Annu. Rev. Pharmacol. Toxicol. 2002. 42:501–25

ALTERED DNA METHYLATION: A Secondary Mechanism Involved in Carcinogenesis

Jay I. Goodman and Rebecca E. Watson
Department of Pharmacology and Toxicology, Michigan State University, East Lansing, Michigan 48824; e-mail: goodman3@pilot.msu.edu, watsonr5@pilot.msu.edu

Key Words DNA methyltransferase, imprinting, maintenance methylase, 5-methylcytosine, risk assessment

■ **Abstract** This review focuses on the role that DNA methylation plays in the regulation of normal and aberrant gene expression and on how, in a hypothesis-driven fashion, altered DNA methylation may be viewed as a secondary mechanism involved in carcinogenesis. Research aimed at discerning the mechanisms by which chemicals can transform normal cells into frank carcinomas has both theoretical and practical implications. Through an increased understanding of the mechanisms by which chemicals affect the carcinogenic process, we learn more about basic biology while, at the same time, providing the type of information required to make more rational safety assessment decisions concerning their actual potential to cause cancer under particular conditions of exposure. One key question is: does the mechanism of action of the chemical in question involve a secondary mechanism and, if so, what dose may be below its threshold?

THE SECONDARY MECHANISM CONCEPT—WHY BOTHER?

In the broad sense, carcinogens may be classified as acting through a genotoxic or nongenotoxic mode of action (1, 2). A genotoxic mode is operative in those situations where the chemical or a metabolite interacts with DNA directly. Nongenotoxic carcinogens act through a mode of action that does not involve direct DNA damage. The nongenotoxic compounds can be viewed as acting through a secondary mechanism of carcinogenesis (3, 4). This term implies that the carcinogen produces a tumor response through the obligate action of a secondary factor, e.g., gastrin-mediated stomach neoplasia produced with long-acting histamine H_2 antagonists; target organ toxicity, as in bladder tumors secondary to urinary tract calculi (5); and physiologic disturbances, such as endocrine tumors demonstrated to be secondary to chemically induced hormone imbalance (6). In each of these examples, the secondary factor is responsible for the tumor response independent of the chemical per se (3). The cancer process is linked to the involvement of a key step(s) involving a biochemical/molecular alteration produced at some threshold

of exposure. In addition to the theoretical implications regarding mechanisms underlying carcinogenesis, the practical implication is that the data demonstrating that a chemical is acting by a secondary mechanism can be used to set a safe exposure level. The some-risk-at-all-dose assumption typically made for carcinogens is not required when the induction of tumors is a consequence of a classical toxic event seen only at some threshold of exposure (4).

Research aimed at discerning the mechanisms by which chemicals can transform normal cells into frank carcinomas has both theoretical and practical implications. As we increase our understanding of the mechanisms by which chemicals affect the carcinogenic process, we learn more about basic biology while, at the same time, providing the type of information required to make more rational safety assessment decisions concerning their actual potential to cause cancer under particular conditions of exposure, e.g., does the chemical in question act by a secondary mechanism and, if so, what dose may be below its threshold? This review focuses on the roles that DNA methylation plays in normal and aberrant gene expression and how, in a hypothesis-driven fashion, altered DNA methylation may be viewed as a secondary mechanism involved in carcinogenesis.

CARCINOGENESIS IS A MULTISTAGE, MULTISTEP PROCESS

Cancer cells manifest six essential alterations in cell physiology that collectively dictate malignant growth: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (7). Carcinogenesis is a multistage, multistep process consisting of at least three experimentally defined stages: initiation, promotion, and progression (8–10). The terms initiation and promotion refer to modes of action (3).

The first stage, initiation, involves a heritable alteration to the genome that facilitates the clonal expansion of initiated cells in response to a promotion stimulus. Operationally, the next stage, promotion, entails the clonal expansion of initiated cells, i.e., cells that may proliferate in response to promoter treatment. In experimental models, it is possible to demonstrate that a chemical exerts a carcinogenic effect by acting at a particular stage(s). For example, many (but not all) genotoxic chemicals have the potential to act as mutagens and are effective initiating agents. Information is abundant on mechanisms of action of genotoxic carcinogens and the role that mutations play in carcinogenesis (11). However, carcinogenesis is more than mutagenesis; many carcinogens are not mutagens and vice versa. In this context, it is instructive to note that a mismatch repair deficiency has been identified in phenotypically normal human cells. The people who donated these cells had numerous mutations in a variety of tissues but very few tumors were evident (12). Promoting agents are capable of facilitating the clonal expansion of initiated cells, potentially leading to a tumor by increasing cell proliferation and/or by decreasing apoptosis (13, 14). The potential exists for the development of spontaneous mutations (some of which may result in initiation) during periods

of cell proliferation, e.g., compensatory hyperplasia in response to necrosis (15–17) and following treatment with promoting agents. Finally, the progression stage of carcinogenesis is characterized by changes in ploidy and autonomous clonal expansion.

The experimentally based description of the three stages of carcinogenesis should not, in our view, be read as connoting that mutation only occurs during initiation, that mutation is the one and only basis for initiation, or that promotion only involves stimulating proliferation of and/or inhibiting apoptosis of initiated cells (13). In fact, heritable alterations to the genome (e.g., point mutation, rearrangements, deletions, chromosome loss and altered methylation) take place at multiple points in the carcinogenic process (18–20). Three hallmarks of carcinogenesis serve to keep our focus on the biology of the process: (*a*) the clonal evolution of tumor cell populations involves a stepwise selection of sublines that are increasingly abnormal and have a selective growth advantage over adjacent normal cells—most of the variants are eliminated (21); (*b*) operationally, the promotion stage is reversible (22); and (*c*) tumors arising in a single organ in response to treatment with a particular carcinogen frequently exhibit different phenotypes, which indicates that multiple pathways may lead to carcinogenesis (23). The numerous heritable alterations to the genome involved in the sequential clonal expansion of precancerous cells that lead to a frank malignancy are illustrated in Figure 1. This is a modification of an earlier scheme (24) that pictured mutation as the genetic mechanism underlying carcinogenesis. We employ the term "critical event" to connote the possibility of heritable epigenetic changes, e.g., altered methylation, being involved in addition to mutation.

THE IMPORTANCE OF EPIGENETIC MECHANISMS

Inheritance Should be Considered on a Dual Level

Inheritance must be considered on a dual level. That is, we should distinguish the transmission of genes from generation to generation or in the somatic sense (i.e., inheritance of DNA base sequence) from the mechanisms involved in the transmission of alternative states of gene activity following cell division. Epigenetics is the term used to describe the latter. It may be defined as the study of mechanisms responsible for the temporal and spatial control of gene activity, e.g., changes in gene expression during development, segregation of gene activities such that daughters of an individual cell have different patterns of gene expression, and mechanisms to permit the somatic inheritance of a specific set of active and quiescent genes. DNA methylation (5-methylcytosine content of DNA) is one epigenetic mechanism by which gene activity may be regulated (25).

Epigenetics and Carcinogenesis

There is, particularly among toxicologists, an excessive focus upon mutagenesis as the (read the one and only) mechanism underlying carcinogenesis. An

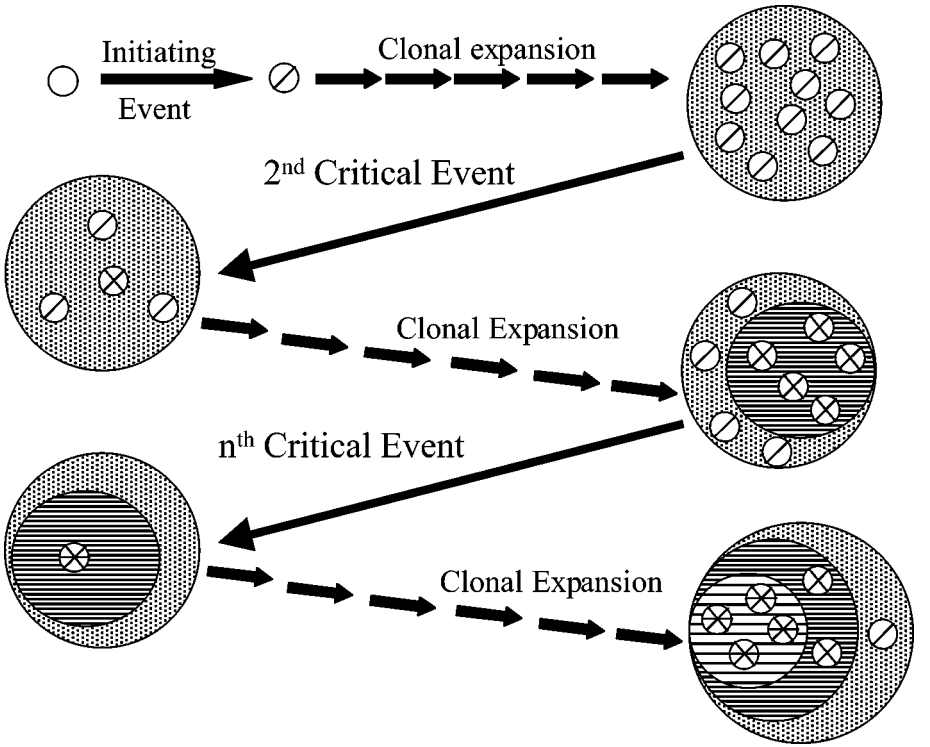


Figure 1 Initiation and cell proliferation in multistage carcinogenesis. The critical events referred to involve heritable changes in the genome. This diagram has been adapted from Swenberg et al. (24) to illustrate that epigenetic changes such as altered DNA methylation, in addition to mutation, may play a key role in carcinogenesis. Each line through a cell represents a critical event. Altered DNA methylation may be a mechanism underlying selective clonal expansion, i.e., hypomethylation may facilitate an aberrant increase in expression of oncogenes and/or hypermethylation may silence tumor suppressor genes. Either of these events could provide a cell with a selective growth advantage over the surrounding cells.

increased emphasis upon epigenetic mechanisms of carcinogenesis is appropriate (20, 26, 27). Epigenetic regulation of gene expression is based upon a modulation of transcription by heritable mechanisms superimposed on that conferred by the primary DNA sequence (28). DNA methylation is an example of such a mechanism, and altered DNA methylation may play a key role in carcinogenesis, as an epigenetic nongenotoxic mechanism (29–35).

