

DNA methylation and gene silencing in cancer: which is the guilty party?

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The DNA methylation pattern of a cell is exquisitely controlled during early development resulting in distinct methylation patterns. The tight control of DNA methylation is released in the cancer cell characterized by a reversal of methylation states. CpG island associated genes, in particular tumour suppressor or related genes, are often hypermethylated and this is associated with silencing of these genes. Therefore methylation is commonly convicted as a critical causal event in silencing this important class of genes in cancer. In this review, we argue that methylation is not the initial guilty party in triggering gene silencing in cancer, but that methylation of CpG islands is a consequence of prior gene silencing, similar to the role of methylation in maintaining the silencing of CpG island genes on the inactive X chromosome. We propose that gene silencing is the critical precursor in cancer, as it changes the dynamic interplay between *de novo* methylation and demethylation of the CpG island and tilts the balance to favour hypermethylation and chromatin inactivation.

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Introduction

Methylation of cytosine residues at CpG dinucleotides occurs by the addition of a methyl group to the carbon-5 position of cytosine through the action of the *de novo* DNA methyltransferase enzymes. So far three active DNA methyltransferase genes, *DNMT1*, *DNMT3a* and *DNMT3b* have been identified in mammalian cells (Bestor *et al.*, 1988; Okano *et al.*, 1998). Deletion of any of these genes in mice is lethal suggesting that methylation has an indispensable function in mammals (Li *et al.*, 1992; Okano *et al.*, 1999). The methylation profile of the cell is exquisitely controlled during development. The methylation pattern is transmitted to the daughter DNA strand during DNA replication by the action of the maintenance methyltransferase activity (Holliday and Pugh,

1975; Riggs, 1975). Methylation patterns are established in the early embryo with initial demethylation of the parental DNA in the first few cell divisions after fertilization, followed by *de novo* methylation of specific CpG sites between the eight cell stage and blastocyst implantation (Howlett and Reik, 1991; Mayer *et al.*, 2000; Monk, 1990; Reik *et al.*, 2001) (Global methylation changes in the early embryo are summarized in Figure 1). It was initially thought that methylation patterns, once established, were faithfully maintained at each cell division but with the advent of genomic sequencing it has become clear that the methylation state of any one CpG site is not always maintained. An apparent interplay between *de novo* methylation and demethylation at each cell division gives rise to a heterogeneous pattern of methylation for any one molecule (Warnecke *et al.*, 1998; Warnecke and Clark, 1999). However, even though heterogeneity can occur at individual CpG sites within a region, the actual regions of DNA that become methylated in the early embryo are shared.

Within the global DNA methylation changes that occur in early development there are regional specific differences in the methylation patterns that are established (Figure 1). In fact not all CpG sites are susceptible to methylation in the early embryo. The genome appears to be compartmentalized with respect to CpG methylation (Bird, 1992). The methylated compartments appear to coincide with regions of gene inactivity, whereas the unmethylated compartments coincide with regions of gene activity. Repeated sequences which comprise up to 35% of the genome are generally hypermethylated, as are gene coding regions and promoters of tissue specific genes (Yoder *et al.*, 1997). In contrast, CpG sites in CpG islands remain unmethylated through development. CpG islands have a frequency of CpG dinucleotides approximately five times greater than the genome and as a whole comprise 1–2% of the genome. They are approximately 200 bp to several kb in length, spanning the promoter and first few exons of most house-keeping genes (Gardiner-Garden and Frommer, 1987). It has been estimated that there are 29 000 CpG islands in the genome and approximately 50–60% of all genes contain a promoter-associated island (Antequera and Bird, 1993). Even though most CpG islands are protected from methylation in the early embryo, some CpG islands undergo differential allelic methylation, for example CpG islands on the inactive X chromo-

