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REVIEW

DNA methylation, imprinting and cancer

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It is well known that a variety of genetic changes influence the development and progression of cancer. These changes may result from inherited or spontaneous mutations that are not corrected by repair mechanisms prior to DNA replication. It is increasingly clear that so called epigenetic effects that do not affect the primary sequence of the genome also play an important role in tumorigenesis. This was supported initially by observations that cancer genomes undergo changes in their methylation state and that control of parental allele-specific methylation and expression of imprinted loci is lost in several cancers. Many loci acquiring aberrant methylation in cancers have since been identified and shown to be silenced by DNA methylation. In many cases, this mechanism of silencing inactivates tumour suppressors as effectively as frank mutation and is one of the cancer-predisposing hits described in Knudson's two hit hypothesis. In contrast to mutations which are essentially irreversible, methylation changes are reversible, raising the possibility of developing therapeutics based on restoring the normal methylation state to cancer-associated genes. Development of such therapeutics will require identifying loci undergoing methylation changes in cancer, understanding how their methylation influences tumorigenesis and identifying the mechanisms regulating the methylation state of the genome. The purpose of this review is to summarise what is known about these issues.
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I. Regulation of DNA methylation in normal development

Introduction

In the mammalian genome, cytosine residues may be methylated at the 5′ carbon. This occurs most commonly at 5′-CG-3′ dinucleotides but cytosine methylation can be found at 5′-CA-3′ or 5′-CT-3′ residues.[1] Since 5′-CG-3 is palindromic, the cytosine on one or both DNA strands may be methylated (hemi-methylated and homo-methylated respectively). DNA methylation is an epigenetic modification that does not alter the primary sequence of DNA and is critical for normal development, gene expression patterns and genomic stability (see Table 1). DNA methylation patterns are dynamic – unmethylated sequences can become methylated and methyl groups can be lost. For example, differences in methylation patterns have been described between oocytes and generally more highly methylated sperm DNA; post-fertilisation development is characterised by waves of genome-wide demethylation in early stages and subsequent remethylation before implantation; CpGs within promoters of many genes on the inactive X-chromosome in female cells are methylated and may be part of the X-inactivation mechanism; differentially methylated regions (DMRs) have been described for imprinted genes where the allele from one parent is methylated but the other is not (see below for more details). Of particular relevance to this review

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Table 1 DNA methylation patterns

Process	*Consequence*
	Normal
Demethylation and remethylation during embryonic development:	• A wave of demethylation is followed by reestablishment of methylation patterns in early embryogenesis and results in reprogramming of the genome.
X-chromosome inactivation:	• Silencing of genes on inactive X chromosome resulting in dosage compensation.
Genomic imprinting:	• Differentially methylated regions are regulatory sites ensuring parent-of-origin specific expression of imprinted genes.
Tissue specific methylation:	• Regulation of tissue specific expression.
Age related methylation:	• Hypermethylation of specific CpG islands in an age-dependent manner in colonic tissue results in gene silencing.
Repeat methylation:	• Silencing of interspersed repetitive retrotransposons such as ALU or LINE1 elements. Hypothesized to protect from deleterious gene insertion events. • Contributes to genomic stability by hypermethylation of heterochromatic satelite repeat sequences.
	Abnormal
Process	*Consequence*
Hypermethylation of CpG islands:	• Inactivates tumour suppressor genes and other cancer related genes. • Areas of frequent chromosomal breakage correlate with DNA hypermethylation.
Hypomethylation:	• Chromosomal instability caused by hypomethylation of normally methylated satellite repeat sequences or other heterochromatic sequences. • Oncogene activation due to point mutations in methylated CpG dinucleotides or activation of normally silent genes. • Gene deletions due to mitotic recombination or chromosomal loss.
Coding region hypermethylation:	• 5-methylcytosine is deaminated and results in a C-T point mutations leading to altered amino acid sequence and or premature stop codons.
Methylation changes in differentially methylated regions:	• Dysregulation of imprinted genes resulting in either biallelic expression or loss of expression in imprinted genes.

is the fact that perturbations in DNA methylation have been associated with abnormal developmental processes including cancer.

