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New insights on the role of DNA methylation from a global view

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1. ABSTRACT

In mammals, DNA methylation is a crucial epigenetic modification with key functions during development. Cellular processes that are regulated by DNA methylation comprise X chromosome inactivation, gene imprinting, genomic stability and transcriptional regulation. Generally, the methylation status of the majority of target sites is reliably propagated during mitosis. However, advances in genome-wide DNA methylation analysis at base-resolution have discovered a substantial amount of differential DNA methylation between normal cells of different tissue-origin. Moreover, aberrant DNA methylation changes are linked with a significant number of human diseases, particularly with cancer. Sites of differential and aberrant DNA methylation include regulatory DNA sequences, such as CpG islands in promoters and distal *cis*-regulatory elements, like enhancers. In this review, we will discuss novel aspects of DNA methylation dynamics, during normal development and in association with diseases.

2. INTRODUCTION

The establishment and maintenance of cell-type specific gene regulatory programs is vital for normal development of multicellular organisms. The "memory" of cellular gene expression patterns is kept through epigenetic mechanisms, particularly the modification of chromatin by DNA methylation.

In vertebrates, DNA methylation predominantly targets the fifth carbon residue of cytosines within the context of CpG dinucleotides in a symmetric manner (1-3). Genomic DNA methylation patterns have a crucial role in development, X chromosome inactivation, transcriptional regulation, genomic stability and gene imprinting (4, 5). A strong inverse correlation of DNA promoter methylation and transcription has imposed the perception that DNA methylation is always linked to repression of gene promoters and repetitive sequences (6, 7). However, mounting evidence substantiates the role of DNA methylation across gene bodies, in alternative intragenic gene promoter usage (8), modulation of intronic enhancers (9, 10) and alternative splicing (11-15).

Ensuring epigenetic inheritance, CpG methylation is maintained during replication by the activity of DNA methyltransferase (DNMT) 1 (16). DNMT1 is the major "maintenance" methyltransferase, as it recognizes preferentially hemi-methylated DNA as a substrate and copies the pattern reliably to the daughter strand (17). On the other hand, DNMT3a and DNMT3b are classified as "*de novo*" methyltransferases and are indispensable for the establishment of DNA methylation patterns during early embryonic development (18, 19). However, there is evidence that the classification of DNMTs into "maintenance" versus "*de novo*" is not absolute, and that enzymes share both activities to some extent *in vivo* (20-22). Particularly at repetitive sequences DNMT3A/3B fill in for DNMT1 and restore incompletely maintained DNA methylation levels (21, 23). The model of stable persistence of DNA methylation throughout development by the cooperative action of DNMT enzymes, had to be further revised after the discovery of enzymes that actively remove DNA methylation. The

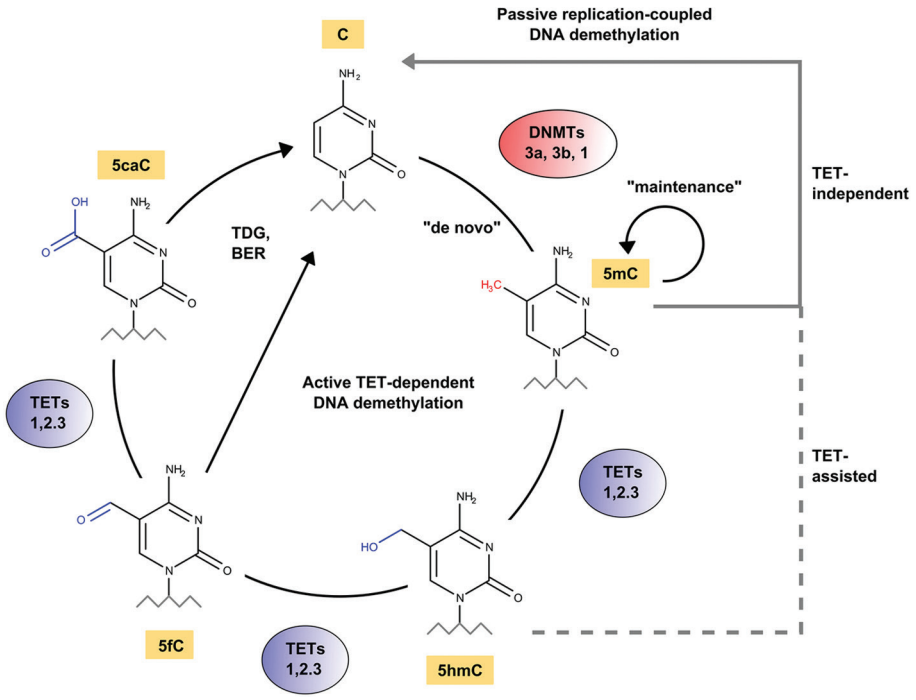

Figure 1. Pathways of active and passive DNA demethylation. DNA methyltransferases (DNMTs) catalyze the methylation of cytosine by transferring a methyl group to the position C5. Ten-eleven translocation (TET) enzymes can catalyze the oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC). Further TET-dependent oxidative reactions lead to the successive conversion of 5hmC into 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). 5fC and 5caC are recognized and removed by thymine-DNA glycosylase (TDG), and the created abasic site is repaired by the base excision repair (BER) pathway, generating an unmodified cytosine. Dilution of modified cytosines, 5mC or 5hmC, during DNA replication can also yield unmodified cytosine, through a mechanism termed passive DNA demethylation, which is either TET-independent or TET-assisted, respectively. Replication-coupled dilution of 5fC and 5caC by passive DNA demethylation are not depicted.

identification of Ten-Eleven Translocation (TET) proteins, that catalyze the conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) (24), revealed that vertebrates possess several mechanisms of active DNA demethylation (25, 26) (Figure 1). The active DNA demethylation pathway leads to a further oxidation of 5hmC by TET enzymes and a final removal and replacement by unmodified cytosine through thymine DNA glycosylase (TDG)-mediated base excision repair (BER) (27, 28). The implications of the three members of the TET family (TET1, TET2 and TET3) and their oxidation products as potential epigenetic marks during pluripotency, cell differentiation, and epigenetic reprogramming are reviewed elsewhere (29, 30).

