The progressive nature of malignant neoplasms and their inherent heterogeneity ultimately limit our ability to understand and treat them. The fundamental concepts of tumor progression elaborated by Foulds (1) and Nowell (2) have as their central themes the idea that increasing genetic alterations generated by random somatic mutational events are responsible for this heterogeneity and progression of tumor cells to increasingly malignant and less responsive states. However, recent information on the unexpected flexibility of the eukaryotic genome with regard to gene amplification, rearrangement, deletion, and methylation makes the concept of somatic mutation-induced tumor progression perhaps too simplistic. For example, not all neoplastic cells show a higher mutational frequency than normal cells (3) and the rate of metastatic variation can be as much as 2 orders of magnitude higher than the rate of mutation in the same cells (4).

Thus, processes such as amplification, rearrangement, deletion, and methylation, which are components of normal cellular ontogeny, become increasingly attractive candidates for mediators of tumor cell progression. Importantly, these changes can be ephemeral in nature and can be used to explain the rapid changes in metastatic abilities which occur in several experimental systems (5, 6). In this article, I will expand upon the suggestions of several authors (7–10) that changes in DNA methylation, which are known to be implicated in eukaryotic gene control, may play a central role in the generation of heterogeneity and phenotypic instability in cancer.

DNA Methylation Is Implicated in Eukaryotic Gene Expression

The evidence that the methylation of specific cytosine residues in DNA controls the expression of some, but not all, eukaryotic genes has strengthened over the last few years. There are several features of eukaryotic DNA methylation which make it a particularly attractive component of a multilevel control mechanism (8). Methylation patterns are symmetrically distributed in CpG doublets (11) and are tissue specific (12, 13). The methylation status of specific CpG sites is maintained after DNA synthesis by methyltransferase enzymes, a fact that ensures that methyl groups on CpG doublets are found in the 5' region of genes. Bird et al. (25) have estimated that about 30,000 such clusters are unmethylated occurs when they are located on inactive X-chromosomes. Wolf et al. (27) have recently found a complete concordance between lack of glucose-6-phosphate
dehydrogenase activity and hypermethylation of CpG clusters on inactive X-chromosomes. Clusters on hypoxanthine phosphoribosyltransferase are undermethylated and hypersensitive to nucleoside digestion on the active X-chromosome whereas the same clusters are hypermethylated on inactive X-chromosomes (32). Interestingly, CpG rich clusters are not invariably located 5’ to the coding regions of genes; those associated with g6pd occur in the 3’ region of the gene. Assuming that this CpG cluster is involved in regulating expression of g6pd, its position suggests the possibility that CpG clusters function as enhancer-like elements. Since methylation of CpG rich clusters prior to introduction into eukaryotic cells can inactivate housekeeping genes (19) we are left with the exciting concept that methylation of CpG sites in enhancers might block enhancing activity.

The existence of CpG rich clusters in association with housekeeping genes and their undermethylation when associated with active rather than with inactive X-chromosomes are powerful evidence that these sequences have an important role in gene expression. Also, the fact that housekeeping genes can be inactivated if the clusters are methylated before introduction into eukaryotic cells (19) is further evidence for their potential importance. However, they are not only confined to housekeeping genes since a cluster occurs on the 2(1) collagen gene (24) which is not generally considered to fulfill a housekeeping function.

5-Azacytidine

Another series of studies which strongly supports a causative role for methylation in suppressing gene activity comes from experiments using the nucleoside analogue 5-aza-Cyd. 5-aza-Cyd was originally developed as a cancer chemotherapeutic agent (33) but also has marked effects on the stability of the differentiated state of cultured cells (34, 35). The analogue is thought to act by incorporation into DNA where it functions as a powerful inhibitor of the methylation of newly incorporated cytosine residues (36). It is quite clear that the fraudulent nucleoside must be incorporated into DNA in order to inhibit methylation (37, 38). The result of this incorporation is a loss of active DNA methyltransferase activity (38–40) which is due to the formation of a tight noncovalent complex (40) or a covalent linkage (41) between the enzyme and 5-azacytosine residues in DNA.