Establishment of methylation patterns (DNMTs and their actions)

Methylation is established by *de novo* methyltransferases (see Figure 1). Replication of homomethylated DNA produces hemimethylated DNA in which one strand of the DNA remains methylated and the newly synthesised is unmethylated. Hemimethylated DNA can become homomethylated by maintenance methyltransferases which place a methyl group at the 5′-CG-3 complimentary to a methylated 5′-CG-3. The first DNA methyltransferase identified, DNMT1, was shown to have both *de novo* methylation[2] as well as maintenance activity, although the *de novo* activity is much weaker than the maintenance activity. In cells lacking DNMT1 maintenance methylation is not abolished indicating other DNMTs have maintenance activity.[3] Two more potent *de novo* methyltransferases have been identified: DNMT3A and DNMT3B. Neither is required for maintenance activity since ES cells deficient for either DNMT maintain the pre-existing methylation patterns.[4] The importance of each of these enzymes for normal development has been demonstrated in mouse mutants that lack the individual genes. Mouse embryos homozygous for mutant alleles of *Dnmt1*, *Dnmt3a* or *Dnmt3b* die early in development either before birth (*Dnmt1*, *Dnmt3b*), or a few weeks after birth (*Dnmt3a*). These experiments dramatically revealed the importance of DNA methylation in normal development, but also identified individual target sequences for each of the enzymes. For example, DNMT3B is required for the methylation of centromeric minor satellite repeat sequences and both DNMT3A and DNMT3B are involved in methylation of C-type retroviral repeats, IAP repeats, major satellite DNA and the differentially methylated region in *Igf2*. Sequence specificity of DNMT3A and DNMT3B seen in *in vivo* experiments was not seen in *in vitro* assays indicating the requirement for additional factors that direct methyltransferase activity.[5] Proteins with homology to the other DNMTs (DNMT2 and DNMT3L) and alternatively spliced forms of DNMT1 and DNMT3B with altered function have been reported,[6,7] however their functions are not completely understood.

DNA demethylation may occur actively by an enzyme with demethylating activity, or passively by several rounds of replication in the absence of maintenance methyltransferase activity. There is evidence that both processes occur. Active demethylation occurs predominantly on the paternally transmitted chromosomes in zygotes while maternal chro-

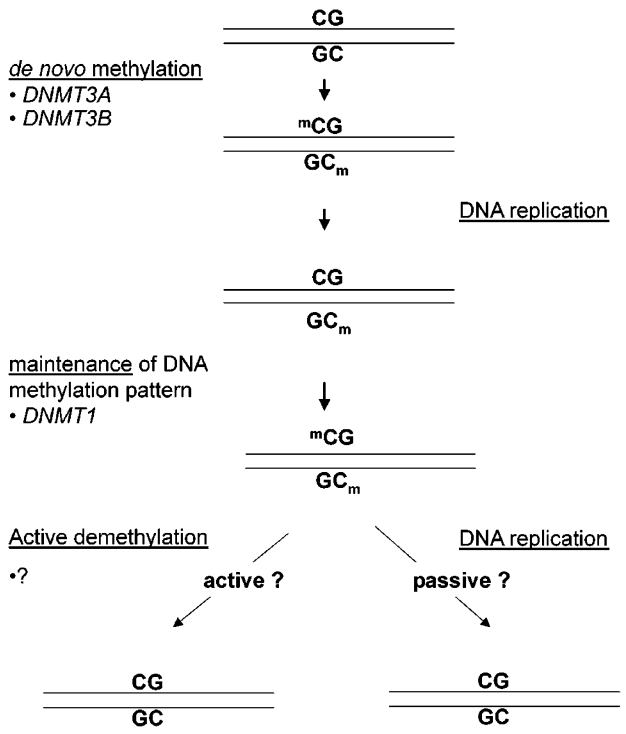

Figure 1 Enzymes and reactims involved in the establishment of DNA methylation patterns (see text for explanations).

mosomes undergo passive demethylation in later cleavage stages.[8]

Trans-acting regulatory factors

Identifying the factors that regulate establishment and maintenance of DNA methylation in both normal and cancer genomes will be important for identifying targets of therapeutic intervention. Few details are known about *trans*-acting factors regulating methylation in mammals and other organisms. While the DNMTs are clearly central to methylation establishment and maintenance, additional *trans*-acting factors regulating the timing and targets of their activity must exist, otherwise, methylation patterns would be static and homogenous across the genome. In *Neurospora crassa*, a histone methyltransfease is required for DNA methylation.[9] The observations that DNMT1 can be detected in complexes with Rb, E2F1 and HDAC1[10] and also with MBD2 and MBD3 localised to replication forks in late S phase[11] highlight these associated factors as potential regulators of methyltransferase activity. These results also link DNA methylation to sequence-specific DNA binding activities and cellular activities regulating growth control. Also, mutations in ATRX (see below) have multiple effects on methylation implicating this factor in methylation regulation.

