Altered methylation patterns in cancer cell genomes: Cause or consequence?

Stephen Baylin[1,3] and Timothy H. Bestor[2,3]

[1]The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore, Maryland 21231
[2]Department of Genetics and Development, College of Physicians and Surgeons of Columbia University, 701 West 168th Street, New York, New York 10032
[3]Correspondence: sbaylin@jhmi.edu (S.B.); thb12@columbia.edu (T.B.)

CpG islands are associated with at least half of all cellular genes and are normally methylation-free. Dense methylation of cytosine residues within islands causes strong and heritable transcriptional silencing. Such silencing normally occurs almost solely at genes subject to genomic imprinting or to X chromosome inactivation. Aberrant methylation of CpG islands associated with tumor suppressor genes has been proposed to contribute to carcinogenesis. However, questions of mechanisms underlying the cancer changes and the precise consequences for tumorigenesis exist in the field, and must continue to be addressed before the importance of abnormalities in genomic methylation patterns in carcinogenesis can be fully understood. In this article, two workers in DNA methylation, one concentrating on cancer biology and the other on developmental biology, address recurrent questions about cancer epigenetics from different perspectives. The goal is to highlight important controversies in the field which can be productive targets of ongoing and future research.

The past several years have seen a surge of interest in the area of epigenetics in general, and in particular the position of this mode of heritable gene expression states in cancer. Our understanding of chromatin with respect to the components that specify for states of gene expression is growing rapidly, and this body of knowledge is establishing a base from which to understand abnormal as well as normal gene expression events. In this regard, an especially active field in cancer research is concerned with patterns of aberrant gene promoter hypermethylation that have been associated with loss of transcription of a growing list of genes in virtually every type of human cancer (Jones and Laird, 1999; Baylin and Herman, 2000). Much evidence has accrued, particularly for classic tumor suppressor genes, to solidify the concept that this heritable alteration in gene expression states constitutes an alternative mechanism to coding region mutations, or genetic alterations, for providing loss of tumor suppressor gene function in cancer.

Despite the above, it is perhaps a good time to reflect upon what we know and do not know about the impact of epigenetic changes in cancer. As publication after publication accrues reflecting the remarkable interest and research activity on the role of epigenetic abnormalities in tumorigenesis, it is important to realize that areas of potential confusion, and healthy skepticism, exist, and must be given due attention. These revolve around the precise functional implications for these changes in actually driving tumor progression. Are some, or even many, altered epigenetic states simply epiphenomena that are downstream from other changes that really drive tumorigenesis? Does the promoter hypermethylation actually initiate the states of gene silencing, or is this, again, a response pattern to a more fundamental problem? These are some of the questions posed.

The present piece is aimed at eliciting dialog surrounding the continued debate about the position of epigenetic abnormalities in the biology of cancer. Stephen Baylin, the cancer epigeneticist, operates from the stance of the hypothesis that epigenetic changes are as important as genetic ones in tumorigenesis and that they complement one another in fueling the process. However, he considers viewing the areas of potential confusion and skepticism as very constructive processes. For it is the raising of, and careful response to, these issues that can serve to refine the experimental approaches which are the only way to verify or refute the hypotheses involved—and, most importantly, which may generate the approaches and accompanying data that teach us how to translate the growing body of findings into means to better manage the diseases that continue to ravage humankind.

Timothy Bestor, the geneticist and epigeneticist, particularly for studies of developmental biology, remains more skeptical about the process, at least from a perceived lack of hard genetic data to substantiate its importance and causes.

The format of this essay will be to first pose a series of questions regarding the areas that biologists, geneticists, and cancer researchers find confusing about reported epigenetic alterations in cancer. In some instances, these concerns reflect their skepticism about epigenetic changes as fundamental steps in tumorigenesis. It is hoped that in discussing these questions, which are substantial and important for ongoing research, workers in the field, and those watching the field, will benefit in the educational sense. In this vein, it is also understood that the goal will not be to "answer" the questions. Rather, in briefly reflecting upon them, sometimes debating them from the perspective of existing data, hopefully both authors and readers will benefit.