Despite CpG methylation, non-CpG methylation has been found enriched predominantly in embryonic and pluripotent cells (31, 32). Initially thought to be basically absent in differentiated cells, there is emerging evidence due to advances in high-throughput whole genome bisulfite sequencing techniques that non CpG methylation is present in almost all human tissues, showing substantial tissue-specific variation (33-35). Especially in the brain non-CpG methylation may have a pivotal role, as this modification accumulates postnatally during neuronal maturation and persists through lifetime (35, 36). However, substantial understanding about the functional role of non-CpG methylation needs to be awaited.

Therefore, this review will focus here on our knowledge on CpG methylation in mammalian genomes. We will address novel aspects of CpG methylation changes, particularly in the context of cancer, and discuss novel insights that have been gained through whole genome studies, enabled by recent advances in the application of high throughput sequencing (37).

3. CpG ISLANDS

On a genome-wide level 70-80% of all CpGs are methylated (1, 38). The methylated cytosine base, however, carries a mutagenic potential, giving rise to C to T mutations through spontaneous deamination (39). Therefore, the bulk of the human genome is devoid of CpGs, except for the occurrence of so called CpG islands which are composed of sequences of elevated GC base composition and high CpG density. The estimated number of CpG islands in the human genome can vary significantly and depends in large part on the arbitrary thresholds applied to define them, such as sequence characteristics (length, CG content), absence of DNA methylation, and

the optional masking of repeat sequences (40, 41). Using high stringency parameters, the number of CpG islands in the human genome was estimated at about 29,000 (40). On average 1,000 bp long they coincide frequently with transcription start sites (TSSs) of annotated gene promoters. More than 60% of all protein coding genes display CpG island containing gene promoters. More commonly found in house-keeping gene promoters, they can also drive tissue-specific gene expression (42, 43). A recent aspect is that a nucleotide sequence-intrinsic nucleosome instability may lead to a lower histone occupancy at CpG islands compared to other promoters and thereby enable gene activation without needing to overcome a strong chromatin barrier (44).

In the overall methylated CpG-poor genome, these stretches of CpG-rich clusters are distinguished by an unmethylated state that is generally maintained throughout normal development. Thus, a major question concerning CpG islands has been, how they keep free of DNA methylation. Previously, genome engineering approaches integrating CpG island sequences at specific genomic loci in murine embryonic stem cells (ESCs) analyzed the contribution of nucleotide sequence motifs to recapitulate the endogenous unmethylated state at an ectopic target site (45, 46). While CpG density was only a good predictor of the unmethylated state for most GC-rich sequences, transcription factor (TF) binding sites was another determinant accounting for variation of DNA methylation among fragments with lower CpG density (45). Furthermore, an overall GC-rich base composition is inevitable to protect against DNA methylation (46). In regards to the latter, recent studies established that the GC base composition is involved in the formation of extended R-loops that are mechanistically linked to DNA methylation resistance (47-49). Concomitant with transcription, as the transcription bubble moves forward, a DNA:DNA:RNA triple-helix is generated involving a DNA:RNA hybrid formed between the nascent RNA transcript and the antisense DNA strand. Transcribed regions that exhibit a strand asymmetry in the distribution of Gs and Cs, termed GC-skew, favor the formation of elongated DNA:RNA hybrids, termed R-loops (up to 600 bp in length) (49). A bioinformatic analysis of the entire human genome revealed that GC skew is a common feature of methylation-resistant CpG islands, conserved among vertebrates (47-49). In agreement with an active role of R-loops in the protection against DNA methylation, R-loop containing CpG islands are used less efficiently as substrates of the *de novo* DNA methyltransferase DNMT3b1 compared with the same CpG islands in the non-R loop orientation (49). This suggests that the underlying DNA sequence plays a major role in specifying DNA methylation patterns. In another study, promoters that were characterized by an unmethylated state in pluripotent cells and underwent specific methylation during differentiation could recapitulate these DNA methylation changes autonomously and transcription-independently, when inserted in an epigenetically inert region (50). Truncation and mutation analyses further defined methylation determining regions within promoters, harboring as crucial determinants TF binding sites.