Initiation of Carcinogenesis May Have an Epigenetic Basis

It is usually assumed that mutagenesis provides the basis for initiation. However, there could be an epigenetic basis as well. Despite the fact that many initiators of carcinogenesis are capable of acting as mutagens under certain experimental

circumstances, one does not have to assume that all of their biological effects stem from mutagenesis. Additionally, it is possible that nonmutagens may act as initiators. Hypermethylation-induced silencing of a tumor suppresser gene(s) (31, 33, 35) and/or hypomethylation-facilitated aberrant increase in expression of an oncogene(s) (31, 32) are plausible mechanisms that could underlie initiation. The involvement of epigenetics in initiation is not mutually incompatible with a role for mutation. Indeed, one or the other, or perhaps both, may play key roles depending upon the particular circumstances, e.g., causative agent, dose, target organ, and species.

Increased Gene Expression Without Mutation May Play a Key Role in Carcinogenesis

The role that mutation plays in carcinogenesis by activating proto-oncogenes to oncogenes and silencing tumor suppresser genes is appreciated widely. It is axiomatic that, in order to affect the phenotype of a cell, a mutated oncogene must be expressed. However, aberrant increased expression of nonmutated genes plays a key role as well (36). Proto-oncogene overexpression may be a mechanism of activation of the ras pathway, alternative to point mutation (37, 38). Overexpression of *myc* as well as *K-ras* can contribute to transformation (39, 40). Furthermore, overexpression of HER2/c-erbB2 receptor tyrosine kinase induces the transformed phenotype of NIH3T3 cells and is required for tumor formation and progression in nude mice (41). In this context, it is important to note that the U.S. Environmental Protection Agency's proposed Cancer Risk Assessment Guidelines include a section (section 2.3.5.3) entitled "Nonmutagenic and Other Effects," which refers explicitly to a role for altered DNA methylation as a basis for the altered gene expression involved in carcinogenesis (42).

DNA METHYLATION

DNA methylation (i.e., the 5MeC content of DNA) is an important determinant of gene activity (43). In contrast to mutation, this does not involve a change in DNA base-coding sequence, i.e., both cytosine and 5-methylcytosine base pair with guanine. Altered DNA methylation which leads to aberrant gene expression due, in part, to affecting the ability of methylated DNA-binding proteins to interact with their cognate *cis* elements (44, 45), may underlie some of the crucial changes in gene expression involved in carcinogenesis. There is a persuasive body of evidence indicating that differential methylation of DNA is a determinant of higher order chromatin structure (46) and that the methyl group provides a chemical signal recognized by transacting factors. Binding or lack of binding of these factors regulates transcription (25), e.g., by interfering with the ability of transcription factors to access their cognate *cis* elements. Thus, DNA methylation is a mechanism whereby cells can control the expression of genes with similar promoter regions in the presence of ubiquitous transcription factors (47). Transcriptional repression

is dependent upon the density of methylation; it is not simply a case of being on or off (44, 48, 49). Furthermore, there is a direct causal relationship between DNA methylation-dependent transcriptional silencing and modification of chromatin. A particular methylated DNA binding protein, MeCP2, recruits histone deacetylase, facilitating the remodeling of chromatin and transcriptional repression (50).

In light of the enzymatic steps involved, it may be expected that modifications in DNA methylation would result from threshold-exhibiting events, though this would have to be determined experimentally for each particular chemical of interest. Under normal conditions, DNA is methylated symetrically on both strands. When DNA replication occurs, 5-methylcytosine is not incorporated directly into the newly synthesized DNA strand (51). Consequently, the new double-stranded DNA contains hemimethylated sites that provide the signal for DNA maintenance methylase to transfer a methyl group from its cofactor, S-adenosylmethionine, to a cytosine residue on the newly synthesized strand. DNA methyl transferase (Dnmt1) is the maintenance methylase responsible for propagating the parental pattern of methylation in daughter cells following cell replication (52). If maintenance methylation does not occur (e.g., owing to a decrease in capacity or fidelity of DNA maintenance methylase activity and/or decreased levels of S-adenosylmethionine) and cell division followed by a second round of DNA replication takes place, then that daughter strand will give rise to double-stranded DNA that has lost a methylated site. This epigenetic change is heritable. Demethylation without DNA replication (53) and de novo methylation (54) may also occur. Additionally, demethylation not linked to DNA replication may occur through the action of a 5-methylcytosine-DNA glycosylase (55) and/or a demethylase that transforms 5-methylcytosine to cytosine (56). Dnmt 3a and 3b are the DNA methylases responsible for de novo methylation (57). Thus, maintaining a normal methylation pattern depends on the interplay between maintenance methylation following DNA replication—demethylation not linked to DNA replication and de novo methylation. A disruption of the normal methylation pattern can disrupt development (57) and affect the phenotype in a fashion that may contribute to carcinogenesis (e.g., silencing of suppressor genes and/or facilitating increased expression of oncogenes). It is important to note the potential to undo alterations in methylation through the actions of the de novo methylases and/or demethylation not linked to replication may provide a mechanism for reversal of aspects of tumor promotion. Operational reversibility is a key feature of the promotion stage of carcinogenesis (8–10). The multiple factors involved in maintaining the normal methylation status of DNA are illustrated in Figure 2.

DNA METHYLATION AND CARCINOGENESIS

Hypomethylation

Changes in DNA methylation are a consistent finding in cancer cells. Hypomethylation is an early event in carcinogenesis (58) and is observed very frequently

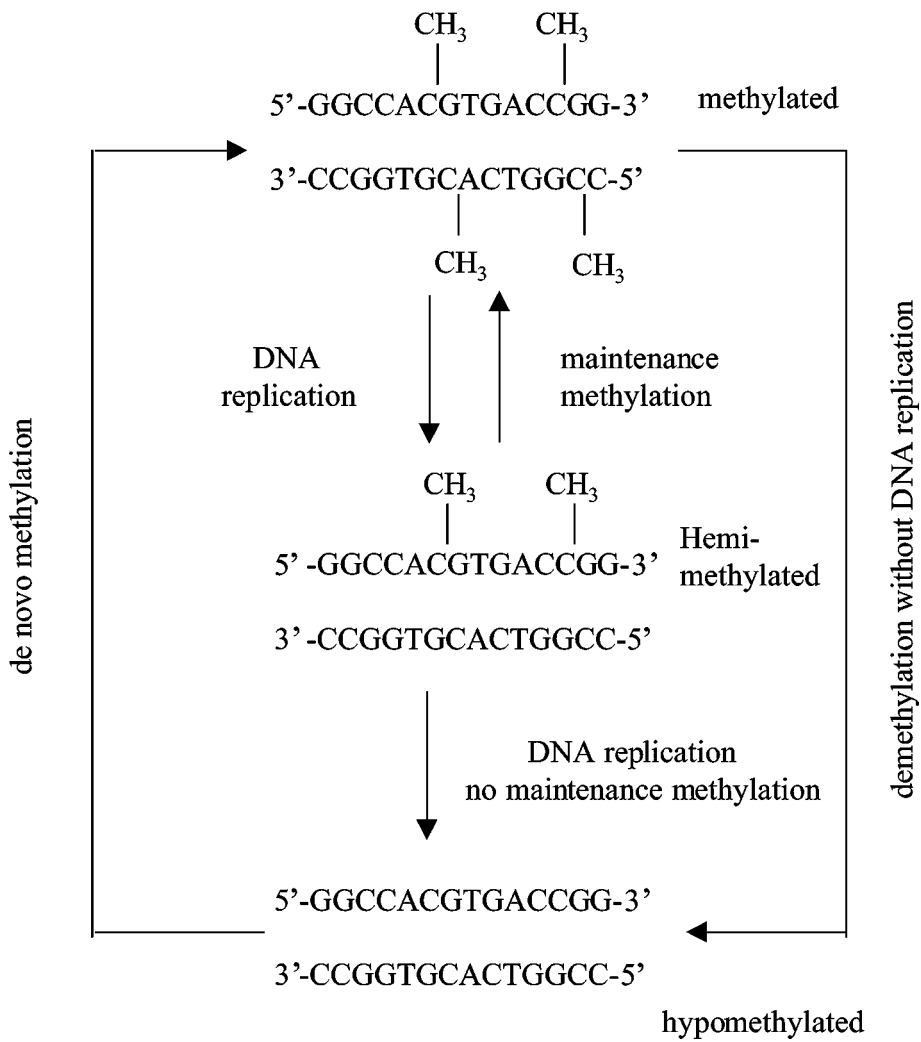


Figure 2 Maintenance of DNA methylation. Newly synthesized DNA is not methylated. Shortly after DNA replication, an S-adenosylmethionine (SAM)-requiring maintenance methylase recognizes hemimethylated sites and methylates cytosine at the 5-position to reestablish the original methylation pattern. A failure to maintenance methylate (e.g., due to decreased levels of SAM and/or inhibition of maintenance methylase during periods of cell proliferation) can result in daughter cells that contain hemimethylated DNA sites. The next round of replication can lead to cells containing hypomethylated DNA, and this is heritable. Furthermore, there are opportunities for demethylation that are not linked to DNA replication and de novo methylation, which does not require a hemimethylated signal. Adapted from Hergersberg (51). Methylcytosine residues are represented as C-CH_3.