The first point discussed is more of a caution that has been raised rather than a question. One danger in the field may be a general reliance on cultured tumor cells, whose genomic methylation patterns are known to rapidly diverge from those found in primary tumor cells. It is methylation abnormalities in primary material that are relevant; methylation differences found in cultured cells that are not demonstrated to occur in primary tumors are of little interest.

Stephen Baylin: It is a perfectly correct view that one should not attempt to judge the potential importance of promoter hypermethylation for any individual gene solely through studies done in cell culture. Rather, cell culture offers only a starting point to

study the implications of the promoter change in terms of degree of gene silencing, effects of induced demethylation on gene reexpression at the mRNA and protein levels, demonstrating the effects of the gene silencing at a functional level, and importantly, studying the mechanisms by which DNA methylation is targeted to promoter regions and how it is coupled to transcriptional silencing. Obviously, all of this knowledge can be useful from many different standpoints. However, for any links to functional relevance to actual cancer progression, it is essential to study the epigenetic status of the gene in question in primary normal and tumor cells. In point of fact, to the author's knowledge, for every gene well characterized for epigenetically mediated aberrant transcriptional silencing in cancer, and especially for those which are classic tumor suppressor genes or which are prime candidates for a role in tumorigenesis, promoter hypermethylation has been well shown for primary as well as cultured tumors (e.g., Herman et al., 1994; Dammann et al., 2000). It should be remembered that the degree of methylation may not always even be more extensive in the culture than in the primary setting, but is, rather, more easily demonstrated from a technical standpoint. The study of primary tumors, even when microdissection techniques are utilized, is often hampered by the presence of normal cells in the sample. Thus, by any assay of methylation status used, especially the most sensitive ones, unmethylated alleles of the gene under study will always be detected. Likewise, even if the gene is completely silenced in the primary tumor cells, some degree of expression is almost always obtained, in assays of steady state mRNA or protein levels, because of the presence of normal cells. One important question for which the data are not often readily available concerns the true incidence for the hypermethylation of a given gene in culture versus the primary setting. For many important genes, the rates may not be that dissimilar. Recent studies from my laboratory and that of my colleague, James Herman, using a microarray approach to identify hypermethylation of gene promoters in colon cancer coupled to loss of gene expression, have shown that as the candidate genes from the screen were validated, there emerged a distinct correlation between the incidence of hypermethylation for a given gene in a panel of cultured colon cancers versus that found for the genes in a substantial sized study of primary colon tumors (Suzuki et al., 2002). It appears, then, that the methylation status in the cultures may often reflect that of the tumor cells in their original state.

Timothy Bestor: The convenience of relatively homogeneous cell populations has led to the common use of cancer cell lines in methylation studies. Permanent lines of cultured cells (whether derived from tumors or normal tissues) can be methylated at the majority of CpG islands associated with tissue specific genes, while these sequences remain unmethylated in all normal somatic tissues regardless of expression status (Jones et al., 1990; Walsh and Bestor, 1999). Maintenance in culture imposes constant and intense selective pressures for rapid cell division, and this leads to the loss or the silencing of genes that restrict proliferation or that encode differentiated functions. While analogous pressures exist within populations of progressing tumor cells, the relationship of the pressures undergone by cells in laboratory culture to those in tumors in situ is difficult to estimate but may be quite different. Gene silencing by CpG island methylation is certainly much more common in cultured cells than in cells from primary tumors; Smiraglia et al. (2001) report that head and neck squamous carcinoma cell lines show hypermethylation at a frequency more than 90-fold greater than the tumors of origin, while colorectal carcinoma cell lines (in which methylation differences between cultured and primary tumor cells were the least pronounced of any of the tumors studied) had a 5-fold increase in focal hypermethylation over the analogous primary tumors. More than 57% of the sequences found to be hypermethylated in cultured tumor cells were not methylated in any of 114 primary tumors.

It is suggested that one danger in the study of cytosine methylation and cancer may be an undue reliance on cultured tumor cells, whose genomic methylation patterns are known to rapidly diverge from those found in primary tumor cells. It is methylation abnormalities in primary material that are relevant; methylation differences found in cultured cells that are not demonstrated to occur in primary tumors may be of little importance to human cancer.

Can an epigenetic abnormality, like a genetic change, ever initiate a cancer?