Even though, the genetic component appears to dictate DNA methylation patterns at CpG islands primarily, epigenetic mechanisms provide another level of regulation. Thus, a bidirectional crosstalk links DNA methylation and histone modifications (51, 52). A chromatin feature counteracting DNA methylation seems to be histone methylation of H3K4, a mark commonly found at gene promoters. To this end, extensive genome-wide chromatin immunoprecipitation (ChIP) sequencing data of histone modifications suggested a strong negative correlation between H3K4me3 and DNA methylation specifically at CpG islands (8, 53). In support of a cross-talk between H3K4 and DNA methylation, the ATRX-DNMT3-DNMT3L (ADD) domain, harbored by DNMT3-related methyltransferases, recognizes the unmodified amino-terminal tail of histone H3 (54-56). However, this interaction is severely inhibited by H3K4me3, and thus H3K4 methylation protects chromatin from DNA methylation functions. Vice versa unmethylated DNA may attract H3K4 methylation activities. Correspondingly, the CXXC Finger Protein 1 (CFP1) that associates with SET1, a H3K4 methyltransferase, and another H3K4 methyltransferase mixed lineage leukaemia protein 1 (MLL1) both possess a CXXC domain that mediates selective binding to unmethylated CpGs (57-61). Therefore, both genetic, as well as epigenetic mechanisms contribute to protect CpG islands against unwanted DNA methylation found anywhere else in the genome. While DNA sequence is a major determinant of the DNA methylation status, epigenetic mechanisms such as crosstalk with histone modifications may stabilize the output and fine-tune the degree.

4. ABERRANT DNA METHYLATION AND CANCER

There is undeniable evidence that the development of various human diseases and epigenetics are inseparable linked. DNA methylation patterns are perturbed in several neurological disorders, such as Multiple Sclerosis and Alzheimer (62-64), and cancer (65, 66). The latter involves severe changes in the epigenetic landscape, involving both genome-wide DNA hypomethylation and site-specific hypermethylation. Whole genome sequencing technologies have enabled the screening for mutations and chromosomal rearrangements in thousands of cancer genomes. As for other epigenetic modifiers, mutations, chromosome translocations and aberrant expression of proteins that catalyze and regulate DNA methylation or demethylation have been frequently found in cancer (67, 68). Until now, mutations or aberrant expression of DNMT and TET

family members have been primarily described in myeloid malignancies, such as myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) (69-71), but also in some solid tumors (72-75). Analysis of pre-leukaemic haematopoietic stem cells (HSCs) strongly suggests that DNMT3A mutations arise frequently in very early stages of AML development and supports the concept that perturbation of DNA methylation networks may actively drive oncogenesis (76, 77). Indeed, studies in mouse models support that mutations in DNMT1 and DNMT3A promote tumor growth of T cell lymphoma and lung cancer, respectively (78, 79). A recent study identified DNMT3A as a haploinsufficient tumor suppressor in chronic lymphocytic leukemia (CLL), revealing that decreased levels of DNMT3A are sufficient for disease induction, even though with a later onset than in case of its lack in DNMT3A-deficient mice (80). Both DNMT3A$^{+/-}$ and DNMT3A$^{\Delta/\Delta}$ CLL cells share a common set of DNA hypomethylated and overexpressed genes that were assigned to a role in cancer. Opposing to its tumor suppressive function in the hematopoietic system (81, 82), DNMT3B can also have an oncogenic role, as its over-expression led to an increased incidence of colon tumors in mice (83). In addition, DNA methylation pathways may be affected by mutations in alleles or deregulation of genes that have not been previously thought to be involved directly. For instance, this can apply to TFs that directly regulate gene expression of enzymes affecting DNA methylation (84) or recruit them to chromatin (85, 86). In AML mutations of the transcription factor Wilms tumor 1 (WT1) occur in a mutually exclusive manner with TET2 mutations (85). Furthermore, hematopoietic cells with WT1 loss of function reveal comparable changes in 5hmC profiles and a differentiation defect that resemble the phenotype observed upon TET2 mutation (86). The recruitment of TET2 by WT1 to its genomic target sites provides a model how WT1 can influence DNA hydroxymethylation and thereby predisposes to the development of AML.

Genetic mutations of epigenetic modifiers might therefore profoundly contribute to alterations of epigenetic patterns, which in turn give rise to aberrant gene expression that can drive malignant cell transformation (87-89). Even though, mutations in a multitude of epigenetic regulators, strongly suggest an active role in driving cancer development, the mechanisms that lead to and the consequences that follow from global alterations of epigenetic patterns are still not very well understood, in particular, when compared with genetic defects. The occurrence of non-random changes of for example DNA methylation in tumors of different tissue origin (as will be discussed later), imply that tissue-specific mechanisms guide the targeted events downstream of genetic mutations. Moreover, an ongoing challenge in the field of cancer epigenetics is to distinguish epigenetic changes that drive carcinogenesis, from events that are solely by-products of a “messed-up” state of chromatin organization and transcriptional regulation in tumor cells.

5. DNA HYPERMETHYLATION OF PROTEIN-CODING GENE PROMOTERS

Hypermethylation of CpG island-associated promoters have been established as a hallmark of cancer (90). Importantly, the aberrant hypermethylation, particularly of tumor suppressor gene promoters, that traditionally coincides with gene repression, offers an alternative mechanism to genetic mutations to achieve loss of function and is a phenomenon in various types of cancer. This type of epigenetic silencing of tumor suppressor genes has been supported by several examples (91-97). Beyond candidate gene approaches an early global analysis of a subset of more than 1,100 CpG islands on 98 primary human tumors confirmed that aberrant promoter hypermethylation occurs non-randomly (98). While a set of CpG islands are targeted more frequently in more than one tumor type, other CpG islands are hypermethylated in a tumor type-specific manner. Once more, this observation would indicate the existence of molecular pathways that differ among cancers of different origins and remain to be identified in the future. In line with a tumor-specific signature aberrant DNA methylation may serve as a valuable molecular biomarker for the detection and diagnosis of cancer.