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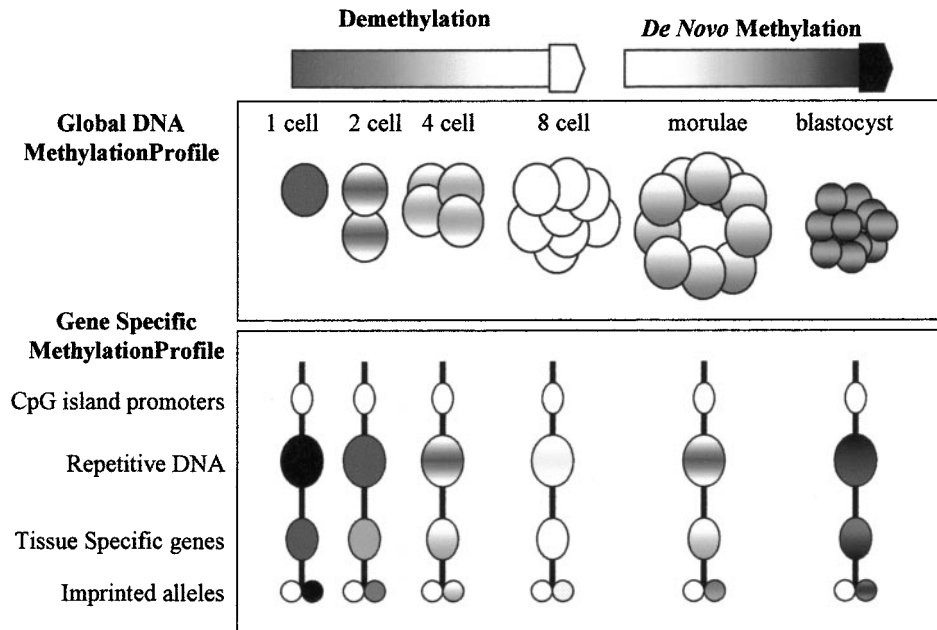


Figure 1 Global and gene specific DNA methylation changes in the early embryo. During early development the methylation profile of the embryo changes with an initial wave of demethylation from fertilization up until the eight cell stage, followed by a wave of *de novo* methylation from the morulae to blastocyst stage (implantation). The global DNA methylation profile shown in the top panel is based on restriction enzyme analysis (Monk *et al.*, 1987) and therefore principally reflects the repetitive fraction of the genome. The lower panel depicts the gene specific methylation profile, with each class of genes or DNA regions being represented as an oval on a contiguous DNA strand. The level of methylation shown is based on genomic sequencing individual candidate genes from these various regions (Warnecke, 1998; Warnecke *et al.*, 1998; Warnecke and Clark, 1999). The intensity of methylation at each stage during development for both the global methylation profile and the gene specific methylation profile is indicated by the degree of shading

some are methylated and CpG islands of many imprinted genes are also differentially methylated (Bird, 1986). Therefore in the one cell the DNA methylation profile for each sequence is finely regulated.

One of the major roles of DNA methylation in mammals is thought to be in control of gene regulation. This is because methylation within gene regulatory regions such as promoters and enhancers generally suppresses their function. For example, most promoter regions that are methylated *in vitro*, either from tissue-specific or CpG island associated genes, show reduced expression after transfection. Methylation induced suppression is thought to occur either by the blocking of transcription factor binding (Iguchi-Arigo and Schaffner, 1989; Molloy and Watt, 1990) and/or by formation of an inactive chromatin state (Bird and Wolffe, 1999; Nan *et al.*, 1998). However, it is still unclear whether methylation directly elicits gene inactivation or is a consequence of gene silencing. For example, CpG islands on the inactive X chromosome are methylated subsequent to gene silencing.

Methylation changes in cancer

In contrast to the normal cell, the methylation pattern in a cancer cell is disrupted with major changes in the methylation compartments of the cells (Baylin *et al.*, 1998; Jones, 1999) (Global DNA methylation changes

that occur in a cancer cell are summarized in Figure 2). The bulk of the genome becomes hypomethylated, in particular the normally hypermethylated and silent regions containing the repetitive elements are substantially demethylated. Conversely the normally unmethylated CpG island-containing genes often become hypermethylated and inactivated.