(19, 47, 59). In addition, most metastatic human neoplasms have significantly lower genomic 5MeC than benign neoplasms or normal tissue, and the percentage of primary malignancies with hypomethylated DNA is intermediate between those of metastases and benign neoplasms (60).

Furthermore, there is a direct relationship between DNA methylation of the promoter region and gene silencing (61). Hypomethylation of a gene is necessary, but not sufficient, for its expression. Therefore, a hypomethylated gene can be considered to possess an increased potential for expression as compared to a hypermethylated gene (62). Hypomethylation may facilitate the aberrant increased gene expression that plays a role in the transformation of a normal cell into a frank malignancy. One must keep in mind the fact that, with the exception of tumor suppressor genes, a mutated gene must be expressed in order to affect phenotype. It is interesting to note two recent publications indicate that marked hypomethylation can inhibit tumorigenesis (63, 64). Since DNA methylation plays a critical role in development (65) and differentiation (30, 66), we believe it is reasonable to suggest that hypomethylation at an intermediate level does play a critical role in carcinogenesis, while excessive hypomethylation may not be compatible with the life of the affected cells (e.g., owing to exceptional deregulation of gene expression). It then follows that the dead cells cannot lead to the formation of tumors.

Decreased Availability of Methyl Groups Causes Liver Tumors in Rodents

Choline and methionine are dietary constituents that provide methyl groups for the synthesis of S-adenosylmethionine, the cofactor for methylation reactions. Diets devoid of choline and deficient in methionine (CMD diets) lead to increased hepatocyte cell proliferation (67), hypomethylation, and increased expression of oncogenes within a period of weeks to a couple of months (68). These changes are reversible when animals are returned to a control diet following several weeks of administration of a CMD diet (69, 70). Further, prolonged administration of CMD diets leads to liver tumor formation in rats (71, 72) and choline deficient diets are tumorigenic to B6C3F1 mice (73). Indeed, this appears to be the only example where removing, rather than adding, something from the diet is carcinogenic (74). The CMD diet leads to liver tumors primarily through promoting effects of the diet (75). Therefore, hypomethylation appears to be a mechanism facilitating liver carcinogenesis in rats and mice administered methyl-deficient diets.

Hypermethylation

Regional hypermethylation may inhibit transcription of tumor suppressor genes (76) and/or lead to genetic instability and loss of tumor suppressor genes (77, 78). Studies involving neural tumors (77) and renal tumors (78) indicate that regional hypermethylation may constitute a molecular change associated with genetic instability, which could lead to allelic loss of the chromosome in question and,

thus, contribute to progression as a consequence of the loss of a tumor suppressor gene(s). Additionally, hypermethylation may silence tumor suppressor genes. Progressive increases in methylation of CpG islands have been observed in bladder cancer (79) and a variety of specific tumor suppressor genes have been reported to be methylated in tumors, e.g., the retinoblastoma gene (80), $p16^{ink4a}$ (81), and $p14^{ARF}$ (82). Furthermore, inactivation of $p16^{ink4a}$ by promoter hypermethylation appears to be an early event in lung cancer (83).

Susceptibility to Carcinogenesis May be Related Inversely to the Capacity to Maintain Normal Patterns of DNA Methylation

Treatment of mice with the rodent carcinogen phenobarbital or placing them on a CMD diet results in hypomethylation in the target organ, liver, which occurs to a greater extent in the tumor-prone B6C3F1 mice as compared to the relatively resistant C57BL/6 strain (31, 70). The B6C3F1 mice appear to be defective with regard to their capacity to maintain normal methylation, and this may, in part, explain their high levels of susceptibility to liver tumorigenesis (70, 84). The observation that C57BL/6 mice have a high capacity to silence expression of transgenes by DNA methylation (85) is consistent with this view. Thus sensitivity to carcinogenesis may be related inversely to capacity to maintain normal patterns of DNA methylation. It is instructive to note that DNA methylation is more stable in human cells as compared to rodent cells (86–90). This is not surprising in light of the fact that the genome of human cells is more stable than that of rodent cells, and it is more difficult to transform human cells as compared to rodent cells (91, 92). Thus, the difference in the relative stability of DNA methylation may, in part, explain this species difference.

INTERRELATIONSHIPS BETWEEN MUTAGENESIS, GENOME STABILITY, AND ALTERED DNA METHYLATION

A role for altered methylation in carcinogenesis is not mutually exclusive with a role for mutation. On the contrary, they are not only compatible, but complementary. For example, a mutation that has a major effect on protein function may require a relatively low level of expression in order to influence phenotype; a mutation that has a minor effect on protein function may require a relatively high level of expression in order to influence phenotype; and an extremely high level of expression of a normal protein may alter the phenotype of a cell. A mutation at codon 12 of *Ha-ras* is not sufficient for transformation of a rat fibroblast cell line: at least one additional event that can lead to increased expression of the gene appears to be required (93).

Both hyper- and hypomethylation may be linked to mutagenesis. Genes that encode enzymes involved in DNA repair may be silenced by hypermethylation. Promoter hypermethylation of O^6-methylguanine DNA methyltransferase, a DNA

repair enzyme that removes mutagenic adducts from the O^6 position of guanine, is an early event in colorectal tumorigenesis and linked to the appearance of G to A point mutations in the *K-ras* oncogene (94). Furthermore, hypermethylation can silence the expression of $p14^{ARF}$ leading to a decreased sequestering of the oncoprotein MDM2 in the nucleolus, thus permitting MDM2 to target p53 for degradation and/or to repress p53-mediated transcriptional activity (82). Interference with the activity of the p53 gene and/or its encoded protein, which acts as a tumor suppressor by inducing temporary growth arrest and DNA repair or apoptosis in response to DNA damage, can facilitate mutagenesis and genome instability leading to tumorigenesis (95). However, it is not just hypermethylation that can result in increased mutation rates. Hypomethylation, too, can lead to elevated mutation rates (96) and p53-dependent apoptosis, which is evidence of genome instability (97). Most of the CpG dinucleotides in the genome are located within parasitic DNA elements or retrotransposons. DNA methylation plays a key role as a genome-defense system to silence expression of these elements and limit their spread within the genome (98). Therefore, hypomethylation may lead to mutation and genome instability by facilitating the expansion of transposable DNA elements (99).

Carcinogen-DNA adducts (100–102) or free radical DNA damage (103) can result in decreased DNA methylation. Additionally, depending on the location of a particular alkylated (O^6-methyl) guanine in relation to a potentially methylatable cytosine residue, DNA methylation may be either increased or decreased (104). Thus, a genotoxic compound may produce an epigenetic change that can persist, even though the DNA damage may be repaired. Additionally, the rate of spontaneous deamination of 5-methylcytosine (to form thymine) is greater than that of cytosine (to form uracil) and therefore, increased methylation can facilitate C:G to T:A transitions (105, 106). Furthermore, in the presence of low levels of S-adenosylmethionine, DNA methyltransferase may be able to catalyze the deamination of DNA-cytosine to form uracil, which also leads to C:G to T:A transitions (105). Thus, the high rate of mutation at CpG dinucleotides may be due, in part, to methyltransferase-facilitated deamination (107).

IMPRINTING

Methylation plays a key role in genomic imprinting, a process by which a gene is monoallelically expressed in a parent-of-origin–dependent fashion (65). Allelic differences in methylation underlie the ability to silence or activate one parental allele of an imprinted gene and not the other (108, 109). Loss of normal imprinting patterns has been associated with several human diseases including Wilms' tumor, cervical carcinoma, and Prader Willi Syndrome (110–112). The insulin-like growth factor 2 gene (*Igf2*) and the cation-independent mannose-6 phosphate/insulin growth factor II receptor gene (*M6P-Igf2r*) are imprinted in the mouse (and both of these genes play a role in tumorigenesis). *Igf2* is expressed

on the paternal allele, while *M6P-Igf2r* is expressed on the maternal allele. In humans, *Igf2* is imprinted while *M6P-Igf2r* is typically not imprinted. *M6P-Igf2r* is required for the activation of TGFβ, which inhibits cell proliferation and facilitates apoptosis. Thus, *M6P-Igf2r* may be regarded as a tumor suppressor gene involved in tumorigenesis in both rodents and humans (113, 114). *Igf2* is involved in tumorigenesis in mice at multiple sites including the liver (115, 116).

Regulation of Expression of *Igf2*

Paternal expression of *Igf2* is necessary for normal growth of the mouse (117). *Igf2* is located on chromosome 7 of the mouse and was found to be imprinted when DeChiara et al. (117) found that a particular dwarfing phenotype associated with *Igf2* was observed only when an *Igf2* nonfunctional mutant allele was paternally inherited. In addition, there is a reciprocal relationship between the paternal expression of *Igf2* and the maternal expression of the *H19* gene (118). *H19* codes for a nontranslatable RNA that may have tumor-suppressor activity (119). Results of an experiment done by Leighton et al. (118) involving a cross of *H19* $-/+$ females with *Igf2*$-/+$ males demonstrated that the growth of *H19*$-/Igf2+$ progeny after birth was increased over wild-type (*H19*$+/Igf2-$), while the growth of *H19*$+/Igf2-$ progeny after birth was decreased. Progeny with the *H19*$-/Igf2-$ genotype demonstrated a growth pattern similar to that of the wild-type mice. In mutant mice lacking methyltransferase activity, expression of *H19* increases owing to expression from the normally silenced paternal allele. This increase in *H19* occurs concurrently with a decrease in the expression of *Igf2* (65). Thus, methylation is necessary for a normal balance of *H19* and *Igf2* expression. Expression of *Igf2* and *H19* was proposed to involve a set of enhancers downstream of *H19* that have the capacity to increase expression at the promoters of both *Igf2* and *H19* (120). Access to these enhancers may be competitive; mice constructed with two such enhancers led to the loss of imprinting and exhibit bilallelic expression of both genes (120). Furthermore, through deletion analysis, an imprint control region (ICR), located approximately 2 kb upstream of *H19*, was required for normal imprinting of *Igf2*. Within the ICR are two nuclease sensitive regions, HS1 and HS2, which are methylated in the paternal but not maternal allele (121). In addition, the vertebrate enhancer blocking protein CTCF was shown to bind to the ICR only if the region is unmethylated (122).