Mechanistically, DNA hypermethylation of only a subset of all CpG islands raises the question what makes them prone to this epigenetic alteration. However, until now the exact mechanisms leading to hypermethylation of CpG island promoters are not understood in detail. In 2007, three studies found that target genes of the Polycomb group (PcG) repressive complex 2 in ESCs frequently acquire hypermethylation during stages of adult cancerogenesis (99-101), occurring in the very early stages of oncogenesis (101, 102). The change from a reversible type of gene repression (by PRC2 and H3K27me3) to a permanently locked state of silencing (by DNA methylation) is also referred to as “epigenetic switching” (103). It has thus been hypothesized that this process keeps cancer cells in a stem-cell like state of clonal expansion, that disables cell differentiation and favors malignant transformation (101, 104). However, it remains an open question what triggers the acquisition of DNA methylation in cancer cells, opposed to normal cells. A likely explanation is an imbalance in the expression of DNA methylation regulating enzymes (69-75) or PRC2 complex components (105-110), as frequently found in cancer. A candidate for protecting promoters, including Polycomb targets, against aberrant DNA methylation is TET1 (111). Other models hold ageing (112, 113) or oxidative DNA damage (114) responsible. Another epigenetic regulator that acts as a protector against hypermethylation of tumor suppressor gene promoters (95-97, 115-117) and imprinted loci (118-120) is the multifunctional 11-zinc finger CCCTC-binding factor (CTCF).

6. ABERRANT DNA METHYLATION AT NON-CODING RNA LOCI

Owing to the development of high-throughput RNA sequencing (RNA-Seq) techniques, the ENCODE (Encyclopedia of DNA Elements) project, amongst other studies, has revealed that the vast majority of the eukaryotic genome is transcribed and therefore provided a revolutionary resource for the discovery of novel, mostly non-coding, transcriptional units (121-123).

In an effort to classify non-coding RNAs (ncRNAs), which represent a multi-functional family of RNA molecules, for which we lack biological roles in the majority of cases, transcript length has been used as the most common feature. Thus, a threshold of 200 nucleotides is generally applied to distinguish small ncRNA, that comprise apart from newly identified transcripts also well-known classes, such as microRNAs (miRNAs), transfer RNAs (tRNAs), ribosomal RNAs (rRNAs) and small nuclear RNAs (snRNAs), from long ncRNAs (lncRNA) (124). The latest reference-based assembly of the human transcriptome built from more than 7,200 RNA-Seq libraries from normal tissues, tumors and cell lines, termed the MiTranscriptome database, led to an estimated number of more than 58,000 lncRNAs that exceeds by far the number of protein coding genes (125). In order to further sub-divide this huge universe of lncRNAs, criteria other than size are frequently consulted, such as association with protein-coding genes, DNA elements (e.g. promoter, enhancer) or repeats (126). Despite a continuous accumulation of lncRNAs with evident biological roles in various cellular processes, a classification based on functionality is still to be awaited.

Despite an evident role of lncRNAs as chromatin regulators shaping the epigenetic landscape by recruiting histone modifying protein complexes (127-131), there is relatively little known about the epigenetic regulation of lncRNA genes on a global scale, for instance by DNA methylation. Previous mapping of CpG islands in human and mouse, estimated that approximately half, termed orphan CpG islands, did not coincide with annotated RefSeq transcripts (132). Promoter-associated characteristics, such as histone H3K4me3 enrichment, RNA Polymerase II (RNAPII) occupancy or transcription initiation led the authors to speculate that orphan CpG islands mark promoters of novel transcripts, that could belong to a great extent to lncRNA genes. To date the annotation of lncRNAs and their promoter regions is still ongoing, which may result in non-consistent estimations of the ratio between CpG island and non-CpG island lncRNAs promoters, and consequently the significance of DNA methylation in controlling lncRNA globally (133, 134). It has been suggested in the context of gene imprinting that CpG island promoter DNA hypermethylation plays a role in allele-specific silencing of lncRNAs, such as Kcnqot1 in the Kcnq1 locus (135). Moreover, in the DNA methylation-sensitive loci of two tumor suppressor genes (TSGs), transcription factor 21 (TCF21) and CCAAT/enhancer binding protein alpha (CEBPA), the expression of both TSGs and overlapping lncRNAs are regulated by CpG island DNA methylation (136, 137). Importantly, the lncRNAs assist directly in keeping CpG island containing promoters of the respective TSG free of DNA methylation. Notably, they achieve this by opposing mechanisms, two lncRNA modes of actions that were termed guide or decoy mechanisms (138-143) (Figure 2). Whereas the TARID lncRNA (TCF21 locus) executes its function by recruiting DNA demethylase activities, the extra-coding CEBPA lncRNA (ecCEBPA) acts by blocking DNMT1 from binding to the CEBPA locus (136, 137). The widespread DNMT1 RNA interactome may suggest that the latter mechanism of DNA methylation modulation could happen on a global scale (137). These data support the idea, that lncRNA genes are not only targets of DNA methylation, but also that their transcript products directly take part in modulating DNA methylation patterns by interacting with proteins of the DNA methylation machinery.