Stephen Baylin: This is a difficult question, not only with respect to reviewing the evidence, but also because of the semantics concerning when a cancer actually starts. Since transformation is a multistep process, there are numerous preneoplastic phases that human cancers evolve through that take many years. Certainly, both losses and gains of methylation, just like genetic changes, can accompany these early steps. Promoter hypermethylation is seen in key genes like *p16* in preneoplastic states, and even in some genes in normal appearing tissues as a function of the key cancer risk state, aging (Jones and Laird, 1999). However, in all of these settings, the cells harboring such epigenetic alterations are not absolutely fated to progress to cancer, and if the DNA methylation changes are functional, the consequences would have to be viewed as permissive in nature for actual cancer and fall into the category of risk changes. Perhaps a better stance from which to view the question is to compare genetic and epigenetic changes for genes which absolutely are connected with cancer by virtue of their mutational status in the germline of families with distinct cancer syndromes. Certain findings are intriguing. For example, *VHL* mutations are largely restricted to renal cancers in familial and nonfamilial clear cell renal cancers, and so is promoter hypermethylation of this gene (Herman et al., 1994). Mutational inactivation of the repair gene *MLH1* is associated in familial and nonfamilial cancers with the microsatelite instability phenotype, and so is promoter methylation of this gene in nonfamilial tumors (Kane et al., 1997; Herman et al., 1998). Recently, two groups have reported a specific microarray pattern for *BRCA1* in familial breast cancers from patients with germline mutations in the gene. The only nonfamilial breast tumors found to share this microarray pattern were those with *BRCA1* hypermethylation (Hedenfalk et al., 2001; van't Veer, et al., 2002).

While the above comparisons may be compelling for the possibility that epigenetic alterations could technically start a cancer, I would conclude that these situations cited do not prove this. One key point is that mutations in a germline setting require complementary steps to give rise to cancers, and the order of these events for cancer formation need not be the same for the familial versus the nonfamilial setting. Thus, the promoter hypermethylation in each of the above genes discussed could be a relatively late event in the noninherited cancer types. Technically, then, the question of cancer origin from an epigenetic change remains an open and very difficult one to prove.

However, the comparisons for the above genes for mutations and promoter hypermethylation do speak strongly to the similar selective advantages and functional consequences for loss of function of the genes produced by their inactivation in association with either genetic or epigenetic abnormalities.

Timothy Bestor: For this particular question, I essentially agree with the points raised by Dr. Baylin.

There is a lack of genetic data that support the causative role of methylation pattern abnormalities in cancer, and especially the current absence of tumor predisposition syndromes caused by mutations in DNA methyltransferase genes. Such genetic data would be convincing and might cement the true importance of the methylation changes in cancer for their functional role.

Stephen Baylin: I heartily agree that it is extremely helpful to consider any genetically derived data that contribute to understanding and documenting the importance of DNA methylation changes in cancer, be they losses or gains. This is a legitimate challenge to ask of promoter hypermethylation and associated gene silencing in cancer in particular, for at least two reasons. First, the critical role of natural genetic changes in cancer is so well documented that it is logical to use these as a comparison point for questioning the position of epigenetic alterations in cancer development. Second, our ability, today, to use genetics in experimental approaches offers perhaps the ultimate tool for verifying or refuting the functional importance of cancer related epigenetic changes. In other words, both the natural and experimental genetics of cancer are then important vantage points from which to view and challenge the importance of epigenetic changes. The question of a lack of cancer predisposition syndromes due to mutations, certainly of the germline type, in DNA methyltransferases (DNMTs) is an interesting one. Predictably, these might be activating ones from the standpoint of increasing promoter methylation. However, cancer cells with promoter hypermethylation of multiple genes simultaneously lose many sites of normal methylation throughout the genome. So, this simple prediction might not hold true. In *Arabidopsis*, DNMT loss-of-function mutations actually cause both widespread losses and simultaneous gains of DNA methylation at selected promoters (Kishimoto et al., 2001; Lindroth et al., 2001). So, perhaps, this scenario might occur in humans. Indeed, germline mutations in both alleles of one of the three known biologically active human DNMTs, DNMT 3b, do occur and give rise to the rare ICF syndrome (Hansen et al., 1999; Okano et al., 1999; Xu et al., 1999). Individuals with this problem, however, may not live long enough to develop cancers. Knockout mice of all three DNMTs are either embryonic or perinatal lethal so, on a germline basis, mutation of any of the DNMTs might not be a likely candidate for tumor predisposition syndromes.