Cis-acting regulatory factors

Information about *cis*-acting regulation of DNA methylation has come from studies of the imprinted loci (see below). The reproducible, parent-of-origin-specific patterns of methylation detectable at those loci provide models for identifying DNA elements that determine whether or not the locus acquires methylation. The *Igf2r* locus is methylated on the maternal allele which is also the expressed allele in mice. A 3 kbp intronic element termed region 2 was shown in transgenic studies to contain the imprinting center needed for imprinted methylation and expression.[12] Subsequent studies identified two sequences within region 2 that regulate maternal allele-specific methylation in the post-zygotic, pre-implantation embryo. One sequence termed the DNS provides a *de novo* methylation signal and is needed for methylation establishment of the maternal allele. A second sequence called the ADS provides an allele discrimination signal that protects the paternal allele from acquiring methylation directed by the DNS. A factor detectable in both androgenetic and gynogenetic ES cells can interact with the DNS while a factor detectable only in androgenetic ES cells interacts with the ADS.[13] Presumably, the methylation patterns seen in later embryos and adults at the endogenous *Igf2r* locus depend upon these *cis*-acting elements.

The *Rasgrf1* locus is methylated on the paternal allele. Expression is also paternal allele-specific in neonatal brain.[14] Deletion of a repeated sequence element (40 copies of a 41-mer) 3′ of the differentially methylated domain prevented establishment of paternal allele methylation in the male germ line. Methylation patterns, once disrupted at the establishment stage, never became established in the soma.[15] Interestingly, repeats and inverted repeats have been described as regulating local methylation in mouse cells[16] and in plants.[17]

At the *H19/Igf2* locus, a region 5′ to the *H19* gene is methylated on the paternal allele. Deletion of 2 kbp of this region did not perturb establishment of methylation of the remaining differentially-methylated sequences, however, established methylation patterns were not efficiently maintained.[18] This highlights potentially important differences between regulation of methylation establishment in the germ line and maintenance of established patterns in somatic tissue. A recent study identified *Dnmt3L* as a key player in the establishment of DMR methylation. *Dnmt3L* is expressed during gametogenesis at stages where genomic imprints are established. Mice lacking this gene do not establish methylation on the maternal allele in DMRs indicating the importance of DNMT3L for the imprinting process.[19]

Further clues about *cis*- and *trans*-acting factors regulating methylation in mammals may be provided by genetic screens in plants and fungi for modifiers of methylation-dependent processes.[17,20–22] Results from these studies demonstrate conservation in plants of factors known to be involved in

DNA methylation in mammals. It is likely that these systems will reveal important clues about how methylation control goes awry in human cancer.

Methylation and transcription

It is now well established that DNA methylation is involved in regulating gene transcription. This can occur by a variety of mechanisms. The interactions of several transcription factors whose binding sites contain CpG dinucleotides has been shown to be methylation-sensitive.[23,24] However, methylated DNA more profoundly affects transcription by interacting with methyl-CpG-binding proteins and associated factors that alter chromatin structure.

The first two methyl-CpG-binding proteins (or protein complexes) to be identified were MeCP1 and MeCP2.[25,26] MeCP2 is a single polypeptide with a methyl CpG binding domain (MBD) and a transcriptional repression domain (TRD).[27] Interestingly, mutations in MeCP2 cause Rett syndrome, one of the leading causes of mental retardation and autistic behaviour.[28] The MBD motif is found in four additional methyl-CpG-binding proteins (MBD1, 2, 3, and 4).[29] One of these (MBD2) facilitates the binding of the multiprotein MeCP1 complex to methylated DNA.[30] How proteins with methyl-CpG-binding activities repress transcription is under active study. There is evidence for a variety of complex mechanisms. One key mechanism involves MBD-mediated recruitment to methylated DNA one of two co-repressor complexes, Sin3 and Mi-2/NuRD, which in turn recruit a core histone deacetylase complex consisting of HDAC1, HDAC2 and two Rb associated, histone-binding proteins, RbAP46 and RbAP48 ([31–33] reviewed in[34,35]). Additional co-repressor complexes exist, however, their role in silencing mechanisms that involve DNA methylation has not been demonstrated. HDACs remove acetyl groups from the lysine residues found at the N-termini of histone H3 and H4 (reviewed in[36]). Their removal results in an increase in the positive charge of the histones which is hypothesised to condense chromatin by enabling a tighter association between the histones and the negatively charged DNA. This may in turn silence transcription by limiting transcription factor binding. The Mi-2/NuRD co-repressor complex which recruits the HDAC core complex also includes factors that remodels chromatin by ATP-dependent mechanisms.[37] These activities can reposition nucleosomes on DNA which may restrict interactions between the DNA and transcription factors.