Noteworthy, the discovery of pervasive transcription supported the idea that bidirectional transcription initiated from head-to-head type promoters is more widespread than previously anticipated in mammals (144-146). Even though many of the antisense transcripts are characterized by low abundance and stability, a significant number of protein-coding gene promoters give rise to stable, spliced and processed lncRNA transcripts. It has been suggested that the expression changes of a significant number of lncRNA-mRNA pairs take place coordinately during differentiation and development (147-149), implying a potential and precise co-regulatory role of lncRNAs over their mRNA partner. However, irrespective of whether lncRNAs execute a crucial role in cancer and other diseases through regulation of mRNAs or in an autonomous manner, their expression is prone to epigenetic regulation. Not surprisingly, the abundance of CpG island-promoters among lncRNA-mRNA gene pairs is higher than with other types of intergenic lncRNAs (133, 150). Thus, it remains to be seen whether DNA methylation could particularly be of importance when targeting lncRNA-mRNA transcription units. In recent years, several well studied examples demonstrated the involvement of lncRNAs in cancer development (151, 152). In addition, comprehensive genome-wide studies assessing the landscape of the human transcriptome across normal versus cancer tissues have compiled a catalogue of lncRNAs with cancer-specific expression patterns (125, 153, 154). For instance, among seven types of cancers in average 15% and 11% of lncRNAs were significantly up- and down-regulated, respectively (153). Furthermore, genome-wide mapping of DNA methylation alterations at more than 2,400 lncRNA promoters in cancer obtained from The Cancer Genome Atlas (TCGA) and corresponding

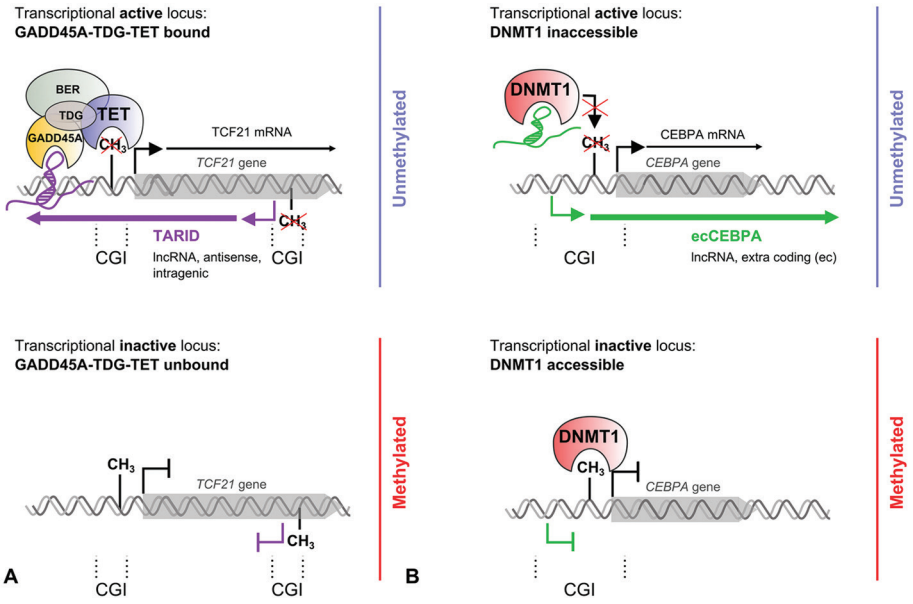

Figure 2. Long non-coding RNA (lncRNA)-mediated regulation of local DNA methylation at tumor suppressor genes (TSGs). A: In normal cells TARID lncRNA is transcribed in the antisense direction of the transcription factor 21 (TCF21) gene (top). TARID recruits GADD45 to the promoter along with TDG and TET enzymes that mediate DNA demethylation at the promoter via base excision repair (BER). In cancer cells, the CpG island (CGI) containing promoters of both TARID and TCF21 are frequently hypermethylated and both genes remain repressed (bottom) (136). B: An extra coding lncRNA (ecCEBPA) transcribed in the sense orientation of the CCAAT/enhancer binding protein (CEBPA) gene binds to DNA methyltransferase 1 (DNMT1) and hinders the access of DNMT1 to the CEBPA promoter (top). In the absence of ecCEBPA transcription DNMT1 can access the CEBPA locus and methylate the promoter (bottom) (137). GADD45: Growth arrest and DNA damage-inducible, TDG: thymine-DNA glycosylase, TET: Ten-eleven translocation.

RNASeq data, identified that roughly 4% of lncRNAs (2,435 analyzed) displayed DNA hypermethylation and decreased expression compared with normal samples (153). Thus, several lines of evidence underline the importance of DNA methylation as part of transcriptional and epigenetic regulation of lncRNAs, specifically as part of tumorigenesis. Most importantly, these global data sets will provide a valuable resource to pinpoint novel cancer-associated lncRNAs in search of biomarkers and targets for treatments in the future.

7. DNA METHYLATION DYNAMICS DURING NORMAL DEVELOPMENT

A common paradigm is that DNA methylation is a static epigenetic mark that, once reestablished after a global removal during early embryonic development (155-157), is largely maintained during normal development. While this is true for the majority of CpGs, this view has been challenged by an unanticipated epigenetic plasticity uncovered by whole methylome sequencing approaches. A comparison of whole-genome DNA methylation patterns across various human tissue and cell types revealed that approximately 15-22% of captured autosomal CpGs vary in a normal developmental context (33, 158). These CpGs clusters are known as differentially methylated regions (DMRs) and overlap with a significant number of distal *cis*-regulatory elements, such as enhancers and TF binding sites (33, 158, 159). Similarly, tissue-specific DMRs are an epigenetic feature in mouse and span at least 6.7% of the genome (159). Importantly, binding sites of cell-type specific TFs are overrepresented among the hypomethylated DMRs in the respective cell of origin (158). For instance DMR overlapping TF binding sites in liver are enriched for motifs of the hepatocyte nuclear factor (HNF) 4G and HNF4A.