Perhaps better candidates would be genes encoding chromatin remodeling or specifying proteins. Again, in *Arabidopsis*, mutations in a remodeling protein of the Swi-Snf type cause shifts in DNA methylation patterns similar to those above for a DNMT (Jeddeloh et al., 1999), and knockout of an ortholog for this protein in mice has recently been reported to cause loss of DNA methylation in embryos (Dennis et al., 2001). Finally, loss of function mutations in histone methyltransferases, in Neurospora (Tamaru and Selker, 2001) and *Arabidopsis* (Jackson et al., 2002), which specifically methylate at lysine 9 residues of histone H3, setting up a posttranslational change critical for establishing transcriptionally repressive chromatin (Jenuwein and Allis, 2001), cause losses of DNA methylation. So, it seems that looking for either germline or somatic mutations in the DNA methylation "machinery" proteins that may be linked to cancer predilection and/or somatic tumorigenesis is certainly a valid consideration. Likely, work is ongoing within this arena.

Even in the absence of association between cancer and the above types of natural mutations, I would maintain, however, that there are in fact several key genetic data points, some of which are provided by nature and others which have recently been experimentally derived, that point to the importance of promoter hypermethylation changes and/or the associated gene silencing. More will be useful, but to date, the following seem compelling. First, the data of Laird et al. (1995) that the knockout of the DNA methyltransferase-1 (*Dnmt1*) gene leads to loss of gastrointestinal tumorigenecity in mice lacking the *APC* gene is intriguing. The leading candidate mechanisms currently proposed by the lead author of the above original study is the elimination of promoter methylation and silencing of tumor suppressor genes (Eads et al., 2002). Some evidence for this is accruing (Eads et al., 2002), but this issue remains to be fully resolved.

Second, the question of selective advantage for epigenetic changes should always be carefully examined, and when juxtaposed to genetic changes, there is now very compelling data for the functional role of promoter methylation in tumorigenesis. In a long-established colorectal cell line, HCT 116, it is well documented that one mutant allele of *p16* is mutated and nonfunctional, but expressed, while the opposite allele is hypermethylated and fully silenced (Myohanen et al., 1998). While this example suffers from being restricted to cell culture, an issue that is addressed above, similar scenarios have emerged in native tumor situations. Thus, studies of E-cadherin hypermethylation in families with germline mutations in this gene, a situation in which members get gastric cancers, reveal that the methylation change in the tumors always acts as a "second hit" by being present, in tumors retaining both E-cadherin alleles, on the nonmutated allele (Grady et al., 2000). Likewise, in a more recent and large study of several inherited cancer types, arising in the setting of germline mutations, hypermethylation of the involved gene in tumors from individuals within the kindreds was never seen in those in which the opposite allele of the germline mutated gene was lost, but was not uncommon in tumors where both alleles were retained (Esteller et al., 2001).

Third, recent evidence juxtaposing a natural genetic event with experimental data in acute promyelocytic leukemia cells gives rise to the consideration of a line of research which could heartily solidify the functional role of promoter hypermethylation and gene silencing in cancer. Thus, translocation of the transcription factor gene, *RAR*α, to create a fusion protein with PML, appears to correlate with hypermethylation of the promoter and gene silencing of a candidate downstream transcriptional target of RARα, *RAR*β. Moreover, the induction of binding of multiple DNMTs to the target promoter accompanies this scenario, as does an altered nuclear localization of these proteins (Di Croce et al., 2002). These events are not seen when RARα acts as a transcriptional activator of the downstream gene rather than a repressor, as it does when in the context of the fusion protein. It must be cautioned that it is not certain that the target gene promoter for RARα is a natural one for RARβ, and much work remains to ferret out the true significance of these above findings.