Bell & Felsenfeld (122) proposed a model in which the *Igf2* promoter has a greater affinity than *H19* for the enhancer. Binding of CTCF to the ICR can block the enhancer from the *Igf2* promoter so that the enhancer binds to the *H19* promoter region instead. In the maternal allele, binding of CTCF to the unmethylated ICR results in the formation of a boundary between the enhancer and the *Igf2* promoter such that *Igf2* transcription is inhibited, and the enhancer acts instead at the promoter of *H19*. In the paternal allele, the ICR is methylated so that CTCF cannot bind and the enhancer can access the *Igf2* promoter, resulting in *Igf2* transcription.

Regulation of Expression of *M6P-Igf2r*

M6P-Igf2r was the second gene found to be imprinted in the mouse (123). *M6P-Igf2r* acts as a tumor suppressor, activating TGFβ to induce apoptosis, thereby halting aberrant cell proliferation (124). *M6P-Igf2r* is located on murine chromosome 17 and is maternally expressed in a reciprocal fashion with paternally expressed antisense (AS)-RNA (123, 125). The *M6P-Igf2r* region was examined for the presence of methylated regions that could play a role in the imprinting process. Two such regions were found: differentially methylated region 1 (DMR1) and differentially methylated region 2 (DMR2). DMR1 is located in the promoter region and is methylated after fertilization (126). DMR2 is an intronic 3-kb CpG island region methylated in the maternal allele at the final stages of egg maturation. Using deletion analysis, a 113-bp imprinting box within the DMR2 was shown to be necessary for imprinting, and in particular for the methylation of four *HpaII*-sensitive sites (H1–H4) within the DMR2 (126). Within this imprinting box are two *cis*-acting elements: an allele discrimination signal (ADS) in the first 6 bp of the sequence, and a de novo methylation signal (DNS), located in the last 8 bp of sequence (126). Allele-specific protein binding to these regions was identified using band-shift assays with nuclear extracts from normal, parthenogenetic, and androgenetic embryonic stem cells. An allele discriminating protein (ADP) binding to the ADS sequence was detected in normal and androgenetic stem cells but not in parthenogenetic cells. A de novo methylation signal protein (DNP) binding to the DNS sequence was detected in all three types of embryonic stem cells (126). Thus, it appears that in the paternal allele, the ADP protein binds to the ADS, inhibiting DNP binding to the DNS, so that methylation of the DMR2 region is inhibited. Under this circumstance an AS-RNA transcript may be produced that can silence *M6P-Igf2* transcription. In the maternal allele, methylation of the DMR2 region prohibits ADP binding to ADS, so DNP is free to bind to DNS, triggering methylation of the DMR2 region. AS-RNA is not transcribed, and transcription takes place (126).

The discussion of the role of methylation in imprinting serves to illustrate the point that one cannot simply say that expression of a gene is related inversely to its degree of methylation: it depends upon the methylation status of a particular region. Methylation of the promoter silences expression. However, in the case of *Igf2* and *M6P-Igf2r*, methylation of a region downstream of the promoter, the ICR and DMR2 regions, respectively, is required for expression of the imprinted allele, and failure to maintain methylation at these sites would silence expression.

CELL PROLIFERATION AND CARCINOGENESIS

Cell proliferation is a fundamental component of the multistage process of carcinogenesis (17). It plays a key role in expanding clones of initiated cells and, in addition, cell replication may contribute to carcinogenesis by facilitating mutagenesis

(15). This can occur either by causing the fixation of promutagenic DNA damage prior to repair or as a consequence of a normal error occurring during DNA replication. In addition, during periods of cell replication, there exists the possibility for heritable decreases in DNA-5MeC (i.e., hypomethylation) owing to a limitation in the capacity for and/or fidelity of DNA maintenance methylation. This event is expected to exhibit a threshold and could result in a heritable epigenetic increase in the potential for gene expression.

We do not anticipate a simple one-to-one relationship between the level of aberrant cell proliferation and the possibility for altered methylation of DNA. The ability to maintain the nascent pattern of methylation is dependent on a complex relationship between the capacity and fidelity of DNA maintenance methylase (including the accessibility of CpG regions to the enzyme), the amount of S-adenosylmethionine, and the level of cell proliferation. A role for hypomethylation leading to increased oncogene expression in tumorigenesis is not mutually exclusive with a role for mutation. In this context, it should be noted that spontaneous deamination of 5MeC to thymine often results in C:G to T:A transitions (105, 106). While methylated CpG islands can be hot spots for these point mutations, they are not all expected to function equally well in this capacity because chromatin structure can result in resistance to deamination (127). In addition, enzymatic methylation of cytosine in DNA can be altered by DNA alkylation, e.g., adjacent O^6-methylguanine residues. Loss of methylation may occur if O^6-methylguanine residues occur in CpG doublets of the newly synthesized daughter strand opposite parental hemimethylated sites during DNA replication (104, 102).

Alterations in DNA Methylation Play a Variety of Roles in Carcinogenesis

For a number of years there has been considerable interest in the role that DNA methylation plays in both normal development (128) and carcinogenesis (64, 129, 130). However, at first glance there may seem to be conflicting reports concerning the role of DNA methylation in carcinogenesis: Hypomethylation facilitates aberrant oncogene gene expression in tumorigenesis (70, 131, 132); hypermethylation leads to inactivation of tumor suppressor genes and marking chromosome regions for deletion (133). Other investigators have downplayed the importance of alterations in gene expression and favor mutation playing the key role. However, carcinogenesis is more than mutagenesis. Further, the traditional view that the key mutations in cancers stem from carcinogen-DNA adducts is too narrow. The current literature provides a compelling basis for suggesting that mutations arising secondary to deamination of 5MeC and/or C are an important source of critical point mutations. Mutation, altered gene expression, hypomethylation, and hypermethylation may all play roles in carcinogenesis; they are not mutually exclusive (31). We do not anticipate a simple one-to-one relationship between DNA methylation and cancer, mutation and cancer, or cell proliferation and cancer, nor do we anticipate all tissues to have identical mechanisms operative. In some situations

hypomethylation may be most important, in others hypermethylation, and in others mutation. Actually there is more harmony than discord here. Focusing attention singly on one mechanism may impede an overall understanding of carcinogenesis, e.g., both hypo- and hypermethylation appear to play key roles in carcinogenesis and which of the two predominates can depend upon species, target organ, and the carcinogen being evaluated. Thus, it becomes important to take a holistic approach. It is instructive to recognize that apparently disparate views can be reconciled in a fashion that provides insight regarding mechanisms underlying carcinogenesis (31). When we juxtapose the view that carcinogenesis is a multistep/multistage process that occurs in a whole animal (9) with the notion that carcinogenesis is more than mutagenesis, it becomes apparent that one should expect DNA methylation to play multiple roles in the transformation of a normal cell into a frank malignancy. There is the potential to undo alterations in methylation through the actions of the de novo methylases and/or demethylation not linked to replication. Thus, changes in methylation status could be involved in tumor promotion as operational reversibility is a key feature of this stage of carcinogenesis (8–10, 131). It is important to understand that there are multiple steps that must be traversed in order to affect a change in DNA methylation, and this is true for increased methylation leading to C:G to T:A transitions as well. Therefore, it appears likely that factors altering normal methylation patterns (e.g., carcinogen treatment) would exhibit thresholds. However, this would have to be demonstrated experimentally for individual chemicals of interest. The multiple factors that combine to regulate DNA methylation are illustrated in Figure 3, and the different ways altered methylation may facilitate carcinogenesis are illustrated in Figure 4.