The mechanism responsible for genomic hypomethylation in cancer is unclear. Several possibilities have been proposed including insufficient dietary folate or genetic lesions in the folate metabolic pathway, since the livers of rats fed folate deficient diets exhibit genome hypomethylation and increased DNA strand breaks and the rats typically develop liver cancer (Pogribny *et al.*, 1997; Stern *et al.*, 2000). In addition it is also possible that hypomethylation is a result of deregulation of putative demethylase enzymes or conversely due to dysfunction of the maintenance activity of the DNA methyltransferase enzymes. Despite the fact that the pathway leading to hypomethylation in cancer is unknown it is clear that hypomethylation is a hallmark of most cancer genomes. It has been proposed that hypomethylation contributes to malignancy by either contributing directly to the activation of oncogenes (Feinberg and Vogelstein, 1983; Holliday and Pugh, 1975), activation of latent retrotransposons (Bestor and Tycko, 1996) and/or chromosome instability (Ehrlich, 2000). Evidence for activation of oncogenes by specific gene

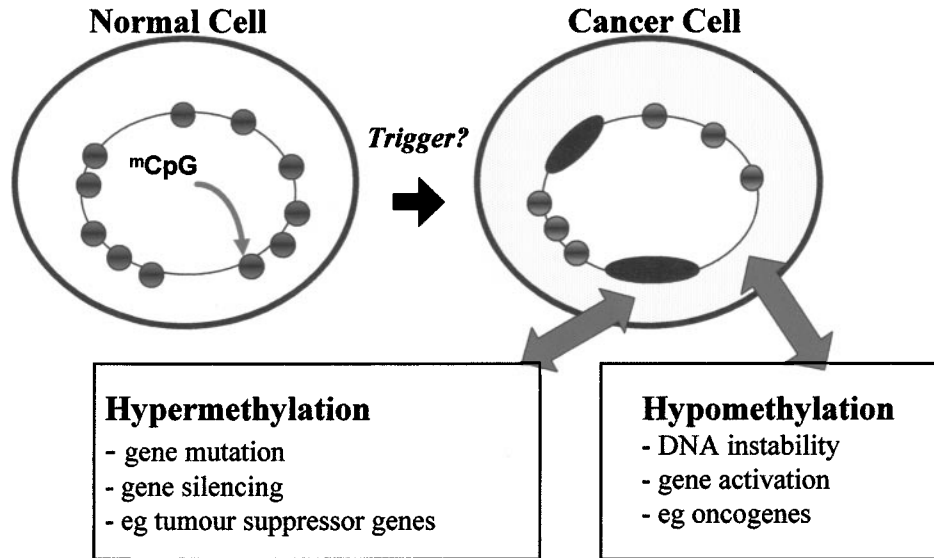


Figure 2 Global DNA methylation changes that occur in a cancer cell. In a normal cell, the methylation at individual CpG sites (^mCpG) is established in a regional specific manner, with some regions that remain unmethylated and others that are hypermethylated. This defined methylation pattern is drastically altered in a cancer cell, with some DNA regions undergoing methylation resulting in hypomethylated areas and some DNA regions undergoing *de novo* methylation resulting in hypermethylated areas. Hypomethylation in a cancer cell is linked with DNA instability and gene activation, whereas hypermethylation in a cancer cell is linked with gene mutation and gene silencing. ^mCpG depicted as shaded circles; hypomethylated areas depicted as a line; hypermethylated areas depicted as ovals