Finally, a very recent collaborative study, involving experimental genetic manipulation of cancer cells, strongly suggests the role of the promoter hypermethylation in epigenetically silencing of genes in cancer and the consequences of this mode of gene inactivation for the tumor phenotype. When both the DNA methyltransferase 1 and 3b genes are fully disrupted in HCT 116 colon cancer cells, virtually all DNA methylation is lost from the cells, and the hypermethylation in tumor suppressor genes, including the wild-type copy of *p16* and an imprinted gene, is relieved with associated gene reexpression (Rhee et al., 2002). Furthermore, a single cell clone in this study where the p16 gene did not demethylate, but the other studied genes did, grows dramatically faster than all of the other double knockout clones which, in turn, grow much slower than the wild-type clones. The product of this gene is a powerful brake for cell growth, and the continued suppressed expression of the wild-type allele may well explain the behavior of this clone.

In summary, there appears to be a growing body of genetic data to support the functional significance of DNA methylation changes in cancer. More data are needed which either complement, refute, or alter the interpretations that have been offered above.

Timothy Bestor: The cause-versus-consequence problem has long been a serious problem in the cytosine methylation field, which has traditionally placed undue reliance on correlative evidence. The finding of a methylated and silent promoter does not require that the methylation be the cause of the silencing; long-term inactivity for other reasons may predispose a gene to de novo methylation. The binding of transcription factors such as Sp1 can cause demethylation of local CpG sites in cycling cells, and even *E. coli* lac repressor bound to lac operator sequences in mammalian cells can induce demethylation of nearby CpG sites (Lin et al., 2000, and references therein). De novo methylation observed at specific genomic foci may reflect not a primary epigenetic silencing event but rather the consequences of loss of expression due to upstream mutational events in signal transduction cascades or transcription factor networks. The association of de novo methylation with a specific gene cannot be taken as evidence that the gene was silenced by methylation; the methylation may have little to do with the primary extinguishing event.

There is at present a lack of genetic data to support the causative role of methylation pattern abnormalities in cancer. While the products of many oncogenes and tumor suppressor genes are involved in signal transduction, control of transcription, DNA repair, cell cycle control, cell-cell interactions, and other processes, none have been shown to be directly involved in the establishment or maintenance of genomic methylation patterns or the specific binding of methylated sites. Somatic mutations in DNA methyltransferase genes have not been reported for any tumor, and the lack of tumor predisposition syndromes caused by mutations in DNA methyltransferase genes is also notable. Were such genetic evidence available, there would be a compelling case for a causative role of methylation abnormalities in cancer. While it has been reported that heterozygosity for mutations in *Dnmt1* in mice modifies the penetrance of the *APC/Min* mutant mice by reducing the number of benign polyps in colonic epithelia (Eads et al., 2002), it has not been shown that the methylation status of any sequence related to cancer is detectably altered in such animals, nor has heterozygosity at *Dnmt1* been shown to modify the penetrance of mutations that predispose to malignant tumors. In one of the few other cases where genetic data linked methylation and malignant disease, the Dnmt1 gene was shown to be amplified in Friend murine erytholeukemia cells (Bestor et al., 1988). However, this is very likely to be due to coamplification of *Dnmt1* with the erythropoietin receptor (*Epor*) gene, which is located very close to *Dnmt1* on proximal mouse chromosome 9 and which is involved in leukemic transformation via activation of Epor by retroviral envelope glycoprotein gp55 (reviewed by Ruscetti, 1999). Amplification of *Dnmt1* in this case was most likely due to proximity to the target gene, and no direct evidence for a role of Dnmt1 in carcinogenesis has appeared. Overexpression of Dnmt1 in tumors is small in extent, if it occurs at all (Eads et al., 1999; Lee et al., 1996; Schmutte et al., 1996). Confirmation of cases in which a primary defect in a methylation-related gene can be demonstrated to be essential for tumorigenesis would remove much of the existing uncertainty as to the importance of cytosine methylation in the etiology of cancer.

At present, there is a lack of a plausible mechanism that might lead to the de novo methylation of CpG islands in somatic cells. Is promoter methylation the cause of silencing, or is it the consequence of a loss of expression due to mutations in genes that encode regulatory factors? In some tumor types (colorectal and urogenital) there is a global reduction in methylation levels with evidence of focal increases. A testable hypothesis that could explain this situation is urgently needed.