This description is surely an over-simplification. First, the chromatin remodelling and histone deacetylation activities are interdependent. It has been observed that the remodelling activity of Mi-2/NuRD is required for Mi-2/NuRD-dependent deacetylation by the core HDAC complex ([37] reviewed in[38]). Second, DNA methylation may require HDAC activity or components of the chromatin remodelling apparatus. In support of this are the observations that treatment of *Neurospora crassa* with the HDAC inhibitor trichostatin A (TSA) can lead to loss of DNA methylation,[39] mutations in *ATRX*, a member of the SWI/SNF chromatin remodelling family, causes regional hyper- and hypomethylation in the genome[34] and DNMTs form complexes with HDACs.[5,10,40,41] Third, adding to the varied interrelationships among silencing mechanisms is the fact that HDACs recruit sequence-specific transcriptional repressors that contribute to silencing, so transcriptional repression by HDACs may not rely solely upon DNA methylation to identify loci to be repressed. Similarly, acetylation- and methylation-mediated repression may function independently. This is supported by studies using the demethylating agent 5-aza-dC and the HDAC inhibitor, TSA. *In vitro* studies show that for several repressed loci, both drugs were needed for maximum de-repression – one alone would not suffice.[30] Taken together, these observations highlight the complex interactions among DNA methylation, histone acetylation, chromatin remodelling and sequence-specific silencing mechanisms that collectively attenuate gene expression.

While methylation is commonly associated with transcriptional repression, there are at least two cases at imprinted loci where methylation results in transcriptional activation. At the imprinted loci *H19/Igf2* and *Rasgrf1*, the differentially methylated domains possess enhancer-blocking activity that restricts enhancer to promoter interactions, preventing promoter activation ([42,43] and Yoon *et al*. unpublished). Binding of the factor CTCF, which is required for the blocking activity, is methylation-sensitive (Figure 2). Methylation of the CTCF site blocks binding and restores enhancer to promoter interactions which activates expression of *Igf2* or *Rasgrf1*. Biallelic *lgf2* expression seen in cancers may involve interference with this mechanism. The mechanism by which CTCF prevents transcription may involve the binding of SIN3A and associated HDACs to an internal 11 Zn finger-containing region of CTCF and possibly additional co-repressors to the C-terminus.[44]

There may be additional mechanisms by which DNA methylation affects transcription. *In vitro* studies have shown that methylation at cytosine residues can cause several changes in DNA structure (reviewed in[45]). Methylation has been shown to increase the helical pitch of DNA,[46] alter the rate constants for cruciform formation, lower the free energy of Z-DNA formation, and promote helix unwinding at B/Z-DNA junctions.[47] If these DNA structural changes occur *in vivo* and affect binding of transcription-regulating factors is not known.

Regardless of the mechanisms by which methylation regulates gene expression, a large number of genes respond to methylation levels. In studies using fibroblast cells in which a *Dnmt1* deletion was induced, up to 10% of over 5000 genes examined underwent significant changes in expression upon induction of the deletion.[48]