The general view is that the genome is characterized by a bimodal methylation pattern. That applies to a genome-wide level, with almost the entire sequence uniformly methylated, and only interspersed by CpG islands as relative short unmethylated segments. Accordingly, at the nucleotide level the majority of CpGs is thought to be completely methylated or unmethylated in each cell of a uniform cell population. However, a recent study published as part of the NIH Roadmap Epigenomics Project (160) identified by genome-wide DNA methylation profiling of 23 primary cell and tissue samples, a significant number of domains of intermediate DNA methylation (IM), of which the majority was restricted to one or a certain subset of tissues/cell-types (161). Individual CpGs were methylated to an extent of 58%. Importantly, approximately half of IM loci coincided with regions previously identified as DMRs (158, 161). Previously, already other mammalian methylomes have exhibited regions of intermediate DNA methylation, though with lower levels ranging from 10 to 50% and

therefore termed low-methylated regions (LMRs) (162). Both IM and LM regions reveal a significant enrichment of enhancer regions. Even though inter-individual and cross-species conservation (161) argue for a biological function of intermediate DNA methylation as an epigenetic signature, the importance remains to be further elucidated. Preliminary data, point toward a quantitative regulation of gene expression and exon usage that could be necessary within a cell population in a context-dependent manner. Hence, dynamics of DNA methylation levels at specific loci between cells of the same and different origin will be a more relevant aspect of DNA methylation biology in the future.

8. DIFFERENTIAL DNA METHYLATION IN CANCER

Since more than three decades DNA hypomethylation (163-165), has been discovered to be a hallmark of cancer. The alterations occur both on a global level (166, 167), as well as at gene-specific loci that encompass regulatory sequences such as promoters of oncogenes and germline-specific genes (165, 168-170). An increase of genomic instability, reflected for instance by chromosomal rearrangements, has been attributed to the high frequency of DNA hypomethylation that occurs recurrently in cancer particularly at repetitive sequences (90, 171, 172). In support of this model elevated levels of DNA hypomethylation induced by mutations in DNTM1 and DNMT3B enzymes are associated with chromosomal instability (79, 173-175). Thus, in mice the expression of DNMT1 from a hypomorphic allele promoted tumor development accompanied by chromosome abnormalities (79, 174). In DNMT1 null murine ESCs deletions at two specific gene loci were predominantly the result of mitotic recombination events or chromosomal loss (173). Furthermore, lack of DNA methylation can lead to an transcriptional activation and mobilization of retrotransposons particularly in cancer and thereby harbor mutagenic potential (176, 177). A frequent insertion of long interspersed nuclear element (LINE) 1 in genes with tumor suppressor function emphasizes the potential role in tumor development (176). In line with this, colon cancer-associated hypomethylation of LINE1 retrotransposons correlates independently of other tumor characteristics with a poorer survival rate (178). Nonetheless, direct mechanistic links between DNA hypomethylation and genomic instability are still largely missing.

While in the past, candidate approaches have been used to look for sites of DNA hypomethylation, cancer epigenome mapping of whole genomes has provided deeper insights in the origin of global hypomethylation in recent years. Inspection of solid tumors of various origin (colon, lung, breast, thyroid, kidney, brain) highlighted broad domains in the kilobase (kb) to Megabase (Mb) scale of hypomethylation that comprised for up to one third to more than half of cancer genomes and therefore can account for decreased DNA methylation (179, 180). In agreement with a decrease in DNA methylation leading to genomic instability, mutation rate quantified as single nucleotide variation (SNV) was significantly elevated in hypomethylated domains (180). Interestingly, these blocks of hypomethylation largely overlap with well-delineated domains that organize the genome of non-malignant cells, such as partially methylated domains (PMDs) (31), large organized chromatin lysine 9 modifications (LOCKs) (181) and nuclear lamin-associated domains (LADs) (182). Notably, loss of epigenetic stability in these domains through DNA hypomethylation resulted in a stochastic hypervariability of increased expression of genes that are consistently silenced in normal samples and are implicated in tumor heterogeneity and tumor invasion (179).

Until now, cancer epigenetics focused mainly on methylation changes at repetitive sequences, as sites of DNA hypomethylation and at CpG islands, present mostly at gene promoters, that are predominantly targeted by DNA hypermethylation. However, recent advances in next-generation sequencing have further enabled the comprehensive genome-wide analysis of methylomes and precise mapping of epigenetic alterations in normal and tumorigenic tissues (65). Recently, this draws our attention to novel genomic features as sites of differential methylation that may allow to further decode the interplay between cancer genomes, epigenomes and transcriptomes. Initially, it was shown that in colon cancer the majority of differential methylation compared to normal tissue samples takes place, not at CpG islands, but at lower CpG density adjacent regions within 2kb of TSSs, termed CpG island shores (183). A large proportion of cancer differential methylation regions (cDMRs) overlap with DMRs that display tissue-specific methylation variation (tDMRs) (183). To give an example, colon cDMRs showed a significant enrichment for tDMRs that distinguished liver from other tissues. Among cDMRs loss of DNA methylation was more common at CpG shores than gain, and gene expression alterations correlated strongly in an inverse manner (179, 183). Cross-species conservation and a marked enrichment of gene ontology (GO) terms associated with pluripotency, development and cell cycle, emphasize a functional importance (179, 183). Besides gene expression as such, alternative transcription may be regulated by differential DNA methylation by controlling promoter usage (183). In summary, these observations match with a model that proposes epigenetic changes to primarily affect tissue-specific differentiation programs and thereby contribute to cancer development. Another CpG island shore related pattern of DNA methylation alteration was discovered in methylomes of medulloblastomas (180). The most prevalent regions of hypomethylation that correlated with gene activation were found downstream of transcriptional start sites (TSS) of the respective genes. While in average

the correlation was highest around 2 kb downstream of the TSS, these domains could stretch across several kbs coinciding with gene body methylation. Noteworthy, about 19% of subgroup-specifically expressed genes were marked by this aberrant methylation pattern, among them RUNX2, a WNT-activated TF solely overexpressed in the WNT subtype of medulloblastoma.