Stephen Baylin: I agree that the above are absolutely, and perhaps the most, fundamental questions in the field. Among them are some that have long confounded understanding of the role of methylation in transcriptional control, not only in cancer cells, but in mammalian cells in general. The ones concerning transcription embody the chicken and egg question of whether the DNA modification is the cause of silencing, or the consequence. This does not negate the importance of the promoter hypermethylation associated with aberrant gene silencing in cancer if the process it is associated with is involved with gene inactivation that is important for the tumor phenotype. However, for all working in the field, the questions are pivotal and emblematic of the type of constructive skepticism and debate that should be central to designing experimentation which could hold the key to full understanding of the processes under discussion.

Could it be possible that the answer to the above questions may well encompass an unexpected paradigm? Recent data suggest that all of the active mammalian DNA methyltransferases (DNMTs) may be capable of directly silencing transcription separately from catalyzing methylation (Fuks et al., 2000, 2001; Robertson et al., 2000; Rountree et al., 2000; Bachman et al., 2001). Might it then be possible that gene silencing and methylation are so integrally coupled that this has confounded attempts to order them temporally by direct experimental approaches? In other words, the interaction of the DNMTs with any promoter might always help initiate silencing and sometimes couple this with methylation. In the truest sense, then, the silencing might come first, but barely. This hypothesis, even if it holds true, would not negate the possibility that mutations in regulatory factors could underlie the abnormal methylation patterns, including those in promoters associated with gene silencing, in cancer. The proteins involved might be those that help target DNMTs properly to chromatin or associate with them once they do. If so, such mutations could explain, at once, the

fact that cancer cells have global loss of methylation and regional gains, if failure to target some sites might allow access to aberrant ones. Again, the studies cited previously in *Arabidopsis* outlining mutations in DNMTs themselves and in chromatin remodeling proteins, which lead to losses and gains of DNA methylation, well illustrate the potential for this scenario. Such a finding in human tumors would not, in and of itself, answer the chicken and egg question. However, it would be incredibly exciting and would help complete a circle in which, during tumor progression, genetic changes could induce epigenetic ones, which can in turn, as for silencing of the *hMLH1* gene (Kane et al., 1997; Herman et al., 1998), produce further genetic changes.

In terms of the types of mutations that could reset the cancer genome for chromatin patterns, one must now consider heavily the recent exciting data concerning the role of the histone code in establishing chromatin patterns. Certainly, methylation of lysine 4 and 9 of histone H3 tails is intimately associated with transcriptionally active versus repressive chromatin, respectively (Jenuwein and Allis, 2001). Further, as previously noted, data in *Neurospora* and *Arabidopsis* have emerged firmly indicating that mutations in enzymes mediating the lysine 9 methylation, histone methyltransferases (HMTs), lead to altered DNA methylation. While the alterations reported, to date, involve losses of the DNA modification, might it not be reasonable to suspect that gains might also ensue if specific lysine 4 or 9 HMTs, or proteins with which they associate, are altered? The tools appear to be at hand to frame reasonable hypotheses concerning the concomitant losses and gains of DNA methylation in cancer cells and to use these to provide a molecular basis for a key aspect of the neoplastic phenotype.

Timothy Bestor: De novo methylation of CpG islands could represent a simple epimutation in which accidental methylation of CpG sites in islands that are normally unmethylated causes the heritable transcriptional silencing of the associated gene. This could contribute to tumor progression if the silencing event endows the cell with a growth advantage in the tumor cell population. In this model, the affected gene presents no features that predispose it to methylation, and de novo methylation is essentially a stochastic event. Given the nearly complete lack of understanding of the cues that designate single copy sequences for de novo methylation at any stage of development, the random model remains valid. However, experiments on methylation and silencing of the *hMLH1* gene suggest that additional factors are involved.