demethylation in cancer is poor, however, hypomethylation has been reported in the body of some oncogenes including cMYC and H-RAS (Vachtenheim *et al.*, 1994). Hypomethylation however has been reported to be responsible for the activation of the MAGE and related genes (De Smet *et al.*, 1999). These genes are germline-specific and the promoters of the genes are normally methylated and silent in all adult somatic tissue but can become aberrantly activated in a number of tumours. The most abundant retrotransposons in the human genome are LINE elements or long interspersed nuclear elements. Loss of promoter methylation and transcriptional activation of LINE elements have been reported in a variety of cancers (Alves *et al.*, 1996; Thayer *et al.*, 1993). The deleterious effects of hypomethylation of LINE retrotransposons in cancer has been linked with both mutational disruption of genes as well as the potential disruption of nearby gene expression. The link between chromosome instability and genome hypomethylation in cancer has come from studies in mouse ES cells that have a homozygous deletion of the methyltransferase *Dnmt1* (Chen *et al.*, 1998). The mutant ES cells have a significantly increased mutation rate primarily involving genomic deletion. Moreover chromosome instability is a feature of ICF patients, a genetic disorder in humans caused by mutations in the DNA methyltransferase *DNMT3b* (Xu *et al.*, 1999).

In the milieu of general hypomethylation, the cancer cell also gains regional specific areas that are hypermethylated. Hypermethylation is thought to contribute to cancer progression because of its potential for induction of mutations particularly in

the coding regions of genes as well as its potential for gene inactivation (Figure 2). The regions that are often hypermethylated are the CpG island regions that span the promoters of house-keeping genes and tumour suppressor genes. It is now well established that multiple genes are concurrently methylated in the one cancer cell (Melki *et al.*, 1999). Genes involved in cell cycle regulation, DNA repair, drug resistance, detoxification, differentiation, apoptosis, angiogenesis and metastasis have all been identified as being susceptible to hypermethylation in different cancers (Costello and Plass, 2001). Over half of these genes cause familial forms of human cancer when mutated in the germline and thus the selective advantage for loss of function is very clear (Baylin and Herman, 2000). However many of the hypermethylated genes in cancer are not defined tumour suppressor genes and for some of these genes the promoter methylation may be the only type of gene inactivation found in cancers, since mutations have not been observed (Baylin *et al.*, 2001). Since methylation of the associated CpG islands corresponds with inactivation of these genes in the tumours, hypermethylation has been included as an alternative mechanism to elicit allelic gene silencing of tumour suppressor genes in cancer, in addition to silencing caused by genetic mutation and/or deletion (Jones and Laird, 1999). It is clear that hypermethylation is a significant alteration in the cancer genome, however the mechanism responsible for eliciting this change is not well understood.

Not only does the methylation profile of a cancer cell change but expression of the DNA methyltransferase enzymes is often modulated. *DNMT1*

expression is elevated 2.5–3.7-fold in colon tumours (Lee *et al.*, 1996); Schmutte *et al.*, 1996) and 4.2-fold in leukaemia (Melki *et al.*, 1998) and *DNMT1* activity appears to increase progressively with advancing stages of both human colon and lung cancer (Belinsky *et al.*, 1996; Issa *et al.*, 1993). *DNMT3A* and *DNMT3B* showed 4.4- and 11.7-fold elevated transcription in acute myeloid leukaemia (AML) (Mizuno *et al.*, 2001) and significant over expression of *DNMT3B* was reported in solid tumours (Robertson *et al.*, 1999). The increased expression of the DNMTs may be functionally important in cancer, as even a 2.2-fold average increase in expression of *DNMT1* has been shown to be associated with the transformation of NIH3T3 fibroblasts (Wu *et al.*, 1993). Furthermore, reduced *DNMT1* activity in mice that are genetically predisposed to colonic adenomas markedly decreases the frequency of colon tumours (Laird *et al.*, 1995). The relationship of the various DNA methyltransferases and their exact role in directing the methylation pattern of a normal cell is yet to be addressed, therefore it is still unclear what role a change in expression may have on modulating the methylation profile of the cancer cell.

