrate genome is probably of little consequence to vertebrate development simply because *Drosophila* had not been proven to have DNA methylation. The reasoning was that *Drosophila*, like vertebrates, is a higher eucaryote with complicated developmental pathways, so if it could accomplish all that differentiation without DNA methylation, how can vertebrate development use DNA methylation as an important gene regulator? In the 1980's, this was an often-quoted idea despite the fact that early *Drosophila* embryos with their syncytial development are dramatically different from early vertebrate embryos and have a much smaller genome. Furthermore, it was already clear in the 1970's that despite the many common themes in molecular biology among diverse organisms, considerably different genetic pathways can yield similar biochemical outcomes. For example, very many bacterial strains use *dam* methylation (at the N^6 position of the A in GATC) to direct DNA mismatch repair as well as to regulate gene expression and the initiation of DNA replication [18, 19], but most bacterial strains do not have *dam* methylation [20, 21]. These *dam* methylation-negative bacteria can use asymmetrical nicks in the DNA generated during discontinuous DNA replication of one strand to direct mismatch repair [22, 23]. Even the premise that *Drosophila* had no genomic m^5C was disproved. Recently it was clearly demonstrated that *Drosophila* has small amounts of this methylated base in its genome although this methylation is not essential for differentiation [24-26], unlike the case for vertebrates (see below).

Vertebrate DNA methylation at transcription control regions appears to often modulate gene expression or help maintain an already established inactive state, rather than simply acting as an on-off switch. However, most methylation of vertebrate genomes is not in such transcription control elements [5, 7], and methylation of these elements does not always control gene expression *in vivo* [12-16]. Moreover, the inverse correlations between expression and methylation that are seen for many gene regulatory regions [27-29] could be consequences of changes in gene expression rather than regulators of such changes. Among the most convincing examples of changes in DNA methylation causally involved in initiating transcription control are studies of imprinted genes (see below).

One of the controversial subjects in this field is whether vertebrate DNA methylation has mainly a protective role in limiting expression of foreign DNA elements and endogenous transposons [30] or also is important in the regulation of the expression of diverse vertebrate genes involved in differentiation [27]. Studies that implicate DNA methylation in establishing tissue-specific gene expression patterns are often done with cultured cells. While extensive *in vitro* methylation of normally unmethylated promoter regions almost invariably leads to inhibition of gene expression, there is a need to mimic the same methylation pattern seen *in vivo*. This exacting requirement was accomplished in some studies [31].

Another model system for studying DNA methylation are transgenic mice with knockout of one of the DNA methyltransferase genes or hypomorphic alleles of these genes as well as embryonic fibroblasts from these mice embryos [32-40]. Complicating studies of the transgenic mice are the multiple activities of all studied vertebrate DNA methyltransferases [41-48]. This precludes conclusions about the functionality of vertebrate DNA methylation just from gene knockout experiments. However, other gene knockouts also globally affect DNA methylation [49, 50], and results from those transgenic animals can be used to complement those from the DNA methyltransferase mutants.

Another important tool for studies of the functionality of DNA methylation is the use of the methylation inhibitors 5-azacytidine or the more specific 5-azadeoxycytidine [51, 52]. A caveat for studies with these inhibitors is that their incorporation into DNA leads to DNA–protein cross-links, inhibition of DNA replication, and mutation [53, 54], as well as to DNA hypomethylation. Despite these reservations about individual experimental approaches, an overview of various kinds of studies convincingly demonstrates the biological importance of vertebrate DNA methylation to normal development [55].

DNA METHYLTRANSFERASES AND THE IMPORTANCE OF DNA METHYLATION IN VERTEBRATES

Two advances reported in 1992 greatly aided DNA methylation research. The first was a methodological breakthrough allowing much easier analysis of DNA methylation at any DNA site of interest, namely bisulfite-based genomic sequencing of m^5C residues by direct display of the methylated base [56], which also gave rise to various valuable techniques for analysis of the bisulfite-treated DNA by polymerase chain reaction (PCR) or PCR plus restriction digestion. The basis for these methods is that methylation of cytosine residues in DNA confers protection against deamination by sequential bisulfite and mild alkali treatments [57, 58].