Furthermore, several studies detected concordantly that aberrant methylation in cancer recurrently takes place at distal regulatory elements (184-187). This supports the idea that understanding the regulatory network between enhancer methylation and gene expression could help better understand the molecular mechanisms of cancer biology. In fact, recent analyses of DNA methylation at enhancer sites in cancer discovered a strong inverse correlation between altered DNA methylation levels at enhancers and expression of associated genes (185, 188). Strikingly, the expression profiles of the majority of genes could be better predicted by enhancer methylation than by promoter methylation. Furthermore, the gene expression correlated-enhancer methylation sites were strongly enriched for known breast cancer-associated risk loci. Among the genes whose expression correlated with the epigenetic state of these loci were for example Cyclin D1 (CCND1) and ESR1 (185). The gene product of the latter, estrogen receptor alpha (ERalpha), itself a key factor regulating breast cancer, binds enhancers in a methylation-dependent manner. These observations offered a striking regulatory link between enhancer methylation at cancer risk loci and cancer-driving genes.

Particularly in cancer with a low frequency of somatic mutations, differential DNA methylation is a promising candidate for subtyping cancer and help finding novel molecular mechanisms that could explain tumor subgroup features such as drug resistance, aggressiveness and disease outcome (186, 189, 190). A recent comprehensive study investigated the methylomes of low-grade and high-grade prostate cancer in comparison with benign prostatic tissue (189). This analysis led to the identification of a set of about 4,900 differentially methylated foci that are exclusively hypermethylated in aggressive prostate cancer. Strikingly, high-grade DMR proximal genes were specifically enriched for gene ontology terms associated with cytoskeleton organization and cell motility. Additionally, aggressive prostate-specific foci of hypermethylation that strongly correlated with decreased gene expression were associated with poor outcome. Conclusively, differential DNA methylation changes evidently occur at disease-related genomic sites with high relevance to tumor progression and cancer severity.

In summary, on a genome-wide level there is mounting support that genomic features other than promoters are the predominant sites of DNA methylation deregulation in cancer cells, among them promoter neighboring, gene body overlapping and distal regulatory regions. DNA methylation plasticity at these sites may fundamentally be involved in disease progression, tumor heterogeneity and cancer subtype diversification.

9. MODULATION OF TF BINDING BY DNA METHYLATION

In each cell activities of DNA regulatory elements direct distinct gene expression profiles that are indispensable for cell identity. In order to fulfill this task they are bound cooperatively by cell-type specific TFs. This association is generally accompanied by chromatin remodeling, leading to sites of low nucleosome abundance and high nuclease sensitivity. TF binding site-flanking nucleosomes are prone to be modified by histone modifications that specify the functional role of the respective DNA regulatory elements (e.g. H3K4me3 for promoters or H3K27ac for enhancer elements). Thus, in the course of the Encyclopedia of DNA Elements (ENCODE) project (191, 192), a large collection of putative *cis*-regulatory elements was delineated across the human genome. Parameters that were taken into consideration for the identification were TF occupancy (ChIP-Seq, DNase I footprinting) and chromatin features (DNase I hypersensitivity (DHS) and histone modifications) (192-194). TF occupied *cis*-regulatory elements are further marked by DNA methylation depletion *in vivo* (162, 193, 194). At present it still remains largely unclear how TF binding induces or maintains a hypomethylated DNA state at DNA regulatory elements and thereby gives rise to cell type specificity and how gain of DNA methylation brings about decommissioning. As so often with epigenetics the issue of cause or consequence is a matter for debate. In other words, is DNA methylation responsible for the silencing of chromatin or is the epigenetic mark a downstream effect of other regulatory events that initiate repression. There are two general scenarios to explain the anti-correlation between DNA methylation and TF occupancy. First, DNA methylation actively evicts TFs from their binding motifs or, second, DNA methylation closes in on abandoned regulatory elements “at second instance”.

In many studies CTCF was investigated as a paradigm to answer questions addressing the dynamics of TFs at regulatory elements and their interplay with DNA methylation. On the one hand, CTCF has been of special interest, because the canonical recognition motif contains CpG dinucleotides and its binding has been shown to be methylation-sensitive at some genomic loci (14, 97, 195-197). On the other hand, the involvement of CTCF as a key organizer of chromatin architecture makes it a prime suspect in ensuring epigenetic stability (198, 199). Furthermore, a recent study established a role for CTCF as a haploinsufficient tumor suppressor gene, since mice lacking only one

allele were prone to tumor development and progression (200). Interestingly, hemizygous deletion of CTCF led to a change of DNA methylation patterns at distinct genomic loci and an increase in CpG methylation variance.