Several lines of evidence support the idea that inhibition of DNA methylation is indeed the mechanism of drug action: (a) biologically active doses of 5-aza-Cyd are strong inducers of gene expression and potent inhibitors of DNA methylation; (b) the effects on cell differentiation are specific for position 5 of cytosine and can be mimicked by other analogues such as pseudocytosidine and 5-fluorodeoxycytidine which also inhibit methylation and change the differentiated state of cells (36); (c) the changes in gene expression are often heritable for many generations in the absence of further drug treatment; (d) the activation frequencies observed are sometimes 5–6 orders of magnitude greater than those expected for the activity of mutagenic agents (42); (e) the effects of 5-aza-Cyd on methylation have been localized to genes which become transcriptionally activated (43); (f) genes which have been inactivated by methylation before introduction into eukaryotic cells can be reactivated by 5-aza-Cyd treatment (44); (g) in an experiment which

How Well Are Methylation Patterns Copied?

The presence of maintenance DNA methyltransferases ensures that established methylation patterns can be faithfully copied. The existence of tissue specific patterns of methylation implies that this is the case during normal development. However, the mechanisms governing the acquisition of new methyl groups or the loss of preexisting methyl groups are not well understood. What has become clear in recent years is that substantial changes in genomic methylation including loss of methyl groups and de novo methylation may occur in cultured cells. If DNA methylation plays an important role in controlling gene expression then it is important that we understand how methylation patterns are changed and altered during normal cellular development and possibly during tumor formation and progression.

Early experiments using DNA molecules transfected into recipient cells suggested that methylation patterns could be inherited with a high (48, 49) but not absolute fidelity (15). Thus, methylation patterns in rapidly dividing cells may not be copied with complete accuracy. Shmookler-Reis and Goldstein (50, 51) found considerable variability in DNA methylation patterns during the serial passage of human diploid fibroblasts and considerable interclonal variations in methylation patterns for expressed and nonexpressed genes in 8 clones isolated from a mass culture of human diploid fibroblasts. Different clone specific patterns of DNA methylation including increased methylation were found indicating a striking degree of interclonal heterogeneity, particularly for those genes not normally expressed in diploid fibroblasts (e.g., g-globin and B-globin). Considerable heterogeneity with regard to X-chromosome DNA methylation has been found in normal euploid human cells during replication in culture (52). These changes in methylation patterns seen in cultured cells may have important implications in the generation of new cellular diversity.

We have found considerable decreases in DNA methylation in aging but not in immortal cells in culture (53). When normal diploid fibroblasts from mice, hamsters, and humans were grown in culture, DNA methylation decreased markedly with the greatest rate of loss of 5-methylcytosine observed in mouse cells which survived the least number of divisions in culture. On the other hand, the immortal cell lines had more stable rates of methylation.

These data therefore show that methylation patterns are not inherited with high degree of fidelity which was previously thought. The mechanisms for the selective loss of methyl groups
Methylation Changes in Cancer Cells

A large body of evidence shows that methylation levels and patterns are deranged in tumor cells (reviewed in Ref. 8). Undermethylation of DNA has been seen in a large number of animal and human tumor cells (64–66) and might contribute to aberrant gene expression. However, hypomethylation in the DNA of tumors freshly excised from children with a variety of neoplasms is not always observed (67) although some tumors, particularly neuroblastomas, were significantly hypomethylated compared to human fibroblasts. These results suggest that significant changes in the overall level of DNA methylation can occur in tumors although it is not possible to generalize these findings. The relationship between decreased levels of overall DNA methylation and gene expression is not known, although it is clear, but not widely recognized, that tissue specific differences in overall genomic methylation levels exist (68).