Why are some genes methylated more than others?

Recent cancer ‘methylomic’ studies predict that hundreds of CpG islands could be methylated in a

tumour cell (Costello *et al.*, 2000). However, it is clear from both the genome-wide methylation studies and candidate gene approaches that each tumour type may have its own set of cancer cell-type specific genes that are more susceptible to methylation. Thus each cancer-type may have the potential to be typed or classified according to its methylation profile (Gitan *et al.*, 2002). Cancer-type methylation differences are highlighted using a candidate gene approach in Figure 3, which shows the methylation profile of eight genes between two distinct cancer types, namely Acute Myeloid Leukaemia (AML) and prostate cancer. In prostate cancer *GSTP1* is methylated in over 95% of tumours (Lee *et al.*, 1994; Millar *et al.*, 1999), but remains unmethylated in AML (Melki *et al.*, 1999). In contrast *p15*, *E-cadherin* and *calcitonin* are commonly methylated in AML but are rarely methylated in prostate cancer. Similarly, *Rb* is unmethylated in both prostate cancer and AML but is commonly methylated in Retinoblastoma tumours (Stirzaker *et al.*, 1997). The different methylation specificities according to cancer type poses the question: ‘Why are some genes more methylated than others in different cancers?’ To address this question we need to address the converse question: ‘What is the mechanism that protects CpG islands from methylation in the normal cell and therefore what goes wrong in the cancer cell?’

CpG islands commonly span the promoter region of house-keeping genes and it is important that these genes remain transcriptionally active throughout devel-

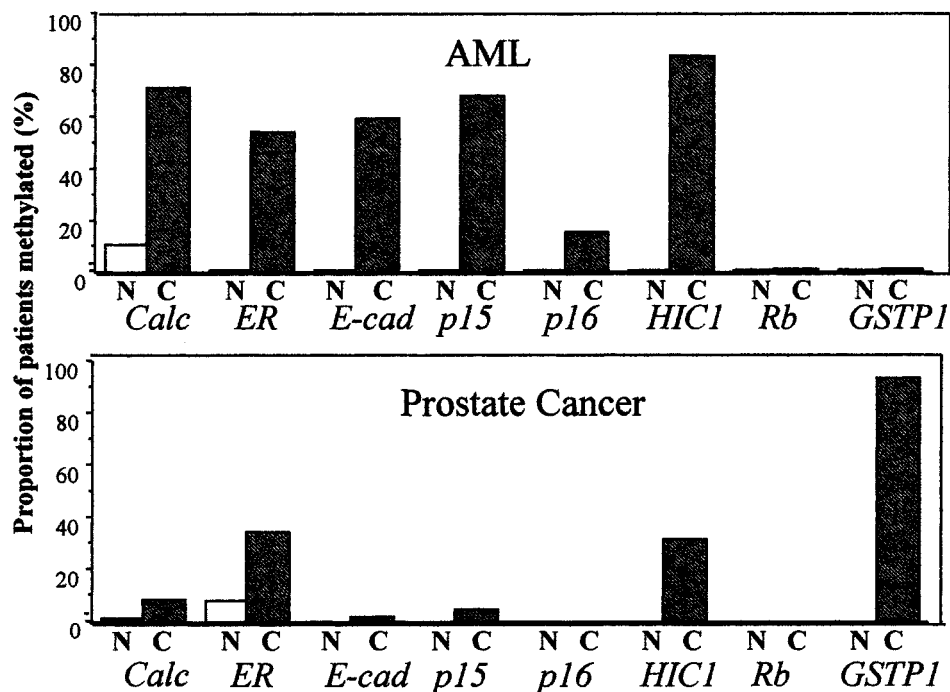


Figure 3 Cancer-specific methylation pattern. The proportion of AML and prostate cancer patients methylated in eight different tumour suppressor or tumour related genes is plotted. Methylation profile for AML was measured by direct bisulphite sequencing DNA isolated from bone marrow from 12 normal patients (N) or from 20 patients with cancer (C) (Melki *et al.*, 1999). Methylation profile for prostate cancer was measured by bisulphite sequencing DNA isolated from the prostate from 18 patients with cancer (C) or from matched normal (N) prostate cells (Clark *et al.* submitted)