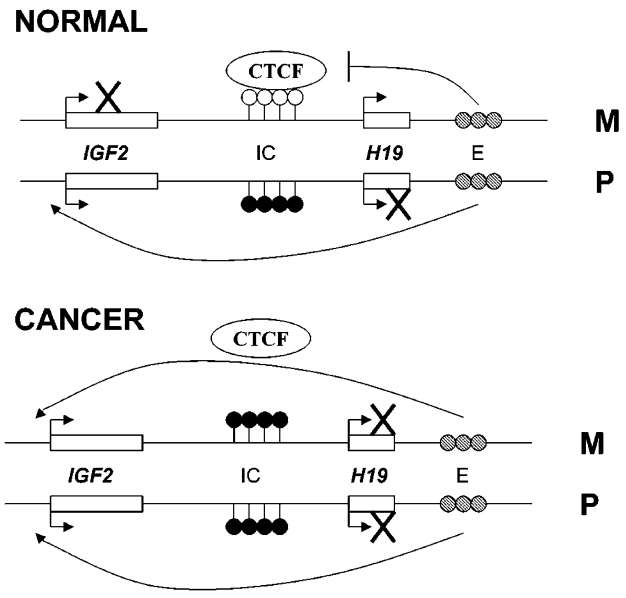

Figure 2 Methylation and the regulation of imprinted *IGF2/H19*. *IGF2* and *H19* are two imprinted genes located within the same chromosomal domain. *IGF2* is normally transcribed from the paternal (P) allele while *H19* is transcribed from the maternal (M). Imprinted expression in both genes is regulated by an imprinting center (IC) in which the maternal allele is unmethylated (open circles) and the paternal is methylated (filled circles). The imprinting center contains CTCF binding sites to which CTCF binds only if they are unmethylated. Binding of CTCF blocks the activity of the enhancer element (E) located downstream of *H19* and restricts activity to *H19* expression. Methylation of the CTCF binding sites prevents the binding of CTCF and allows the enhancer to activate *IGF2*. Biallelic expression of the CTCF binding sites in cancer results in *IGF2* overexpression.

Compartmentalisation of the genome by DNA methylation patterns

The human genome is divided into several functionally different compartments with different base composition. Compartments include gene rich or gene poor regions, intergenic sequences, interspersed repetitive elements, and middle repetitive sequences such as the centromeric repeats. Further compartmentalisation comes through epigenetic modifications including different densities of chromatin condensation and DNA methylation patterns. CpG dinucleotides, the major target sequences for DNA methylation, are underrepresented in the genome compared to other dinucleotides. However, some GC-rich sequence stretches contain a higher frequency of CG dinucleotides. For example, CpG islands are GC-rich sequences between 0.2 and 2 kb in size and contain a higher than expected number of CpG dinucleotides (CpG to GpC ratio >0.6) and have a GC-content of more than 50% (reviewed in[49]). The majority of CpG islands are found in the promoter regions of genes. The majority of all CG dinucleotides in the genome are found repetitive elements such as rDNA, satellite sequences and centromeric repeats, and are usually unmethylated.

Genomic imprinting and the involvement of DNA methylation

The importance of specific DNA methylation patterns for developmentally appropriate gene expression is most clearly demonstrated for the imprinted loci. Normally, genes are expressed from both the maternal and the paternal alleles. At the imprinted loci, only the maternal or the paternal allele is expressed (for reviews see:[50–53]). Over 50 imprinted loci have been found in mammals (see http://cancer.otago.ac.nz/IGC/Web/home.html and http://www3.ncbi.nlm.nih.gov/Omim/ search term 'imprint'). This restriction may be limited to specific tissues or times during development. The methylation status of the DNA surrounding an imprinted locus also displays a pattern that is unique to each allele. The locations of the differentially methylated domains or regions (DMDs or DMRs) are variable and the expressed allele may show both hypo- and/or hypermethylated domains ([15,54,55] reviewed in[56]). These parental allele-specific methylation patterns may be established in primordial germ cells and are detectable in gametes, alternatively, they may appear in the zygote after fertilisation. Evidence indicates that the allele-specific patterns of methylation are what direct allele-specific expression.[15,57] In the case of the *H19/Igf2* and *Rasgrf1* loci, the DMRs have enhancer blocking activity and bind CTCF in a methylation-sensitive manner[42,43,58] (and Yoon unpublished result). As described earlier, CTCF bound to an unmethylated DMR represses enhancer to promoter interactions needed for *Igf2* and *Rasgrf1* expression and this block is relieved allowing expression when the DMRs are methylated and CTCF binding is prevented.

Other allele-specific phenomena can be observed at imprinted loci. These include differences in the timing of replication[59] and chromatin structure[55,60,61] of the two parental alleles. These results reveal that whatever the mechanism of genomic imprinting, it influences several DNA-based phenomena and not just gene transcription.

II. Methylation changes in cancer

For many years, genetic changes that alter primary DNA sequence were thought to be the mechanism by which critical gene activities were lost in cancer. While these largely irreversible mutations are very common in cancer, it is now well established that DNA methylation plays a significant role in loss of gene function (reviewed in[62,63]). Either homozygous methylation or methylation in combination with one of the genetic alterations has been described for multiple tumour suppressor genes and candidate cancer genes. Methylation can provide one of the hits postulated in

Knudson's two hit hypothesis to inactivate tumour suppressor genes. Hypermethylation events in usually unmethylated CpG islands are found in a large number of cancer genes. Hypo- and hypermethylation events are found in the same tumour samples (reviewed in[64]) indicating a defect in the regulatory mechanisms that participate in the establishment and maintenance of methylation patterns.