A subset of colorectal carcinoma cell lines showed microsatellite instability and did not express the *hMLH1* DNA repair gene, although the sequence of both *hMLH1* alleles was normal (Veigl et al., 1998). The 5′ CpG islands of both alleles were found to be methylated, and hMLH1 expression could be induced by culture in the presence of 5-aza 2'-deoxycytidine (azaC), a specific inhibitor of DNA cytosine methyltransferases. However, withdrawal of azaC was followed by silencing and remethylation of both alleles, and reexposure to azaC again caused transient expression of hMLH1 transcripts. The rapid and concerted remethylation of *hMLH1* upon removal of the methylation inhibitor in these experiments is difficult to reconcile with the random de novo methylation model, which predicts a slow and stochastic process. The demethylation induced by azaC was sufficient to allow full access of transcription factors, as shown by the activity of the promoter after demethylation. The locus either underwent alterations of sequence or of heritable chromatin configurations that predisposed them to silencing, or the initial silencing event was wholly stochastic and some unidentified remnant of the silent state was sufficient to overcome the reactivated promoter and cause resilencing upon withdrawal of demethylating agent. It should also be noted that in this case transcriptional activity was insufficient to protect the promoter region from de novo methylation; as noted below, the binding of factors such as Sp1 to cellular sequences or even lac repressor to lac operator transgenes can cause demethylation of local sequences. The *hMLH1* silencing studies are not compatible with the simple stochastic de novo methylation hypothesis, and fundamental questions about the mechanism of methylation and gene silencing in cancer are raised by these studies.

What does the hypomethylation side of the coin contribute to tumorigenesis?

Stephen Baylin: Certainly, there is an excellent body of literature documenting both global and gene specific loss of methylation in cancer (e.g., Feinberg and Vogelstein, 1983; Feinberg et al., 1988). It remains, however, to be proven exactly what the consequences of this change are with respect to specific events in tumorigenesis. The work previously discussed concerning mutations of DNMT3b underlying the ICF syndrome amply suggests the potential role of methylation loss for chromosomal instability, and that has been a major hypothesis for the consequences of genomic hypomethylation in cancer. Another hypothesis, as I'm sure Dr. Bestor will address, is the possibility for unwanted transcription of repeated elements from areas of methylation loss in cancer cells. This area of epiegentic change in cancer is certainly of continued potential importance and warrants continued clever experimentation to demonstrate its exact significance(s) for tumorigenesis.

Timothy Bestor: Most solid tumors present with aneuploid and severely rearranged karyotypes, and some (notably colorectal and urogenital carcinomas; reviewed by Schulz, 1998) are also characterized by global genome hypomethylation. While many sources of genome destabilization have been identified, the role of methylation patterns in genome stability has received little attention. The large majority of cytosine methylation lies within repeated sequences (Yoder et al., 1997), primarily the transposons that make up more than 45% of the human genome; demethylation would be expected to unmask repeats and increase the frequency of rearrangements caused by homologous recombination between nonallelic repeats. Results of experiments in embryonic stem cells homozygous for mutations in Dnmt1 support this conclusion; mutation rates at introduced marker loci were elevated 5- to 10-fold in demethylated genomes, and the mutations were primarily rearrangements rather than point mutations (Chen et al., 1998). The global demethylation characteristic of certain tumor types may predispose to genome instability via homologous recombination between unmasked repeats rather than favoring dysregulation of gene expression, as was originally envisioned.

Conclusions

Stephen Baylin: It is hoped that, in the above discussions, I have kept to the promise of not "answering" questions that have not yet been answered. Obviously, human nature dictates that some personal biases will come through. Those of us in cancer research who would be, or are, called "epigeneticists" are, perhaps, seduced into thinking we have come a long way considering the virtual explosion in interest for this field over the last few

years. Perhaps we are still just starting and only continued well-focused research will provide data that will be the substrate for history to put the story, especially for cancer, in its proper perspective. In so doing, things may appear quite different in the future from the view we have today.

Timothy Bestor: Are disturbances of genomic methylation patterns directly involved in carcinogenesis, and if so, what fraction of tumor incidence involves such abnormalities? These questions are very difficult to answer at this time, due in large part to the lack of plausible and testable candidate mechanisms that could lead the cell to participate in the irreversible silencing of genes that are required for normal cellular function. Progress will depend on a better understanding of the factors that silence expression of genes that are in the presence of the full complement of factors normally required for their expression.

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