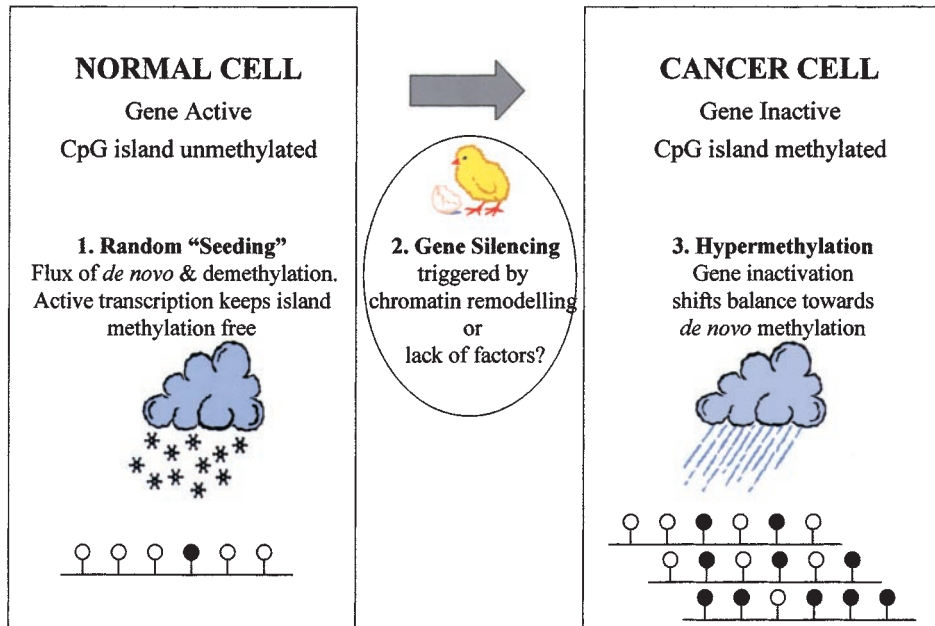


Figure 4 Hypermethylation model (seeding and silencing). It has always been a conundrum as to what comes first the chicken or the egg, that is 'Does methylation cause gene silencing or does gene silencing cause methylation?' We propose that in cancer, gene silencing comes first and triggers hypermethylation of the CpG island associated gene. In a normal cell, CpG island-associated genes are actively transcribed and remain essentially unmethylated, whereas in a cancer cell the genes are silent and associated CpG island is hypermethylated. Our model suggests that in a normal cell, CpG islands are subjected to a steady, but light 'snowfall' or 'seeding' of *de novo* methylation, but remain methylation free due to demethylation promoted by active transcription of the gene. Whereas in a cancer cell, gene silencing is a critical precursor and that in combination with 'seeding' methylation shifts the balance in the weather conditions and triggers a 'snow storm' of methylation

opment and differentiation. Since methylation of CpG islands is associated with gene silencing it makes sense that the CpG islands remain methylation free. However, CpG islands are rich in CpG sites, the target for DNA methyltransferase, and yet during early development these CpG sites remain unmethylated, even when the bulk of the genome is undergoing *de novo* methylation, including the CpG islands spanning genes on the inactive X or imprinted alleles. It is thought that the CpG islands spanning the house-keeping type genes are protected from methylation through either (1) the process of active transcription, including the possible steric hindrance of transcription factor or DNA polymerase binding, (2) active demethylation via RNA transcripts from CpG islands possibly in combination with 5MeC-glycosylase (Jost *et al.*, 1997), (3) replicating timing or (4) local chromatin structure that may inhibit access to the DNA methyltransferase (Kass *et al.*, 1997). The importance of gene activity, however as the mode of protection is exemplified by the fact that the CpG islands on the inactive X chromosome are silenced prior to methylation (Pfeifer *et al.*, 1990). Therefore, CpG islands are not intrinsically 'unmethylatable' in the early embryo but active transcription appears to be a major protective factor.