The second advance was the finding that insertional inactivation of the most plentiful DNA methyltransferase in mammals was embryonic lethal in mice [32]. Knockout of the other two main murine DNA methyltransferase genes also leads to embryonic lethality or death soon after birth [35]. Given the necessary participation of all these genes (*DNMT1*, *DNMT3A*, and

DNMT3B) in setting and retaining DNA methylation patterns in vertebrates, it would be easy for DNA methylation to have been lost during vertebrate evolution if it did not play essential roles. Although the enzymes encoded by these genes have other non-catalytic functions, such as acting as repressors and recruiting histone deacetylases [41-48, 59], these functions were apparently secondarily acquired during evolution in the portions of the enzymes outside the catalytic domain. In contrast to the rest of the sequence, the C-terminal catalytic domain is shared by all prokaryotic and eukaryotic DNA C-5 methyltransferase genes [60].

Evidence that the DNA methyltransferase function itself is necessary for normal phenotypes comes from studies of a rare specific type of chromosome instability syndrome called ICF (immunodeficiency, centromeric region instability, facial anomalies) [61]. ICF is usually linked to mutations in both alleles of *DNMT3B* in the C-terminal portion of the protein which contains the catalytic domain. This domain is catalytically active even when the rest of the protein is deleted [62]. The mutations result in a minor decrease in overall DNA methylation, which includes large decreases in satellite DNA methylation in several of the satellite DNA sequences to which the chromosome abnormalities are targeted [61, 63, 64]. Although DNMT3B has repressor activity that is independent of its DNA methyltransferase activity, repression involves portions of the protein that do not overlap the catalytic domain [48]. DNMT3B also forms a complex with DNMT1 and DNMT3B and with small ubiquitin-like modifier 1, but these interactions involve the N-terminus of DNMT3B [65, 66]. It is in one of ten motifs conserved among all cytosine-C5 methyltransferases and present in the DNMT3B C-terminal domain that many ICF-causing missense mutations are found, which decrease DNMT3B's enzymatic activity [62]. These findings suggest that it is the loss of DNA methyltransferase activity and not some other function of the protein that is responsible for the syndrome. The involvement of DNA hypomethylation in the phenotype of ICF is supported at the cytogenetic level because the ICF-specific rearrangements in mitogen-treated lymphocytes are the same in frequency, spectrum, and chromosomal specificity as those we found in a normal pro-B lymphoblastoid cell line treated with the DNA methylation inhibitors 5-azacytidine or 5-azadeoxycytidine [67, 68].

DNA METHYLATION MODULATES EXPRESSION OF SOME VERTEBRATE GENES DURING DEVELOPMENT AND FUNCTIONS IN GENE IMPRINTING AND X-CHROMOSOME INACTIVATION

Enough thorough studies have now been reported to show that many tissue- or development-specific changes in methylation at vertebrate promoters, enhancers, or insulators regulate expression and are not simply inconsequential by-products of expression differences [55].

First, there are mechanistic links that have been established between DNA methylation and regulation of gene expression. DNA methylation can affect histone modifications and chromatin structure, which, in turn, can alter gene expression [27, 59, 69]. Increases in DNA methylation can affect chromatin structure by increasing binding of sequence-nonspecific methylated DNA binding proteins, which can recruit histone deacetylases or other proteins to down-regulate transcription [27].

Alternatively, increases or decreases in methylation of DNA sequences can alter their interactions with sequence-specific DNA binding proteins that bind less or more avidly to their CpG-containing recognition sites when those sites are methylated [70-72]. During methylation of hemimethylated DNA sequences in newly replicated DNA, a recruited DNA methyltransferase can alter transcription by interacting itself with histone deacetylases or other transcriptional repressors [59]. As described above, these interactions involve methyltransferase domains other than the catalytic domain. Methylation of special DNA elements called insulators can control long-distance interactions of chromatin in *cis* by preventing insulator activity and thereby allowing positive interactions of an enhancer on one side of the insulator with a promoter on the other side [29, 73, 74].

Although CpG-rich mammalian promoters are often constitutively hypomethylated, many show tissue-specific differences in DNA methylation [75, 76]. There is probably much overshooting in the control of establishment of tissue-specific methylation patterns so that only a small percentage of tissue-specific DNA methylation modulates gene expression. For various genes with tissue-specifically methylated promoters, there is evidence that changes in DNA methylation help regulate expression. For example, the *ALF* gene, which specifies a germ cell-specific TFIIA subunit, and a testis-specific lactate dehydrogenase gene are expressed almost exclusively in testes or germinal epithelium [77, 78]. For both of these genes, hypomethylation of the promoter in the expressed cells *in vivo* and experiments *in vitro* implicate DNA methylation in downregulation of expression or in maintaining the inactive state. Other genes that display tissue-specific promoter hypomethylation and expression and for which there is evidence that these *in vivo* methylation differences are causally involved in regulation of the gene's expression include the following: the myometrium-specific oxytocin receptor gene, the liver-specific tyrosine aminotransferase gene, the astrocyte- and astrocyte precursor-specific *GFAP* gene, and the cytokine-encoding *IFN-γ* gene [71, 79-86].