Global hypomethylation in human malignancies

Methylation changes in human malignancies were first reported almost 20 years ago.[65] Studies by Ehrich[65] and Gama-Sosa,[66] using HPLC to determine the 5′-methylcytosine content in genomic DNAs, demonstrated a reduction of the overall amount of 5′-methylcytosine relative to normal tissues. Subsequent studies demonstrated a positive correlation between the degree of hypomethylation and the increasing malignancy grade or a correlation[66–68] between tumour size and histological grade suggesting that hypomethylation may be a useful biomarker with prognostic significance.[69] While global assays show that genome-wide levels of methylation decrease in many tumours, they do not provide information on where the hypomethylation occurs.

Hypermethylation in cancer

While global hypomethylation is detectable in the cancer genome, regions of hypermethylation are commonly found. Locus-specific methylation changes were initially studied using methylation sensitive restriction enzymes to digest genomic DNAs. A major breakthrough was made with the finding that sodium bisulphite treatment converts unmethylated cytosines to uracil whereas methylated cytosines remain unmodified.[70] This treatment generates different sequences based on the methylation status of a gene. Subsequent PCR reactions using bisulphite-treated DNA can be performed to detect specifically the methylated sequences (MsPCR, Ms SNuPE).[71,72] Alternatively, PCR products can be cloned and sequenced to identify methylated regions. A major emphasis in the past was the investigation of promoter methylation in known tumour suppressor genes. The list of genes that are found to be inactivated by DNA methylation events is growing rapidly and includes genes involved in apoptosis, angiogenesis, cell-cycle, differentiation, DNA repair, metastasis, signal transduction and transcription. Defects in a number of these genes have been identified as the underlying cause of familial cancer syndromes (reviewed in[64]).

The effect of DNA methylation on transcription in cancer cell lines show that hypermethylation most commonly causes gene silencing. Treatment of these cell lines with 5′–aza-2′-deoxycytidine results in demethylation of previously methylated sequences and subsequent activation of the gene under investigation.

CpG island hypermethylation

More recently, two methods were introduced that allow scanning for CpG island hypermethylation in tumour samples. Differential methylation hybridisation (DMH) is an array based approach in which CpG island sequences are spotted in high density onto nylon membranes.[73] These arrays are subsequently hybridised with pools of PCR products derived from CpG islands that were amplified from genomic DNAs following *Bst*UI digest. *Bst*UI is a methylation sensitive restriction enzyme and its recognition sequence is frequently located in CpG island sequences. PCR products are only amplified when the target sequence is methylated and thus undigested. Array profiles from normal and tumour tissue can be compared and methylated sequences are visualised by stronger signal intensities in the tumour profile compared to the normal tissue profile. This technique was used to study methylation in breast cancer and it was shown that overall levels of CpG island methylation correlate with histological grades. Poorly differentiated tumours appeared to show more hypermethylated CpG island sequences than moderately and well differentiated tumours.[74]

Restriction Landmark Genomic Scanning (RLGS), a two-dimensional gel-electrophoretic technique, allows the assessment of methylation patterns in up to 2000 CpG islands per gel by using rare-cutting, methylation sensitive restriction enzymes (e.g. *Not*I or *Asc*I)[75]. RLGS profiles display unmethylated CpG islands, whereas methylated CpG islands are not displayed. Aberrant methylation in primary tumours or cancer cell lines is identified by comparing tumour RLGS profiles to profiles from matching normal DNAs. Loci aberrantly methylated in tumours are easily cloned using arrayed boundary libraries and subsequent database searches allow to link the methylation events to genes or ESTs.

Genome-wide scans for methylation changes in CpG islands showed that the frequencies of methylation are different between tumour types.[75] Breast, head and neck and testicular tumours showed either no or relatively low frequencies ($<1\%$) of methylated CpG islands. Other malignancies including acute myeloid leukemias, colon cancer and brain tumours showed an overall much higher frequency of methylation ranging up to 10% of all CpG islands tested. Patterns of methylation were not random, suggesting the existence of mechanisms that either allow the preferential methylation of certain CpG islands or a selective pressure that favours the growth of cells with specifically methylated CpG island sequences. The non-random nature of CpG island methylation patterns was underlined by the finding of several hypermethylated CpG islands in the major breakpoint cluster region for medulloblastomas on chromosome 17p11.2, suggesting a possible link between chromosomal instability in this region and hypermethylation events.[76] Acute myeloid leukaemias showed a prevalence of hypermethylated CpG islands on chromosome 11, again supporting the finding that methylation patterns are not random.[77] Careful inspection of the methylated target

sequences allowed the identification of sequences that were methylated in several different tumour types and others that were methylated in a tumour-type specific manner. This finding is in line with reports by other groups that have found BRCA1 promoter methylation only in breast and ovarian cancers, VHL promoter methylation only in clear cell renal carcinomas and hemangioblastomas, while other tumour suppressor genes including p16, DAPK, MGMT, have been found methylated in multiple tumour types.[64]