So why are some CpG island genes more susceptible to hypermethylation than others in different cancer types? If in a cancer cell there is a general deregulation

of the entire methylation and/or protective machinery we might expect a random profile of genes methylated in any one cancer and not the apparent cancer specific subsets. It is possible that cancer specific subsets arise through the activation of DNA methyltransferase enzymes or co-factors with sequence specificity, however such specificity in the DNMTs has not yet been identified. A more attractive proposal is that down regulation or silencing of particular genes in different somatic cell types, renders them more susceptible to *de novo* methylation, especially in a cancer cell which often has elevated DNA methyltransferase activity.

What triggers hypermethylation?

It is not easy to address this question in tumour tissue because DNA hypermethylation is an early event in tumourgenesis and therefore, once the tumour is large enough to detect, the aberrant methylation process has already occurred and the hypermethylated genes are already silenced. What is the evidence to support the proposal that gene inactivation may precede and in fact trigger hypermethylation in cancer?

- (1) Hypermethylation of the CpG island promoter is associated with gene silencing in tumours. When the methylation pattern of individual cells from the tumour are analysed, a variable pattern of

- methylation is often observed (Melki *et al.*, 1999, 2000; Millar *et al.*, 2000; Stirzaker *et al.*, 1997). Some molecules show very little methylation whereas other molecules can be extensively methylated. However regardless of the extent of the methylation, the gene appears to be silenced in the tumour. For example, variable levels of methylation are seen in the *Rb* CpG island in retinoblastoma tumours (Stirzaker *et al.*, 1997), in the *p15* gene in leukaemia (Aggerholm *et al.*, 1999; Cameron *et al.*, 1999; Melki *et al.*, 1999) and *GSTP1* in prostate cancer (Millar *et al.*, 1999, 2000) and yet all of these genes are commonly silenced in these specific tumours. One explanation would be that these genes are often inactivated in these cell types, possibly due to variable levels of regulatory factors, and that inactivation renders them susceptible to *de novo* methylation. However *de novo* methylation in cancer does not appear to be processive, it appears to occur randomly and accumulates with passage number, thus leading to a variable pattern of methylation both in individual cells and in the tumour.
- (2) Silenced genes are often associated with mutation, gene deletion and hypermethylation of the promoter region of the gene. However, genes can also show variable levels of expression in a cell without any apparent genetic or epigenetic mutation, possibly due to variable levels of regulatory factors. Mosaic or variable expression is often observed when normal tissues or tumour tissues or cell lines are stained for gene expression (Morgan *et al.*, 1999). It is possible that in a cancer cell the subset of genes that are susceptible to methylation are those genes that show variability in expression. For example, the *E-cadherin* CpG island-associated promoter is commonly silent in leukaemic blast cells, however only some cells show methylation across the *E-cadherin* CpG island (Melki *et al.*, 2000).
 - (3) The p16^{INK4} gene encodes a tumour suppressor gene that is often inactivated and methylated in many human tumour cell lines and primary tumours. The p16^{INK4} gene is also inactivated in human mammary epithelial cells (HMECs) that escape selection and is necessary for *in vitro* life span extension (Noble *et al.*, 1996). We and others have shown that p16^{INK4} silencing in HMECs that have escaped selection is associated with hypermethylation of the CpG island spanning the promoter of the p16^{INK4} gene (Huschtscha *et al.*, 1998). Interestingly, the cells in colonies that initially escape selection, and stain negative for p16^{INK4} expression, show very little methylation across p16^{INK4} promoter (Melki *et al.*, manuscript in preparation). Extensive hypermethylation occurs only after multiple passages of the HMECs. In this example, p16^{INK4} expression appears to be silenced prior to methylation. However, even though the gene appears to be silenced prior to hypermethylation the gene can be reactivated by treatment with