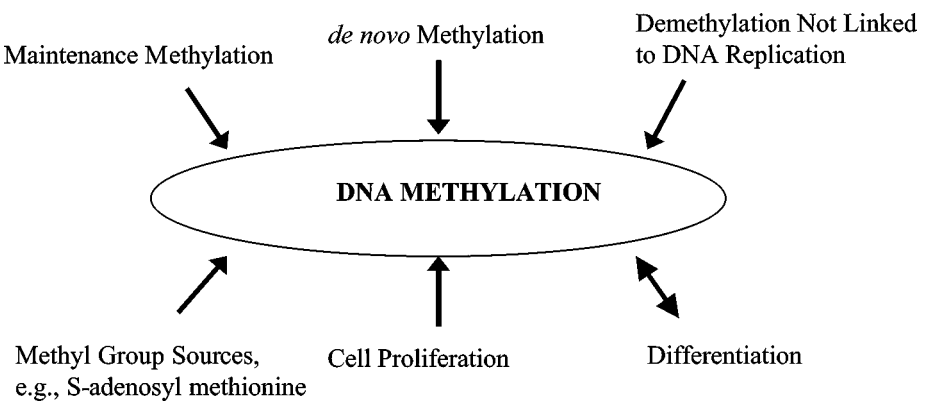


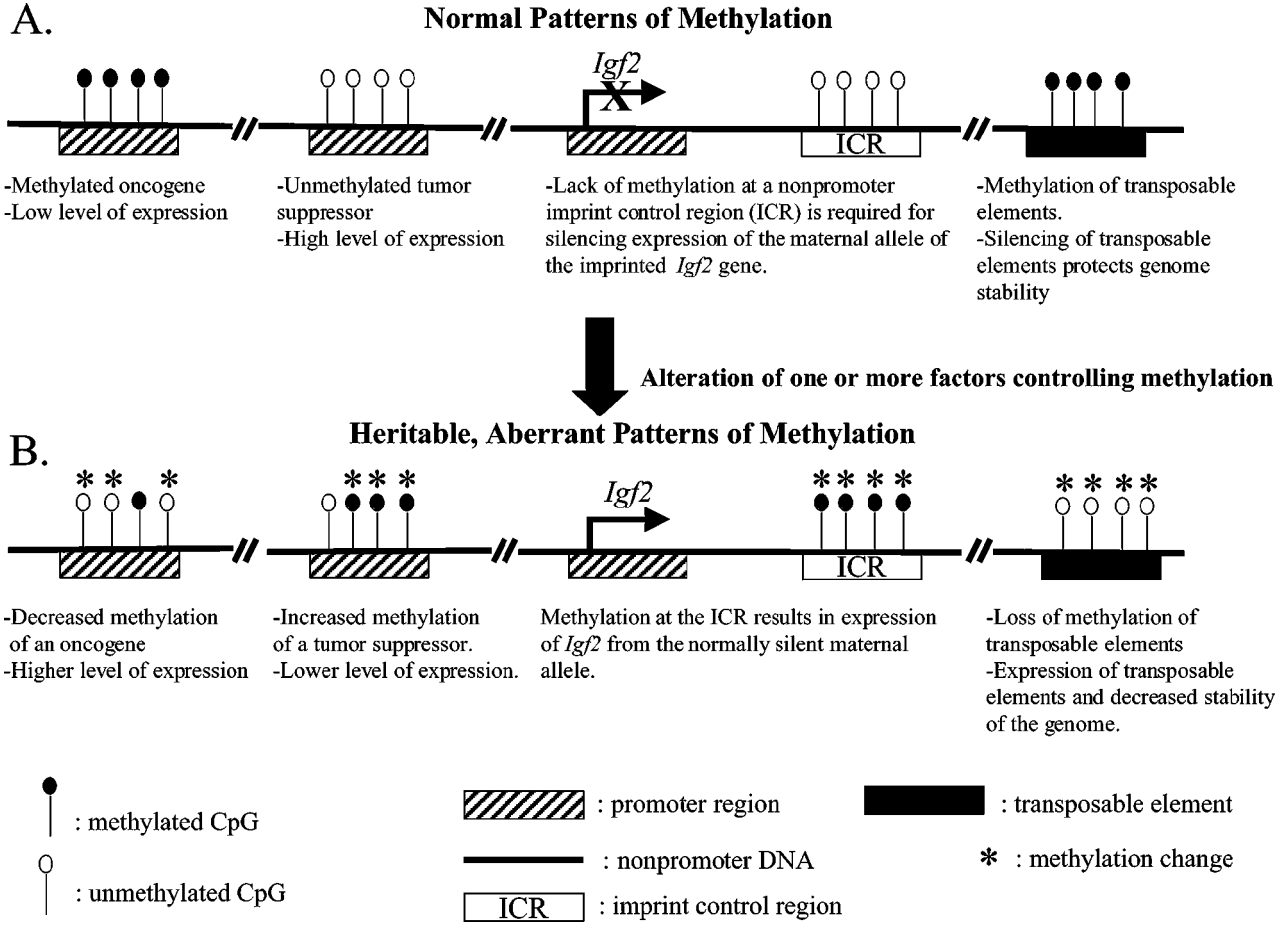
Figure 3 Multiple factors controlling DNA methylation. A particular pattern of DNA methylation is the product of multiple, interdependent factors. Alteration of one or more of these can lead to major changes in methylation status. The state of differentiation can affect methylation, and methylation status can influence the state of differentiation; thus, the double-headed arrow between methylation and differentiation.

ALTERED DNA METHYLATION SHOULD BE CONSIDERED A SECONDARY MECHANISM UNDERLYING CARCINOGENESIS

There are six basic criteria that need to be taken into account in order to accumulate a body of evidence that is sufficiently robust to confirm that a secondary mechanism underlies the ability of a particular chemical to cause cancer (adapted from 4):

1. A BIOLOGICALLY PLAUSIBLE MECHANISM. The plausibility of the proposed mechanism needs to be established experimentally. This is not intended to connote that one must be able to elucidate each and every biochemical/molecular step in the transformation process. This would entail a level of understanding of carcinogenesis that we are not likely to reach for many years. What is necessary here is a reasonable degree of certainty (not absolute certainty) that the proposed mechanism is operative, under the conditions that the chemical in question is carcinogenic.
2. A NONGENOTOXIC MECHANISM. There are experimental data indicating that genotoxic compounds, i.e., the compound itself or a metabolite of it interacts directly with DNA (1), exhibit thresholds (133a, 133b). However, in the practical sense, it is easier to picture nongenotoxic compounds acting by mechanisms exhibiting thresholds. Therefore, at the present time, we elect to limit our discussion of the secondary mechanism concept to nongenotoxic compounds.
3. EXPERIMENTAL MEASUREMENT OF A MARKER. There needs to be a marker showing that the putative mechanism is operative.
4. ESTABLISH A NO-EFFECT LEVEL. An experimental no-effect level is necessary to establish a safe dose, i.e., a dose below the threshold.
5. DATA SUPPORTING HUMAN RELEVANCE. Since there are secondary mechanisms that occur uniquely in rodents, e.g., male rat kidney tumors that arise secondarily to α2u-globulin-induced nephropathy (134, 135), it is necessary to indicate that the proposed mechanism is likely to be relevant to humans in order to base a safety assessment decision on the threshold for its occurrence.
6. DATA INDICATING THAT CARCINOGENESIS CAN BE BLOCKED BY INHIBITING THE MECHANISM. Our confidence that the proposed mechanism is essential is increased if it can be shown that inhibition of the mechanism blocks tumor formation.

Altered DNA methylation is a mechanism underlying carcinogenesis that can fit the criteria for a secondary mechanism presented above. However, altered DNA methylation per se is not indicative of carcinogenesis, e.g., a change in DNA methylation should not be employed as a short-term test for carcinogens. As discussed above, DNA methylation is a biologically plausible mechanism involved in the regulation of normal gene expression and the aberrant gene expression that leads


A.
Normal Patterns of Methylation
Igf2
ICR
-Methylated oncogene
-Low level of expression
-Unmethylated tumor suppressor
-High level of expression
-Lack of methylation at a nonpromoter imprint control region (ICR) is required for silencing expression of the maternal allele of the imprinted Igf2 gene.
-Methylation of transposable elements.
-Silencing of transposable elements protects genome stability
Alteration of one or more factors controlling methylation
B.
Heritable, Aberrant Patterns of Methylation
Igf2
ICR
-Decreased methylation of an oncogene
-Higher level of expression
-Increased methylation of a tumor suppressor.
-Lower level of expression.
Methylation at the ICR results in expression of Igf2 from the normally silent maternal allele.
-Loss of methylation of transposable elements
-Expression of transposable elements and decreased stability of the genome.
: methylated CpG
: unmethylated CpG
: promoter region
: nonpromoter DNA
ICR : imprint control region
: transposable element
* : methylation change

to carcinogenesis. Furthermore, changes in methylation status can arise through nongenotoxic mechanisms, e.g., decreased levels of S-adenosylmethionine, inhibition of the activity of one (or more) of the DNA methyltransferases. The literature pertaining to DNA methylation discussed in this review describes a variety of ways to measure DNA methylation. A no-effect level for a chemical that has the potential to alter methylation can be established experimentally. For example, tumor promoter–induced alteration in DNA methylation exhibits a clear dose-response relationship. Administration of a tumor-promoting dose of phenobarbital (PB) in the drinking water (500 ppm) for 14 days resulted in hypomethylation of *raf* in the liver of the tumor-prone B6C3F1 mouse, while the methylation status of the gene was not affected in the relatively resistant C57BL/6 mouse (132). It is important to note when B6C3F1 mice were administered drinking water containing 20 ppm PB the methylation of *raf* was not affected. Additionally, there is a substantial body of data supporting the human relevance of altered DNA methylation playing a causative role in carcinogenesis [i.e., hypomethylation (60, 56, 136–138); hypermethylation, which results in the silencing of tumor suppressor genes (79–83, 133, 139); and hypermethylation, which leads to increased cytosine to thymine point mutations (140, 141)]. Moreover, there is a considerable body of literature that indicates increased availability of methyl group donors may protect against experimental carcinogenesis (74, 142–146).

It is important that for each particular carcinogen of interest, the questions as to the role of altered DNA methylation in its mechanism of action, and whether or not altered methylation may be viewed as a secondary mechanism, should be approached in a hypothesis-driven fashion. It is not possible to pinpoint every particular step where altered methylation may play a role in carcinogenesis because much remains to be learned before we have a complete knowledge of the carcinogenic process. Is altered DNA methylation a secondary mechanism by which carcinogens act? In order to consider this, one must acquire a sound overall understanding of the ability of a carcinogen to affect relevant change(s) in methylation status in target organs under conditions that lead to cancer (e.g., dose, route of exposure).Through studying the role of altered DNA methylation in carcinogenesis,

←

Figure 4 Illustration of four possible alterations in normal patterns of methylation that may facilitate tumorigenesis. (*A*) Normal methylation patterns. (*B*) Aberrant methylation. A decrease in methylation of the promoter region of an oncogene can result in increased expression, whereas an increased methylation of the promoter region of a tumor suppressor gene can silence its expression. Altered methylation can affect imprinting. An increase in methylation of the imprint control region (ICR) of the imprinted oncogene *Igf2* may lead to expression of *Igf2* from the normally silenced maternal allele in addition to the expression that occurs normally from the paternal allele. Furthermore, a decrease in methylation of transposable elements can lead to their expression and, thus, contribute to genetic instability. Additionally, 5-methylcytosine may deaminate spontaneously to thymine, resulting in a C:G to T:A point mutation.

one may learn more about the basic mechanisms underlying carcinogenesis, which could provide information leading to more rational approaches in the assessment of the probable risk that rodent carcinogens pose to humans. Thus, research aimed at furthering our understanding of the roles that altered DNA methylation play in carcinogenesis can lead to a win-win situation.