Methylated genes

Both strategies, the gene-by-gene approach as well as the genome scanning approach, provided long lists of methylation targets.[64,77–79] The significance of tumour suppressor gene inactivation in tumour progression has been already established. However, this is not the case for the genes identified in the genome scans for aberrant methylation. Genome scans may identify candidate tumour suppressor genes and genes for which no tumour suppressor function has been established. Several current examples should be highlighted. First, RLGS was used to scan hepatocellular carcinomas and identified several RLGS sequences that were methylated in the tumour samples.[80] Cloning of one of these loci (spot 7) identified the promoter region of suppressor of cytokine signalling (*SOCS1*).[81] *SOCS1* is a member of the JAK/STAT pathway where by binding of SOCS1 to JAK2 phosphorylation is inhibited. Silencing of *SOCS1* by promoter methylation results in constitutive activation of the JAK/STAT pathway and subsequent transactivation of target genes. The second example is bone morphogenic protein 3B (*BMP3B*), identified in a screen for aberrant methylation in non-small cell lung cancers.[79] *BMP3B* is a member of the transforming growth factor-β (*TGFβ*) superfamily, a group of secreted polypeptides that regulate a diverse spectrum of developmental processes. Members of this superfamily signal through Ser/Thr kinase receptor which subsequently propagate signals to the SMAD pathway. Methylation of the *BMP3B* promoter was shown to correlate with gene silencing and could be reactivated in lung cancer cell lines by 5′-aza-2′-deoxycytidine. The effects of *BMP3B* silencing for tumour progression need to be demonstrated,[79] however, *BMP3B* is located on chromosome 10q11, an area that shows loss of heterozygousity in lung cancer.

RAS effector homologue (*RASSF1*), a novel lung cancer tumour suppressor candidate gene, is located in a commonly deleted region of chromosome 3p. *RASSF1A*, the major transcript of this gene, is also silenced by DNA methylation in lung tumours.[82] Subsequent reports have also demonstrated the silencing of *RASSF1A* by promoter methylation in several other cancer types.[83,84] Re-expression of *RASSF1A* in lung cancer cell lines resulted in reduced numbers of colonies in colony formation assays, suppressed the anchorage independent growth and most importantly inhibited the formation of tumours in nude mice.[82]

Methylation profiles and clinical diagnostics

Genome scans such as RLGS allow the correlation of thousands of methylation events to clinical data. Frühwald *et al*. identified eight sequences in an RLGS scan that showed statistical significant correlation with survival of medulloblastoma patients.[85] Surprisingly, one of these methylation events correlated with improved outcome. RLGS analysis in AML patients identified a sequence within the *WIT1* gene that was methylated at a higher frequency in relapsed AML as compared to diagnostic samples thus correlating with a chemoresistant phenotype.[86]

Dysregulation of genomic imprinting in cancer

In addition to silencing known and candidate tumour suppressors, aberrant DNA methylation affects expression of imprinted genes. Loss of imprinting (LOI) of *IGF2* and the tightly-linked *H19* locus has been associated with tumorigenesis in a variety of patients. In Wilms' tumour, imprinted expression of *IGF2* is commonly relaxed resulting in maternal allele expression.[87,88] This is associated with increased methylation and reduced expression from the maternal copy of the tightly linked *H19* locus.[89–91] Similar patterns of LOI for either or both of these loci have been seen in patients with hepatoblastoma,[92] uterine leiomyosarcomata,[93] cervical carcinoma,[94] renal cell carcinoma,[95] rhadbomyosarcoma,[96,97] gliomas[98] and colorectal cancer.[99,100] It is not known if LOI of *IGF2* or other imprinted loci is a cause or a consequence of neoplastic transformation, however, the importance of *IGF2* in human cancer is supported by the observation that patients with Beckwith-Wiedemann Syndrome (BWS) express both *IGF2* alleles and are predisposed to several cancers.[101–103]

The mechanisms and methylation events underlying the LOI at *H19* and *IGF2* vary from tumour to tumour, however, maternal allele methylation is commonly acquired at sequences that include the *H19* promoter and *IGF2* enhancer blocker[104,105] which can simultaneously silence *H19* and activate *IGF2* on the maternal chromosome[42] (see Figure 2).