5 deoxy-aza-cytidine (5-AzaC). This suggests that hypermethylation of CpG islands may not initiate gene silencing in cancer, but instead is critical for maintenance of the inactive stage of genes, similar to methylation of genes on the inactive X chromosome (Csankovszki *et al.*, 2001; Mermoud *et al.*, 2002).

- (4) The CpG island promoter of the gene glutathione-S-transferase (*GSTP1*) is silenced and hypermethylated in over 95% of all prostate cancers (Lee *et al.*, 1994). In contrast, to other tumour suppressor genes which are often silenced by mutation or deletion, both alleles of *GSTP1* are inactivated and hypermethylated (Millar *et al.*, 1999). The *GSTP1* gene is a good example of a cancer-type specific gene because it is not often methylated in other tumours. To address why the *GSTP1* CpG island is uniquely susceptible to hypermethylation in prostate cancer but remains unmethylated in the majority of other cancers we have investigated a number of factors that are purported to be involved in triggering hypermethylation (Song *et al.*, 2002). We found that the *GSTP1* gene was not specifically targeted for methylation in prostate cancer cells and unlike the *APRT* gene in CHO cells (Brandeis *et al.*, 1994; Macleod *et al.*, 1994) removal of the Sp1 sites, or indeed other critical transcription factor binding sites, did not trigger hypermethylation of the *GSTP1* island in prostate cancer cells. Intriguingly, silencing of the *GSTP1* promoter was also insufficient to trigger hypermethylation in the cancer cells. We found that a low level of *de novo* methylation at random CpG sites, we termed 'seeding methylation', was required in combination with gene silencing, to initiate hypermethylation of the island. 'Seeding methylation' of the actively transcribed gene promoted demethylation, whereas 'seeding methylation' of the inactive gene promoted extensive *de novo* methylation of the *GSTP1* CpG island. Our results support the proposal that active transcription can play an important role in maintaining the island in an unmethylated state, possibly by promoting demethylation of any CpG sites that become spuriously methylated. Demethylation of these sites could be achieved either through an active process (Schwarz *et al.*, 2000), or through a passive process, for example lack of maintenance during replication.

Hypermethylation model

'Seeding methylation' occurs randomly at a low level in a normal somatic cell. Analysis by bisulphite sequencing has revealed that not all CpG sites in a CpG island are unmethylated in a normal cell (Cameron *et al.*, 1999; Melki *et al.*, 1999). In any one molecule there is often a few random sites of methylation in an otherwise unmethylated region. We propose, as summarized in Figure 4, that in a normal cell there is

dynamic interplay between a low but persistent level of *de novo* methylation of CpG sites within the CpG island versus a persistent process of demethylation promoted by active transcription or indeed active chromatin (Cervoni and Szyf, 2001). However, in the cancer cell, since active transcription can no longer promote demethylation, the dynamic interplay shifts to favour *de novo* methylation of the island and consolidation of gene silencing.

Conclusion

We propose that active transcription protects CpG island from methylation in the normal cell and that the sub-set of genes that are prone to hypermethylation in a specific cancer type are those genes that have previously been silenced, either transiently through

lack of factors or via chromatin remodelling. The jury is not unanimous, but the evidence suggests that methylation is not responsible for causing the gene silencing in cancer, but rather an accessory to the crime with its primary role in maintenance of silencing. We suggest that gene silencing is the critical precursor in cancer as it changes the dynamic interplay between *de novo* methylation and demethylation of the CpG island and tilts the balance to favour hypermethylation and chromatin inactivation.

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