Another set of genes for which studies of animals, humans, and cultured cells clearly demonstrate a role of DNA methylation in the regulation of expression are

those on the X chromosome. DNA methylation is not necessary for establishing silencing of the inactive X chromosome (X_i). However, it seems to be important in efficiently protecting the one X chromosome that needs to stay active from inactivation and for preventing reactivation of many silenced genes on X_i once this inactivation is established [28, 87-89].

Differential DNA methylation is a critical signal for mammalian gene imprinting, which gives the mono-allelic expression of imprinted genes [29]. For most of the studied clusters of imprinted genes, one allele is very highly methylated and the other unmethylated or methylated at only a small percentage of CpGs in a 1- to 5-kb CpG-rich region (differentially methylated region, DMR). The gamete-specific differences in DMR methylation patterns, which are usually at least partially retained during embryogenesis, appear to generally be the primary imprinting mark.

Among the imprinted genes improperly expressed in *DNMT1*$^{-/-}$ mouse embryos are *H19*, whose paternal allele is normally silent, and the nearby *IGF-2*, whose maternal allele is normally silent. In these mutant embryos, the paternal *H19* allele is abnormally activated, and the reciprocally imprinted, paternal *IGF-2* allele is abnormally silenced [29, 90]. Consistent with the *DNMT1* mutation acting through its effect on DNA methylation, this mutation decreases methylation of the paternally imprinted DMR (an insulator) between *H19* and *IGF-2* in mutant embryos. Conversely, hypermethylation of this DMR on the paternal chromosome as a result of engineered strong overexpression of *DNMT1* in murine embryonic stem cells is concomitant with bi-allelic expression of *IGF-2* [40]. In humans, inappropriate methylation of this DMR in the paternal *IGF2*- and *H19*-containing imprinted gene cluster due to *cis*-acting imprinting mutations is found in certain patients with the Beckwith–Wiedemann syndrome as well as in various cancers. Accompanying this hypermethylation is bi-allelic *IGF-2* expression, resulting in abnormally high levels of its encoded mitogen and fetal growth promoting protein. Both losses and gains of methylation in DMRs may contribute to carcinogenesis *via* the resulting abnormal expression of imprinted genes, which requires alteration of only one allele for phenotypic changes [29, 91]. The demonstration of the involvement of Dnmt3L [92] in indirectly contributing to Dnmt3a-dependent DNA methylation necessary for spermatogenesis and imprinting in mice further implicates DNA methylation in gene imprinting [93]. However, unexpectedly it was reported that human DNMT3L RNA is detectable only after birth [94]. This is another example of the complexity of DNA methylation findings.

Controversial enigmas about vertebrate DNA methylation are still arising. Several labs have found that the sperm-derived male pronucleus in the zygote prior to nuclear fusion undergoes active and extensive demethylation within hours of fertilization while the female pronucleus undergoes passive demethylation (demethylation due to the absence of methylation upon DNA replication) during the early cell divisions [95]. Similar findings were obtained for human, pig, and rat embryos.

These results were interpreted as indicating a critical role for massive DNA demethylation in early mammalian embryogenesis. Such zygote- and early blastula-linked demethylation may be related to important functions for gamete-specific DNA methylation patterns, imprinting, and possible epigenetic problems associated with *in vitro* fertilization. However, sheep embryos did not show evidence of demethylation during the first post-fertilization cell cycle or after the first mitosis [95]. Nonetheless in the subsequent several cell divisions there is a considerable loss of DNA methylation although it is not as widespread as in the mouse. Also, *Xenopus* and zebra fish embryos do not display the extensive, very early genomic demethylation seen in murine embryos.

Because the studies of zygote DNA methylation rely heavily on the use of antibodies to m^5C, the question of whether asymmetric pronuclear methylation is an artifact reflecting antibody accessibility has been raised even though there are controls in these studies [95]. Despite these interspecies differences in the extent and timing of demethylation of DNA during early embryogenesis, considerable demethylation has been observed in all studied mammals and so its biological role needs to be carefully assessed.