Substantial hypomethylation of specific sites within growth hormone and globin genes in primary human colonic tumors was observed in 4 of 5 tumors by Feinberg and Vogelstein (69). These studies, which were conducted using colon carcinoma cells, were particularly significant because of the availability of normal mucosal tissue in the vicinity of the tumor which could easily be separated from the underlying stroma. Since the neoplasms themselves were derived from the epithelial cell layer, it was possible to conduct carefully controlled studies when comparing the normal cells to the tumor cells.

Feinberg and Vogelstein (70) extended their studies to the ras gene family in primary colon and lung carcinomas and observed undermethylation in 6 of 8 tumors relative to normal colonic mucosa. Interestingly, the degree of hypomethylation appeared to be greater in metastatic nodules than in the corresponding primaries suggesting that progressive undermethylation might occur during the metastatic process. This was extended in the more recent study by Goeltz et al. (71) who observed altered 5-methylcytosine patterns in a series of genes in 23 neoplasms. Hypomethylation of a specific site in the c-myc gene in human tumor cell lines has also been observed (72) and changes in methylation within specific genes have also been observed in animal tumor cells (73, 74). However, several of the genes investigated in these studies were not expressed even though they were hypomethylated (71). Future work will therefore have to concentrate on genes known to be involved in transformation and progression before the exact significance of methylation changes can be understood.

Nevertheless, these studies demonstrate that considerable heterogeneity of methylation patterns occurs in tumors derived from humans and animals and in cultured cell lines. This suggests that methylation patterns are not as rigorously controlled in these cells as they are in their normal counterparts but says nothing as to the potential relevance of these findings to the phenomenon of tumor progression. More direct evidence that the 2 phenomena are linked comes from studies in which methylation patterns within tumor cells have been artificially changed and these experiments suggest a strong causal relationship between methylation changes and increased phenotypic diversity in tumor cells.

Changes in Malignant Behavior Induced by 5-Azacytidine

5-aza-Cyd is a useful tool for assessing the role of epigenetic phenomena in cancer because several studies have shown that...
the agent is not demonstrably mutagenic in eukaryotic cells (75–
77). The drug is capable of the direct transformation of cultured
cells (78, 79) and is tumorigenic in whole animals (80). However,
recent excitement has been generated by the finding that 5-aza-
Cyd can have substantial effects on malignant behavior.
Frost et al. (81) found that 5-aza-Cyd influenced the expression of
tumor antigens on tumorigenic mouse cell lines which resulted in
some of them becoming nontumorigenic in syngeneic animals.
These experiments were expanded in a later study by Kerbel et
al. (77) who found that phenotypically unstable clones of meta-
static tumor cells could be obtained from the TA3 mammary
carcinoma cell line which in itself is nonmetastatic. A key point
in this study was the fact that the cell lines derived were not
completely stable with regard to their phenotype, which raised
the possibility that epigenetic rather than genetic mechanisms
were responsible for the fluctuation in biological behavior. Olso-
son’s group (82, 83) have also found that 5-aza-Cyd can induce
metastatic potential in nonmetastatic sublines or extinguish met-
astatic potential in cells already metastatic.
These results therefore show that 5-aza-Cyd can have variable
effects on tumor progression and may activate genes necessary
for progression in some cases but serve to activate genes which
repress progression in others. A recent paper suggests that this
might be the case in rat embryo cells transformed by adenovirus
type 5 (84). Progression was not correlated with major changes
in the pattern of integration of viral DNA sequences but was
associated with an increased methylation of integrated viral
sequences other than those corresponding to the transforming
genes of the virus. A single exposure of progressed cells to 5-
aza-Cyd resulted in the stable reversion to the unprogressed
state of the original parental clone. The observations in the viral
system thus suggest that progression is a reversible process,
which might be associated with changes in the state of methyl-
ation of one or more specific genes.
Before leaving the subject of the effects of 5-aza-Cyd on
progression and metastatic potential it should also be remem-
bered that the drug can induce the end-stage differentiation of
tumor cells. Examples of this are the formation of contractile
muscle cells from tumorigenic transformed derivatives of 10T1/2
cells (85) and the end-stage differentiation of Friend cells (40).
The differentiation may result in the formation of cells with no
further division potential so that changes in DNA methylation
patterns can result in the apparently normal differentiation of
malignant cells.