On a genome-wide level analyses of the regulatory landscapes in a cohort of human and murine tissues and cell lines reveal a strong correlation between chromatin accessibility, TF binding and DNA hypomethylation (162, 193, 194, 201). Therefore, to address the question whether CTCF could actively promote DNA depletion at its binding sites, a transgene containing or lacking a CTCF motif was inserted at an otherwise methylated genomic site in murine ESCs (162). The insertion of a site recognized by CTCF led to a local demethylation at the reporter construct, irrespective of the methylation status of the motif itself before occupancy. This predicts that CTCF binding can occur, in line with its role as a master regulator, despite the presence of DNA methylation, at least at CpG poor sequences, and that its presence mediates demethylation. On an endogenous level this concept was confirmed by comparing allele-specific DNA methylation levels at CTCF binding sites that were heterozygously impaired by single-nucleotide polymorphisms (SNPs). Furthermore, the REST TF revealed a similar mode of action. Thus, REST reexpression in $REST^{-/-}$ ESCs reversed the methylated state of its binding site and thereby reestablished the hypomethylation found in wild-type cells. In ESCs and neuronal progenitor cells hypomethylated regulatory sequences were found specifically enriched for 5hmC, an intermediate of TET-catalyzed DNA demethylation, in a cell-type specific manner (202). Importantly, REST TF occupancy was crucial for active DNA methylation turnover. In addition, for the majority of TFs (70%) a negative relationship between TF expression that strongly correlates with TF chromatin occupancy, and binding site DNA methylation was observed (194). Interestingly, both CTCF and REST have been found to interact with TET enzymes (203, 204). Together, these data would support a model in which cell-type specific TF binding can dictate DNA methylation changes by active turnover during differentiation or in disease-related contexts. Additionally, the results above implicate that DNA methylation acquisition succeeds displacement of TFs from regulatory elements. Despite the proactive role of TFs to maintain a hypomethylated state at their binding site, several lines of evidence suggest that DNA methylation can prevent association of TFs to specific genomic loci (205-207). For instance, methylation-sensitive CTCF binding was observed at distinct genomic loci in various regulatory contexts, including an exonic region of CD45 (14), the RB promoter (97) and the imprinting control region (ICR) of the *Igf2/H19* locus (195, 196). In an effort to gain a more global view, CTCF ChIP-Seq profiles were compared to altered DNA methylation patterns (201, 208). This revealed that for 41% (1677) of CTCF binding sites, for which reduced representation bisulfite sequencing (*RRBS*) data were available, DNA methylation and chromatin occupancy correlated significantly (201). The vast majority of sites revealed an strong inverse relationship, with hypermethylation accompanied by CTCF loss, and thereby supported a relevant role of DNA methylation regulating cell type-specific CTCF binding variability. However, this analysis was somewhat limited by the amount of DNA methylation data covering only a subset of CTCF binding sites. If DNA hypermethylation could actively hinder TFs from binding, one would expect that demethylation at these binding sites would allow a recurrence. In order to test this hypothesis, CTCF occupancy was profiled in colorectal carcinoma cells that were depleted of DNA methylation by DNMT1/DNMT3B double knockout or chemical inhibition (208). Despite a strong reduction in genomic DNA methylation, the bulk of established CTCF binding sites was unaffected, and only a small subset of sites with a known capacity to recruit CTCF in other cell types was *de novo* bound. These data contrast with an anticipated major role of DNA methylation patterns shaping the landscape of TFs. However, one has to keep in mind, that CTCF is used here as a paradigm for an armada of TFs. Even though CTCF shares characteristics with classical TFs, its regulatory function as an architectural organizer of the genome outreaches by far basic transcriptional control (198, 199). Therefore, more data sets will be required to draw a conclusive model and it remains to be seen to which extent conclusions can be drawn from CTCF to create a general concept. Furthermore, CTCF recognition sites *in vivo* are diverse and non-canonical binding sites often lack CpGs, which could explain a widespread insusceptibility to DNA methylation. Lastly, in cancer cell lines that underwent a plethora of genetic and epigenetic changes it might not be sufficient to solely diminish DNA methylation at sites of TF vacancy, as other barriers may hinder recurrence at many loci.

10. SUMMARY AND PERSPECTIVE

During normal development DNA methylation patterns are determined in large part by the genetic information of mammalian genomes. Critical determinants encoded in the primary DNA sequence are for instance GC content and CpG density, as well as TF binding sites. In addition, the distribution of DNA methylation is further shaped by a cross-talk with other epigenetic marks, such as histone modifications, and the factors that recruit enzymes of the DNA methylation and demethylation machinery to their target sites. Among the latter factors, lncRNAs have emerged as novel players that will probably keep gaining in importance. However, the exact relationship and hierarchy between DNA sequence, DNA methylation, histone modifications, TFs and other chromatin regulators remain a complex system to unravel. Furthermore, recent advances in measuring genome-wide DNA methylomes at base-resolution have given us insight into the dynamic nature of DNA

methylation in a cell population or during differentiation. However, we mostly lack a profound understanding of the mechanisms that override the normal settings in diseases, such as cancer, and result in aberrant DNA methylation changes on a global scale. A significant amount of differential DNA methylation overlaps with distal *cis*-regulatory elements, like enhancers. The fact that enhancers can contact promoters over large genomic distances by chromatin looping makes it more difficult to correlate DNA methylation variations at these regulatory sequences with gene expression changes as an output. A growing number of high-resolution maps of chromatin-chromatin interactions, providing promoter-enhancer contacts, will help understand the consequences of DNA methylation changes at *cis*-regulatory elements in the future. Finally, from a clinical point of view it would be desirable to use the increasing epigenetic profiling data to improve prognosis, and in the long term sensitivity and specificity of therapeutics.

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