DNA METHYLATION AND CANCER

Abnormal changes in DNA methylation postnatally are a major factor contributing to oncogenesis. Both local increases in DNA methylation and global decreases in genomic methylation are extremely common in human cancer [91]. Abnormal DNA methylation in a variety of human cancers relative to various normal somatic tissues was first described in 1983 by our laboratory in collaboration with Charles Gehrke using HPLC analysis of the total m^5C content of DNA digested to deoxynucleosides [9]. By Southern blotting with a number of gene probes, Feinberg and Vogelstein in 1983 described frequent DNA hypomethylation in colon cancer [96, 97]. Both our study and those of Feinberg and Vogelstein revealed decreases in DNA methylation in cancer (DNA hypomethylation). Earlier reports of animal studies indicated cancer-linked decreases in DNA methylation [5, 98-100] as well as cancer-linked increases [101].

Hypermethylation of CpG-rich promoter regions specifically in human cancers was first reported in 1986 for the calcitonin gene, which is probably subject to *de novo* methylation during carcinogenesis, not because it is a biologically important target, but rather, just because it is caught in a wave of *de novo* methylation of certain

CpG-rich regions [102]. Subsequently, tumor suppressor genes (TSGs) were shown to be frequently hypermethylated in human cancer [103, 104]. Many later studies provide evidence that this hypermethylation is often used to silence one or both TSG alleles during carcinogenesis in a wide variety of tumors with tumor-specific profiles for which TSG promoters are preferentially hypermethylated [105-108].

After the initial demonstrations that epigenetic inactivation of TSG is a major mechanism for TSG silencing, research on DNA methylation changes in cancer widely shifted to studying cancer-linked hypermethylation, often ignoring the many reports [9, 109-117] of cancer-linked hypomethylation. Consistent with that misleading oversimplification, many laboratories looked for increases in DNA methyltransferase mRNA or protein to explain abnormal DNA methylation in human malignancies [118-123].

Recently, it has become more widely appreciated that cancer-linked DNA hypomethylation is just as prevalent as cancer-associated DNA hypermethylation [91]. Early research on DNA methylation and cancer implicated experimentally induced hypomethylation in carcinogenesis or tumor progression [124-130]. Feeding rats and mice methyl-deficient diets resulted in hepatocarcinogenesis, global DNA undermethylation, and protooncogene demethylation although diet effects other than DNA hypomethylation could contribute to tumor formation [131-134]. However, these reports were largely overshadowed for a while by journal articles implicating DNA hypermethylation causally in carcinogenesis [135-138]. Recently reported studies on transgenic mice subject to partial loss of DNA methyltransferase activity [139-141] confirm the earlier studies implicating DNA hypomethylation causally in oncogenesis.

It had been initially demonstrated by Vanyushin's group that the highly repetitive fraction of vertebrate genomes is enriched in m^5C [5]. Studies from our laboratory, which were subsequently confirmed by other laboratories, show that tandem DNA repeats are frequently targeted for hypomethylation, sometimes to a very large extent, in a variety of human cancers [142-149]. One of our studies and a study from Itano et al. [149] revealed that hypomethylation of tandemly repeated sequences is significantly correlated with tumor progression and was an independent marker of poor survival [117, 149], like hypermethylation of gene regions [150]. It had been proposed that DNA demethylation during carcinogenesis occurs prior to *de novo* methylation, and that the functional significance of this demethylation is just that it provokes TSG methylation. Alternatively, it was hypothesized that DNA demethylation during cancer formation is just a defensive attempt to counteract cancer-linked DNA hypermethylation.

However, we have shown that global DNA hypomethylation and satellite DNA hypomethylation in Wilms tumors and ovarian epithelial cancers are not significantly associated with hypermethylation of most promoters of TSGs although both DNA hypomethylation and hypermethylation are linked to cancer [115, 151]. There are a number of possible explanations for how DNA hypomethylation can contribute to tumor formation and progression [91, 152], but the exact mechanisms are much less clear than for TSG hypermethylation and its associated gene repression [45, 153]. By whatever mechanism cancer-linked DNA hypomethylation occurs and whatever its most important biological targets, it should be more widely noted that decreases in DNA methylation induced as part of a therapeutic regimen might contribute to carcinogenesis [139, 140] or tumor progression [117, 149]. Therefore, DNA hypomethylation therapies should be used only when less risky alternatives are not available.

The reference list is necessarily incomplete, and the author apologizes for the omission of other important contributions to the field of DNA methylation.

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This review is dedicated to Boris Vanyushin, a gracious leader in the field of DNA methylation, whose research on animal DNA methylation was the impetus for my laboratory's initial studies of the tissue-specificity and then the cancer-specificity of human DNA methylation.

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