ACKNOWLEDGMENTS

R.E.W. is the recipient of a Predoctoral Fellowship awarded by the International Life Sciences Institute, Health and Environmental Sciences Institute. Appreciation is expressed to Janice M. Thompson for help with the preparation of the manuscript.

Visit the Annual Reviews home page at www.AnnualReviews.org

LITERATURE CITED

1. Weisburger JH, Williams GM. 1981. Carcinogen testing: current problems and new approaches. *Science* 214:401–7
2. Williams GM. 1989. Methods for evaluating chemical genotoxicity. *Annu. Rev. Pharmacol. Toxicol.* 29:189–211
3. McClain RM. 1994. Mechanistic considerations in the regulation and classification of chemical carcinogens. In *Nutritional Toxicology*, ed. FN Kotsonis, M Mackey, J Hjelle, pp. 273–304. New York: Raven
4. Scheuplein RJ. 1995. The use of biological data in addition to the carcinogen bioassay in quantitative risk assessment. See Ref. 147, pp. 347–57
5. Cohen SM. 1998. Urinary bladder carcinogenesis. *Toxicol. Pathol.* 26:121–27
6. McClain RM. 1992. Thyroid gland neoplasia: non-genotoxic mechanisms. *Toxicol. Lett.* 64/65:397–408
7. Hanahan D, Weinberg RA. 2000. The hallmarks of cancer. *Cell* 100:57–70
8. Pitot HC. 1990. Altered hepatic foci: their role in murine hepatocarcinogenesis. *Annu. Rev. Pharmacol. Toxicol.* 30:465–500
9. Dragan YP, Sargent L, Xu Y-D, Xu Y-H, Pitot HC. 1993. The initiation-promotion-progression model of rat hepatocarcinogenesis. *Proc. Soc. Exp. Biol. Med.* 202:16–24
10. Trosko JE. 2001. Commentary: is the concept of "tumor promotion" a useful paradigm? *Mol. Carcinog.* 30:131–37
11. Swenberg JA. 1995. Bioassay design and MTD setting: old methods and new approaches. *Regul. Toxicol. Pharmacol.* 21:44–51
12. Parsons R, Li GM, Longley M, Modrich P, Liu B, et al. 1995. Mismatch repair deficiency in phenotypically normal human cells. *Science* 268:738–40
13. Schulte-Hermann R, Timmermann-Trosiener I, Barthel G, Bursch W. 1990. DNA synthesis, apoptosis, and phenotypic expression as determinants of growth of altered foci in rat liver during phenobarbital promotion. *Cancer Res.* 50:5127–35
14. Bursch W, Oberhammer F, Jirtle RL, Askari M, Sedivy R, et al. 1993. Transforming growth factor-β-1 as a signal for induction of cell death by apoptosis. *Br. J. Cancer* 67:531–36
15. Ames BN, Gold LS. 1990. Chemical carcinogenesis: too many rodent carcinogens. *Proc. Natl. Acad. Sci. USA* 87: 7772–76
16. Cohen SM, Ellwein LB. 1990. Cell

proliferation in carcinogenesis. *Science* 249:1007–11
17. Cohen SM, Ellwein LB. 1991. Genetic errors, cell proliferation and carcinogenesis. *Cancer Res.* 51:6493–505
18. Land H, Parada LF, Weinberg RA. 1983. Cellular oncogenes and multistep carcinogenesis. *Science* 222:771–78
19. Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, et al. 1988. Genetic alterations during colorectal-tumor development. *N. Engl. J. Med.* 319:525–32
20. Zingg J-M, Jones PA. 1997. Genetic and epigenetic aspects of DNA methylation on genome expression, evolution, mutation and carcinogenesis. *Carcinogenesis* 18:869–82
21. Nowell PC. 1976. The clonal evolution of tumor cell populations. *Science* 194:23–28
22. Pitot HC, Dragan YP. 1991. Facts and theories concerning the mechanisms of carcinogenesis. *FASEB J.* 5:2280–86
23. Dragan YP, Pitot HC. 1992. The role of the stages of initiation and promotion in phenotypic diversity during hepatocarcinogenesis in the rat. *Carcinogenesis* 13:739–50
24. Swenberg JA, Richardson FC, Boucheron JA, Deal FH, Belinsky SA, et al. 1987. High- to low-dose extrapolation: critical determinants involved in the dose response of carcinogenic substances. *Environ. Health Perspect.* 76:57–64
25. Holliday R. 1990. Mechanisms for the control of gene activity during development. *Biol. Rev.* 65:431–71
26. Klaunig JE, Kamendulis LM, Xu Y. 2000. Epigenetic mechanisms of chemical carcinogenesis. *Hum. Exp. Toxicol.* 19:543–55
27. Feinberg AP. 2001. Cancer epigenetics takes center stage. *Proc. Natl. Acad. Sci. USA* 98:392–94
28. Holliday R. 1994. Epigenetics: an overview. *Dev. Genet.* 15:453–47
29. Holliday R. 1987. DNA methylation and epigenetic defects in carcinogenesis. *Mutat. Res.* 181:215–17
30. Holliday R. 1987. The inheritance of epigenetic defects. *Science* 238:163–70
31. Counts JL, Goodman JI. 1995. Alterations in DNA methylation may play a variety of roles in carcinogenesis. *Cell* 83:13–15
32. Counts JL, Goodman JI. 1995. Hypomethylation of DNA: a possible epigenetic mechanism involved in tumor promotion. See Ref. 147, pp. 81–101
33. Baylin SB. 1997. Tying it all together: epigenetics, genetics, cell cycle, and cancer. *Science* 277:1948–49
34. Trosko JE, Chang CC, Upham B, Wilson M. 1998. Epigenetic toxicology as toxicant-induced changes in intracellular signaling leading to altered gap junctional intercellular communication. *Toxicol. Lett.* 102/103:71–78
35. Jones PA, Laird PW. 1999. Cancer epigenetics comes of age. *Nat. Genet.* 21:163–67
36. Shastry BS. 1995. Overexpression of genes in health and sickness. *Comp. Biochem. Physiol. B* 112:1–13
37. Mangues R, Kahn JM, Seidman I, Pellicer A. 1994. An overexpressed N-ras proto-oncogene cooperates with N-methylnitrosourea in mouse mammary carcinogenesis. *Cancer Res.* 54:6395–401
38. Clark GJ, Kinch MS, Gilmer TM, Burridge K, Der CJ. 1996. Overexpression of the ras-related TC21/R-ras2 protein may contribute to the development of human breast cancers. *Oncogene* 12:169–76
39. Schwab M, Alitalo K, Varmus HE, Bishop JM. 1983. A cellular oncogene (c-Ki-ras) is amplified, overexpressed, and located within karyotypic abnormalities in mouse adrenocortical tumour cells. *Nature* 303:497–501
40. Lee LW, Raymond VW, Tsao M-S, Lee DC, Earp HS, Grisham JW. 1991. Clonal cosegregation of tumorigenicity with over expression of c-myc and transforming

growth factor α genes in chemically transformed rat liver epithelial cells. *Cancer Res.* 51:5238–44
41. Baasner S, von Melchner H, Klenner T, Hilgard P, Beckers T. 1996. Reversible tumorigenesis in mice by conditional expression of the HER2/c-erbB2 receptor tyrosine kinase. *Oncogene* 13:901–11
42. US Environ. Prot. Agency. 1996. Proposed guidelines for carcinogen risk assessment. *Fed. Regist.* 61:17,960–8011
43. Razin A. 1998. CpG methylation, chromatin structure and gene silencing—a three-way connection. *EMBO J.* 17:4905–8
44. Boyes J, Bird A. 1991. DNA methylation inhibits transcription indirectly via a methyl-CpG binding protein. *Cell* 64:1123–24
45. Samiec PS, Goodman JI. 1999. Evaluation of methylated DNA binding protein-1 in mouse liver. *Toxicol. Sci.* 49:255–62
46. Lewis J, Bird A. 1991. DNA methylation and chromatin structure. *FEBS Lett.* 285:155–59
47. Jones PA, Buckley JD. 1990. The role of DNA methylation in cancer. *Adv. Cancer Res.* 54:1–23
48. Costello JF, Futscher BW, Tano K, Graunke DM, Pieper RO. 1994. Graded methylation in the promoter and body of the O^6-methylguanine DNA methyltransferase (MGMT) gene correlates with MGMT expression in human glioma cells. *J. Biol. Chem.* 269:17,228–37
49. Hsieh C-L. 1994. Dependence of transcriptional repression on CpG methylation density. *Mol. Cell Biol.* 14:5487–94
50. Jones PL, Veenstra GJC, Wade PA, Vermaak D, Kass SU, et al. 1998. Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nat. Genet.* 19:187–91
51. Hergersberg M. 1991. Biological aspects of cytosine methylation in eukaryotic cells. *Experientia* 47:1171–85
52. Bestor T, Laudano A, Mattaliano R, Ingram V. 1988. Cloning and sequencing of a cDNA encoding DNA methyltransferase of mouse cells. *J. Mol. Biol.* 203:971–83
53. Razin A, Szyf M, Kafri T, Roll M, Giloh H, et al. 1986. Replacement of 5-methylcytosine by cytosine: a possible mechanism for transient DNA demethylation during differentiation. *Proc. Natl. Acad. Sci. USA* 83:2827–31
54. Hasse A, Schultz WA, Sies H. 1992. De novo methylation of transfected CAT gene plasmid constructs in F9 mouse embryonal carcinoma cells. *Biochem. Biophys. Acta* 1131:16–22
55. Fremont M, Siegmann M, Gaulis S, Matthies R, Hess D, Jost JP. 1997. Demethylation of DNA by purified chick embryo 5-methylcytosine-DNA glycosylase requires both protein and RNA. *Nucleic Acids Res.* 25:2375–80
56. Bhattacharya SK, Ramchandani S, Cervoni N, Szyf M. 1999. A mammalian protein with specific demethylase activity for mCpC DNA. *Nature* 397:579–83
57. Okano M, Bell DW, Haber DA, Li E. 1999. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* 99:247–57
58. Goelz SE, Vogelstein B, Hamilton SR, Feinberg AP. 1985. Hypomethylation of DNA from benign and malignant human colon neoplasms. *Science* 228:187–90
59. Jones PA. 1986. DNA methylation and cancer. *Cancer Res.* 46:461–66
60. Gama-Sosa MA, Slagel VA, Trewyn RW, Oxenhandler R, Kuo KC, et al. 1983. The 5-methylcytosine content of DNA from human tumors. *Nucleic Acids Res.* 11:6883–94
61. Holliday R, Ho T. 1991. Gene silencing in mammalian cells by uptake of 5-methyldeoxycytidine-5′-triphosphate. *Somat. Mol. Genet.* 17:537–42
62. Vorce RL, Goodman JI. 1989. Altered methylation of ras oncogenes in benzidine-induced B6C3F1 mouse