Imprinted loci other than *H19*/*IGF2* have also been implicated in cancer. In neuroblastomas without *N-MYC* amplification, maternal-specific deletions of 1p36 have been reported.[106] This region was later shown to contain the *p73* tumor suppressor, a p53 homologue which is maternally-expressed.[107] Silencing of the active maternal allele by methylation has been observed in acute leukaemia and Burkitt's lymphoma[108] but it is possible that methylation-independent means of silencing occur in other cancers.[109] *p57KIP2*, a cyclin dependent kinase inhibitor, is expressed primarily from the maternal allele.[110] In lung cancers with 11p15 deletions, the maternal allele is predominantly deleted.[111] In Wilms' tumours, it is substantially silenced,[112] however, it is not clear if this results from aberrant methylation. *NOEY2*/*ARHI* is a maternally-expressed tumour suppressor commonly found to be silenced by gene deletion in breast and ovarian cancers. The methylation status of

intact alleles from tumours is not known.[113] In hepatocellular carcinomas, multiple imprinted loci within the 11p15 region including *CDKN1C*, *SLC22A1L*, and *IGF2* genes were aberrantly silenced.[114]

Causes of aberrant methylation

Little is known about the mechanisms regulating DNA methylation in normal development and less is known about how these mechanisms go awry in cancer. Many correlative studies revealed increased levels of DNMTs in some cancer tissues or cells (reviewed in[115]), however, this was not universal.[116] Furthermore, even when the correlation held, over expression of DNMTs alone was not sufficient for altered methylation patterns.[117] Experimental models have supported the importance of DNMT expression levels in cancer development. In mice of strains susceptible to tumour formation, increased levels of *Dnmt1* and methylated DNA were found in lungs from carcinogen-treated animals when compared to mice of resistant strains. However, in the strains used, there were many other strain-specific differences beyond *Dnmt1* expression levels.[118] In more easily controlled studies using *Min* mice which are predisposed to develop intestinal tumors, experimental reduction in *Dnmt1* levels reduced tumour incidence.[119] This is consistent with earlier observations that *Dnmt1* has transforming activity *in vitro*.[120]

Regardless of the levels of DNMT protein, regulation of DNMT activity may be lost in cancer cells or precancerous lesions. This may be through changes in DNMT-associated factors that may regulate activity or subcellular localization. HDAC2, DMAP1 and TSG101[40] and two MBDs (MBD2 and MBD3)[11] have all been shown to interact with DNMT1 and a histone methyltransferase is important to DNA methylation in *Neurospora*.[9] If any of these are important for regulating DNMT activity in mammals and whether interfering with such regulation contributes to aberrant DNA methylation in cancer is not known.

III. Perspectives

As DNA methylation is important in the regulation of gene expression one could argue that the identification of aberrant methylation patterns could serve as a biomarker with predictive values for future progression or for the response to treatment. Several groups have been interested in these approaches. In a recent study by Esteller *et al*. the authors describe promoter methylation in the DNA repair gene O6-methyl-guanine-DNA methyltransferase (*MGMT*).[121] MGMT repairs alkylated sites in the DNA that otherwise would form cross-links between adjacent DNA strands. Alkylation of the DNA is the underlying principle of chemo-therapeutics such as carmustine that kill tumour cells and are used to treat patients with gliomas. Silencing of *MGMT* by promoter methylation was shown to correlate with the responsiveness of the gliomas to the treatment with alkylating agents. A different approach was used by Palisamo *et al*. in lung cancers where highly sensitive MS-PCR reactions were used to detect *p16* and *MGMT* promoter methylation in sputum of patients up to three years prior to clinical diagnosis).[122] This strategy offers the intriguing possibility of a population based screening for the detection of lung cancer. The identification of a powerful biomarker does not require the knowledge of the effect of DNA methylation on gene transcription. Several studies have identified methylated target sequences with statistically significant values.

The past years have moved the field of DNA methylation to a new level and improved our knowledge of targets affected by aberrant methylation in the cancer genome and how these changes lead to altered gene expression. Using model systems and selected genes, especially imprinted genes, it became possible to design studies that helped to explain the underlying mechanisms that regulate methylation changes. Future work will focus on several unanswered questions to determine how sequences become targets for aberrant methylation in cancer, to determine the regulatory mechanisms that malfunction in this disease and to determine the functional consequences of aberrant methylation. Identification of the factors regulating DNA methylation and demethylation may reveal novel targets for therapeutic intervention. Because DNA methylation does not alter the primary DNA sequence and is reversible, epigenetics-based therapeutics may be able to restore the activity of silenced tumor suppressors even in advanced tumours.

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