Summary

The main thrusts of the arguments that aberrant DNA meth-
ylation is involved in the generation of tumor heterogeneity and
progression can be summarized as follows. The methylation of
specific cytosine residues in DNA is certainly an important com-
ponent in multilevel gene control in eukaryotes. The discovery of
CpG clusters in the flanking regions of genes and their under-
methylation on housekeeping genes, except those located on
inactive X-chromosomes, strongly suggests a controlling func-
tion for modification in these regions. Since methylation plays
an important role in controlling normal cellular development, it
follows that aberrations within this mechanism may be implicated
in the abnormal gene control which characterizes cancer.
Methylation patterns are not copied rigorously in rapidly divid-
ing cells. This may be because there is normally a close coordi-
nation between DNA synthesis, DNA methylation, and DNA
packaging, and changes in the timing of these processes could
conceivably result in hypomethylation at some sites and de novo
methylation at others. Since the greatest variability of methylation
patterns is seen in nonexpressed genes, it is possible that there
is a tendency for cells to activate genes when dividing in an
inappropriate growth environment.
The constant evolution and shuffling of methylation patterns
which occur during division might play a role in the development
of new phenotypes within cell populations. One might predict
that selective pressures within the host would select for those
cells with specific new methylation patterns allowing for the
expression of genes necessary for survival in a particular envi-
ronment. Many experiments have in fact shown that methylation
levels and patterns and indeed methyltransferase levels (57) are
altered in cancer cells. Thus, there is considerable heterogeneity
within tumor populations with regard to this fundamental biolog-
ical control mechanism. The fact that direct intervention by
the use of 5-aza-Cyd can result in dramatic alterations in malignant
potential allows this hypothesis to be tested more critically.
Hopefully, the use of 5-aza-Cyd in defined systems will allow
us to isolate genes which might become activated by drug
administration and which might contribute to metastatic potential. An
understanding of the fundamental aspects of the enzymology
and control of DNA methylation might therefore allow us to make
significant inroads into understanding how heterogeneity is gen-
erated and what we might do about it.

References

3. Elmore, K., Kakunaga, T., and Barrett, J. C. Comparison of spontaneous
mortality rates of normal and chemically transformed human skin fibroblasts.
5. Kerbel, R. S., Frost, P., and Liteplo, R. G. Genetic and epigenetic regulations
of the metastatic phenotype: a basis for resolving the controversy regarding
its selective or random nature and variable phenotypic stability. In: L. Liotta,
C. Rabson, and B. Chambers (eds.), Biochemistry and Molecular Genetics.
variation in cells derived from lung metastases of KHT fibrosarcoma. Invasion
during tumor development: an extension of the miscoding concept. Br. J.
10. Frost, P., and Kerbel, R. S. On a possible epigenetic mechanism(s) of tumor
cell heterogeneity: the role of DNA methylation. Cancer Metastasis Rev., 2:
11. Bird, A. P. Use of restriction enzymes to study eukaryotic DNA methylation. II.
The symmetry of methylated sites supports semi-conservative copying of the
12. Waalwijk, C., and Flavell, R. A. DNA methylation at a CCGG sequence in the
large intron of the rabbit B-globin gene: tissue specific variations. Nucí. Acids
13. McGhee, J. D., and Ginder, G. D. Specific DNA methylation sites in the vicinity
sequences in eukaryotic cells. Proc. Natl. Acad. Sci. USA, 77: 6463–6467,
1980.
15. Wigler, M., Levy, D., and Perouco, M. The somatic replication of DNA methyl-
124, 1983.
DNA METHYLATION AND CANCER


48. Deiers, A., Szpirer, J., Szpirer, C., and Saggoro, D. Spontaneous and 5-
DNA METHYLATION AND CANCER