liver tumors. *Toxicol. Appl. Pharmacol.* 100:398–410
63. MacLeod AR, Szyf M. 1995. Expression of antisense to DNA methyltransferase mRNA induces DNA demethylation and inhibits tumorigenesis. *J. Biol. Chem.* 270:8037–43
64. Laird PW, Jackson-Grusby L, Fazeli A, Dickinson SL, Jung WE, et al. 1995. Suppression of intestinal neoplasia by DNA hypomethylation. *Cell* 81:197–205
65. Li E, Beard C, Jaenisch R. 1993. Role for DNA methylation in genomic imprinting. *Nature* 336:362–65
66. Razin A, Cedar H. 1994. DNA methylation and genomic imprinting. *Cell* 77:473–76
67. Abanobi SE Lombardi B, Shinozuka H. 1982. Stimulation of DNA synthesis and cell proliferation in the liver of rats fed a choline-devoid diet and their suppression by phenobarbital. *Cancer Res.* 42:412–15
68. Wainfan E, Poirier LA. 1992. Methyl groups in carcinogenesis: effects on DNA methylation and gene expression. *Cancer Res.* 52(Suppl.):S2071–77
69. Christman JK, Sheikhnejad G, Dizik M, Abileah S, Wainfan E. 1993. Reversibility of changes in nucleic acid methylation and gene expression induced in rat liver by severe dietary methyl deficiency. *Carcinogenesis* 14:551–57
70. Counts JL, Sarmiento JI, Harbison ML, Downing JC, McClain RM, Goodman JI. 1996. Cell proliferation and global methylation status changes in mouse liver after phenobarbital and/or choline-devoid, methionine-deficient diet administration. *Carcinogenesis* 17:1251–57
71. Mikol YB, Hoover KL, Creasia D, Poirier LA. 1983. Hepatocarcinogenesis in rats fed methyl-deficient, amino acid-defined diets. *Carcinogenesis* 4:1619–29
72. Ghoshal AK, Farber E. 1984. The induction of liver cancer by dietary deficiency of choline and methionine without added carcinogens. *Carcinogenesis* 5:1367–70
73. Newberne PM, deCamargo JLV, Clark AJ. 1982. Choline deficiency, partial hepatectomy, and liver tumors in rats and mice. *Toxicol. Pathol.* 10:95–106
74. Rogers AE. 1993. Chemical carcinogenesis in methyl-deficient rats. *J. Nutr. Biochem.* 4:666–71
75. Sawada N, Poirier L, Moran S, Xu YH, Pitot HC. 1990. The effect of choline and methionine deficiencies on the number and volume percentage of altered hepatic foci in the presence or absence of diethylnitrosamine initiation in rat liver. *Carcinogenesis* 11:273–81
76. Greger V, Debus N, Lohmann D, Hopping W, Passarge E, Horsthemke B. 1994. Frequency and paternal origin of hypermethylated RB1 alleles in retinoblastoma. *Hum. Genet.* 94:491–96
77. Makos M, Nelkin BD, Chazin VR, Cavenee WK, Brodeur GM, Baylin SB. 1993. DNA hypermethylation is associated with 17p allelic loss in neural tumors. *Cancer Res.* 53:2715–18
78. Makos M, Nelkin BD, Reiter RE, Gnarra JR, Brooks J, et al. 1993. Regional hypermethylation at D17S5 precedes 17p structural changes in the progression of renal tumors. *Cancer Res.* 53:2719–22
79. Salem C, Liang G, Tsai YC, Coulter J, Knowles MA, Feng A-C, et al. 2000. Progressive increases in de novo methylation of CpG islands in bladder cancer. *Cancer Res.* 60:2473–76
80. Stirzaker C, Millar DS, Paul CL, Warnecke PM, Harrison J, et al. 1997. Extensive DNA methylation spanning the RB promoter in retinoblastoma tumors. *Cancer Res.* 57:2229–37
81. Myöhänen SK, Baylin SB, Herman JG. 1998. Hypermethylation can selectively silence individual $p16^{INK4a}$ alleles in neoplasia. *Cancer Res.* 58:591–93
82. Esteller M, Cordon-Cardo C, Corn PG, Meltzer SJ, Pohar KS, et al. 2001. $p14^{ARF}$ silencing by promoter hypermethylation mediates abnormal intracellular localization of MDM2. *Cancer Res.* 61:2816–21
83. Belinsky SA, Nikula KJ, Palmisano WA,

Michels R, Saccomanno G, et al. 1998. Aberrant methylation of $p16^{INK4a}$ is an early event in lung cancer and a potential biomarker for early diagnosis. *Proc. Natl. Acad. Sci. USA* 95:11,891–96

84. Counts JL, McClain RM, Goodman JI. 1997. Comparison of effect of tumor promoter treatments on DNA methylation status and gene expression in B6C3F1 and C57BL/6 mouse liver and in B6C3F1 mouse liver tumors. *Mol. Carcinog.* 18:97–106
85. Schumacher A, Koetsier PA, Hertz J, Doerfler W. 2000. Epigenetic and genotype-specific effects on the stability of de novo imposed methylation patterns in transgenic mice. *J. Biol. Chem.* 275:37,915–21
86. Wilson VL, Jones PA. 1983. DNA methylation decreases in aging but not in immortal cells. *Science* 220:1055–57
87. Wareham KA, Lyon MF, Glenister PH, Williams ED. 1987. Age related reactivation of an X-linked gene. *Nature* 327:725–27
88. Migeon BR, Axelman J, Beggs AH. 1988. Effect of ageing on reactivation of the human X-linked HPRT locus. *Nature* 335:93–96
89. Holliday R. 1989. X-chromosome reactivation and ageing. *Nature* 337:311
90. Ono T, Yamamoto S, Kurishita A, Yamamoto K, Yamamoto Y, et al. 1990. Comparison of age-associated changes of c-myc gene methylation in liver between man and mouse. *Mutat. Res.* 237:239–46
91. Holliday R. 1996. Neoplastic transformation: the contrasting stability of human and mouse cells. *Cancer Surv.* 28:103–15
92. Balmain A, Harris CC. 2000. Carcinogenesis in mouse and human cells: parallels and paradoxes. *Carcinogenesis* 21:371–77
93. Finney RE, Bishop JM. 1993. Predisposition to neoplastic transformation caused by gene replacement of H-ras1. *Science* 260:1524–27
94. Esteller M, Toyota M, Sanchez-Cespedes M, Capella G, Peinado MG, et al. 2000. Inactivation of the DNA repair gene O^6-methylguanine-DNA methyltransferase by promoter hypermethylation is associated with G to A mutations in K-ras in colorectal tumorigenesis. *Cancer Res.* 60:2368–71
95. Sigal A, Rotter V. 2000. Oncogenic mutations of the p53 tumor suppressor: the demons of the guardian of the genome. *Cancer Res.* 60:6788–93
96. Chen RZ, Pettersson U, Beard C, Jackson-Grusby L, Jaenisch R. 1998. DNA hypomethylation leads to elevated mutation rates. *Nature* 395:89–93
97. Jackson-Grusby L, Beard C, Possemato R, Tudor M, Fambrough D, et al. 2001. Loss of genomic methylation causes p53-dependent apoptosis and epigenetic deregulation. *Nat. Genet.* 27:31–39
98. Yoder JA, Walsh CP, Bestor TH. 1997. Cytosine methylation and the ecology of intragenomic parasites. *Trends Genet.* 13:335–40
99. Robertson KD, Wolffe AP. 2000. DNA methylation in health and disease. *Nat. Rev.* 1:11–19
100. Wilson VL, Jones PA. 1983. Inhibition of DNA methylation by chemical carcinogens in vitro. *Cell* 32:239–46
101. Wilson VL, Smith RA, Longoria J, Liotta MA, Harper CM, Harris CC. 1987. Chemical carcinogen-induced decreases in genomic 5-methyldeoxycytidine content of normal bronchial epithelial cells. *Proc. Natl. Acad. Sci. USA* 84:3298–301
102. Hepburn PA, Margison GP, Tisdale MJ. 1991. Enzymatic methylation of cytosine in DNA is prevented by adjacent O^6-methylguanine residues. *J. Biol. Chem.* 266:7985–87
103. Weitzman SA, Turk PW, Milkowski DH, Kozlowski K. 1994. Free radical adducts induce alterations in DNA cytosine methylation. *Proc. Natl. Acad. Sci. USA* 91:1261–64

104. Tan N-W, Li BF. 1990. Interaction of oligonucleotides containing 6-O-methylguanine with human DNA (cytosine-5-)-methyltransferase. *Biochemistry* 29: 9234–40
105. Shen JC, Rideout WD, Jones PA. 1992. High frequency mutagenesis by a DNA methyltransferase. *Cell* 71:1073–80
106. Magewu AN, Jones PA. 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.* 14:4225–32
107. Laird PW, Jaenisch R. 1994. DNA methylation and cancer. *Hum. Mol. Genet.* 3:1487–95
108. Jaenisch R. 1997. DNA methylation and imprinting: why bother? *Trends Genet.* 13:436–43
109. Reik W, Walter J. 1998. Imprinting mechanisms in mammals. *Curr. Opin. Genet. Dev.* 8:154–64
110. Huff V. 1998. Wilms Tumor genetics. *Am. J. Med. Genet.* 79:260–67
111. Douc-Rasy S, Barrois M, Fogel S, Ahomadegbe JC, Stehelin D, et al. 1996. High incidence of loss of heterozygosity and abnormal imprinting of H19 and IGF2 genes in invasive cervical carcinomas. Uncoupling of H19 and IGF2 expression and biallelic hypomethylation of H19. *Oncogene* 12:423–30
112. Ming J, Blagowidow N, Knoll J, Rollings L, Fortina P, et al. 2000. Submicroscopic deletion in cousins with Prader-Willi syndrome causes a grandmatrilineal inheritance pattern: effects of imprinting. *Am. J. Med. Genet.* 92:19–24
113. De Souza AT, Yamada T, Mills JJ, Jirtle RL. 1997. Imprinted genes in liver carcinogenesis. *FASEB J.* 11:60–67
114. Mills JJ, Falls JG, De Souza AT, Jirtle RJ. 1998. Imprinted M6p/Igf2 receptor is mutated in rat liver tumors. *Oncogene* 16:2797–802
115. Rogler CE, Yang D, Rossetti L, Donohoe J, Alt E, et al. 1994. Altered body composition and increased frequency of diverse malignancies in insulin-like growth factor-II transgenic mice. *J. Biol. Chem.* 269:13,779–84
116. Westley BR, May FEB. 1995. Insulin-like growth factors: the unrecognized oncogenes. *Br. J. Cancer* 72:1065–66
117. DeChiara TM, Robertson EJ, Efstratiadis A. 1991. Parental imprinting of the mouse insulin-like growth factor gene. *Cell* 64:849–59
118. Leighton P, Ingram R, Eggenschwiler J, Efstratiadis A, Tilghman S. 1995. Disruption of imprinting caused by deletion of the H19 gene region in mice. *Nature* 375:34–39
119. Hao Y, Crenshaw T, Moulton T, Newcomb E, Tycoko B. 1993. Tumor suppressor activity of H19 RNA. *Nature* 365:764–67
120. Webber A, Ingram RS, Levorse JM, Tilghman SM. 1998. Location of enhancers is essential for the imprinting of H19 and Igf2 genes. *Nature* 391:711–15
121. Thorvaldsen JL, Duran KL, Bartolomei MS. 1998. Deletion of the H19 differentially methylated domain results in loss of imprinted expression of H19 and Igf2. *Genes Dev.* 12:3693–702
122. Bell A, Felsenfeld G. 2000. Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene. *Nature* 405:482–85
123. Barlow D, Stoger R, Herrmann BG, Saito K, Schweifer N. 1991. The mouse insulin-like growth factor type-2 receptor is imprinted and closely linked to the Tme locus. *Nature* 349:84–87
124. Jirtle RL, Hankins GR, Reisenbichler H, Boyer IJ. 1994. Regulation of mannose 6-phosphate/insulin-like growth factor-II receptors and transforming growth factor beta during liver tumor promotion with phenobarbital. *Carcinogenesis* 15:2763–67
125. Wutz A, Smrzka O, Schweifer N, Schellander K, Wagner E, Barlow D. 1997. Imprinted expression of the Igf2r gene

depends on an intronic CpG island. *Nature* 389:745–49
126. Birger Y, Shemer R, Perk J, Razin A. 1999. The imprinting box of the mouse Igf2r gene. *Nature* 397:84–88
127. Adams RLP, Eason R. 1984. Increased G + C content of DNA stabilizes methyl CpG dinucleotides. *Nucleic Acids Res.* 12:5869–77
128. Razin A, Kafri T. 1994. DNA methylation from embryo to adult. *Progr. Nucleic Acids Res. Mol. Biol.* 48:53–81
129. Laird PW. 1997. Oncogenic mechanisms mediated by DNA methylation. *Mol. Med. Today* 3:223–29
130. Robertson KD, Jones PA. 2000. DNA methylation: past, present and future directions. *Carcinogenesis* 21:461–67
131. Goodman JI, Counts JL. 1993. Hypomethylation of DNA: a possible nongenotoxic mechanism underlying the role of cell proliferation in carcinogenesis. *Environ. Health Perspect.* 101(Suppl. 5):169–72
132. Ray JS, Harbison ML, McClain RM, Goodman JI. 1994. Alterations in the methylation status and expression of the raf oncogene in phenobarbital-induced and spontaneous B6C3F1 mouse liver tumors. *Mol. Carcinog.* 9:155–66
133. Herman JG, Merlo A, Mao L, Lapidus RG, Issa JP, et al. 1995. Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers. *Cancer Res.* 55:4525–30
133a. Williams GM, Iatropoullos MJ, Wang CX, Jeffrey AM, Thompson S, et al. 1998. Nonlinearities in 2-acetylaminofluorene exposure responses for genotoxic and epigenetic effects leading to initiation of carcinogenesis in rat liver. *Toxicol. Sci.* 45:152–61
133b. Williams GW, Iatropoulos MJ, Jeffery AM, Luo FQ, Wang CX, et al. 1999. Diethylnitrosamine exposure-responses for DNA ethylation, hepatocellular proliferation, and initiation of carcinogenesis in rat liver display non-linearities and thresholds. *Arch. Toxicol.* 73:394–402
134. Lehman-McKeeman LD, Rivera-Torres MI, Caudill D. 1990. Lysosomal degradation of α-$_{2u}$-globulin and α-$_{2u}$-globulin-xenobiotic conjugates. *Toxicol. Appl. Pharmacol.* 103:539–48
135. Dietrich DR, Swenberg JA. 1991. The presence of α-$_{2u}$-globulin is necessary for d-limonene promotion of male rat kidney tumors. *Cancer Res.* 51:3512–21
136. Kim YI, Giuliano A, Hatch KD, Schneider A, Nour MA, et al. 1994. Global DNA hypomethylation increases progressively in cervical dysplasia and carcinoma. *Cancer* 74:893–99
137. Cravo M, Pinto R, Fidalgo P, Chaves P, Gloria L, et al. 1996. Global DNA hypomethylation occurs in the early stages of intestinal type gastric carcinoma. *Gut* 39:434–38
138. Gloria L, Cravo M, Pinto A, de Sousa LS, Chaves P, et al. 1996. DNA hypomethylation and proliferative activity are increased in the rectal mucosa of patients with long-standing ulcerative colitis. *Cancer* 78:2300–6
139. Herman JG, Civin CI, Issa J-PJ, Collector MI, Sharkis SJ, Baylin SB. 1997. Distinct patterns of inactivation of $p15^{INK4B}$ and $p16^{INK4A}$ characterize the major types of hematological malignancies. *Cancer Res.* 57:837–41
140. Schmutte C, Yang AS, Nguyen TT, Beart RW, Jones PA. 1996. Mechanisms for the involvement of DNA methylation in colon carcinogenesis. *Cancer Res.* 56:2375–81
141. Yang AS, Gonzalgo ML, Zingg J-M, Millar RP, Buckley JD, Jones PA. 1996. The rate of CpG mutation in Alu repetitive elements within the p53 tumor suppressor gene in the primate germline. *J. Mol. Biol.* 258:240–50
142. Newberne PM, Suphiphat V, Loeniskar M, de Camargo JL. 1990. Inhibition of hepatocarcinogenesis in mice by dietary

methyl donors methionine and choline. *Nutr. Cancer* 14:175–81
143. Pascale RM, Marras V, Simile MM, Daino L, Pinna G, et al. 1992. Chemoprevention of rat liver carcinogenesis by S-adenosyl-L-methionine: a long-term study. *Cancer Res.* 52:4979–86
144. Pascale RM, Simile MM, Feo F. 1993. Genomic abnormalities in hepatocarcinogenesis. Implications for a chemopreventive strategy. *Anticancer Res.* 13:1341–56
145. Simile MM, Saviozzi M, De Miglio MR, Muroni MR, Nufris A, et al. 1996. Persistent chemopreventive effect of S-adenosyl-L-methionine on the development of liver putative preneoplastic lesions induced by thiobenzamide in diethylnitrosamine-initiated rats. *Carcinogenesis* 17:1533–37
146. Kim YI, Salomon RN, Graeme-Cook F, Choi SW, Smith DE, et al. 1996. Dietary folate protects against the development of macroscopic colonic neoplasia in a dose response manner in rats. *Gut* 39:732–40
147. McClain RM, Slaga TJ, LeBoeuf RA, Pitot HC, eds. 1995. *Growth Factors and Tumor Promotion: Implications for Risk Assessment*. New York: Wiley-Liss