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THE ROLE OF DNA METHYLTRANSFERASE 1 IN GROWTH CONTROL

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1. ABSTRACT

Vertebrate DNA contains in addition to the four bases comprising the genetic information a modified base, 5-methyl cytosine that plays an important role in the epigenome. The methylated bases form a pattern of methylation that is cell specific and is faithfully inherited during cell division. The enzyme DNA methyltransferase 1 DNMT1 is responsible for copying the DNA methylation pattern but other *de novo* methyltransferase as well as demethylases might also be involved. Multiple mechanisms are in place to ensure the coordinate inheritance of the DNA methylation pattern with DNA replication. There is a bilateral relationship between the cell cycle and DNMT1. The expression of DNMT1 is tightly regulated with the cell cycle while the expression of DNMT1 can affect the cell cycle. DNMT1 protein might regulate cell cycle events by mechanisms that are independent of its DNA methylation activity through its multiple protein-protein interactions. The unique position of DNMT1 in the cell cycle is consistent with the hypothesis that it plays an important role in cancer.

2. INTRODUCTION

The DNA of many organisms contains within its covalent structure bases that are modified by methylation. 5-methyl cytosine is the only known modification in vertebrates. This base is unique since it is not replicated exclusively by the DNA ploymerase machinery, but it is inherited by two independent steps. The first step is the replication of the unmodified base during DNA replication. The second step is accomplished by separate enzymatic machinery that includes DNA methyl transferases (DNMT) (1). DNMTs catalyze the transfer of a methyl group from the methyl donor S-adenosyl methionine (AdoMet) onto the 5^{th} position on the cytosine ring (1).

Not all cytosines are modified in the vertebrate genome. Most of the modifications occur in the dinucleotide sequence CpG. Furthermore, the cytosines residing in the dinucleotide CpG are not fully methylated (2-3). The methylated CpGs are distributed in a pattern that is unique for each cell type (3). Distinct patterns of methylation are established during development by a sequence of methylation and demethylation events. Since the pattern of methylation plays an important role in maintaining the distinct gene expression profile of a given cell type (4), it is evidently important that the pattern of methylation is accurately inherited during replication. Thus, replication of mammalian genomes must involve the coordination of two independent tasks, replicating the genomic content and replication of the methylation pattern.

It is clear that vertebrates had to develop specific mechanisms to coordinate these two processes, DNA replication and DNA methylation. This review argues that this requirement for coordinating DNA replication and methylation necessitates that a bilateral relationship is established between DNA methylation enzymes and cellular growth control circuits. That is, cellular growth signals regulate the expression of DNA methylation enzymes whereas the expression of DNA methylation enzymes influences cell growth. This review focuses mainly on DNMT1, the main DNA methyltransferase responsible for replication of the DNA methylation pattern. Pharmacological or genetic alterations in DNMT1 levels result in changes in growth control (5-7). These changes are brought about by two mechanisms, the first involves the biochemical function of DNMT1 which is DNA methylation (8) whereas the second mechanism is a consequence of the growth regulatory roles of DNMT1 (9).

3. DNA METHYLATION MARKS INACTIVE REGIONS OF THE GENOME

3.1. DNA methylation patterns and chromatin.

One of the earliest indications that the pattern of methylation plays an important role in controlling gene expression was the observation of a remarkable correlation between the distribution of methyl groups and the distribution of inactive chromatin (10). The hypothesis that DNA methylation marks inactive genes was further supported by studies of the pattern of methylation of specific genes (2). Numerous studies demonstrated that 5' regions of active genes are hypomethylated whereas inactive genes are hypermethylated (3).

3.2. DNA methylation inhibits gene expression by direct and indirect mechanisms

DNA methylation can interfere with gene expression by direct and indirect mechanisms. First, methylation of a CpG site in the recognition sequence of a transcription factor hinders its interaction with its recognition sequence (11). Interference with transcription factor binding is not an exclusive mechanism for inhibition of gene expression by DNA methylation since the binding sites for many transcription factors do not contain CpGs while some transcription factors are not inhibited by DNA methylation (12). Second, methylation of a region around a transcription regulatory site recruits proteins that bind methylated DNA (13). These methylated DNA binding proteins (MBD) such as Mecp2 recruit co repressors and histone deacetylases that inactivate the chromatin configuration around the gene (14-17). A number of MBDs (1-4) were recently discovered which might all act by a similar mechanism to Mecp2 (18, 19). Two MBDs were shown to posses other enzymatic activities. MBD2 was shown to encode a demethylase (see below) and MBD4 a thymidine glycosylase (20). It is still unclear whether the different MBDs have distinct roles in silencing specific groups of genes or whether their functions are redundant.

3.3. DNA methylation controls gene expression *in vivo*

Ectopic methylation inhibit gene expression (21) while pharmacological inhibition of DNA methylation induces the expression of certain genes (22). Nevertheless, there has been a longstanding discussion in the literature whether DNA methylation plays an important role in differential gene expression *in vivo* (23).

Some recent data lend strong support to the hyopthesis that DNA methylation plays an important role in controlling gene expression *in vivo*. Using an embryo-specific element from the CpG island sequence upstream of the *aprt* gene, an *aprt-globin* transgene whose methylation pattern can be switched *in vivo* was generated (24). Analysis of globin transcription in this system showed that methylation in *cis* inhibits gene expression in a variety of tissues, indicating that DNA modification may serve as a global genomic repressor *in vivo* (24). Recent data utilizing differential expression arrays of fibroblast derived from mice bearing an inducible knockout deletion of *dnmt1* demonstrates that DNMT1 is involved in regulation of expression of at least 10% of expressed genes in fibroblasts (25).

4. MECHANISMS RESPONSIBLE FOR INHERITANCE OF THE DNA METHYLATION PATTERN

4.1. How is the pattern of methylation faithfully inherited?

Since DNA methylation plays a critical role in marking inactive genes, it is clear that the methylation pattern has to be conserved during cell division of a given cell type. This review does not discuss the question of how DNA methylation patterns are generated during development and cellular differentiation. The focus of this review is on the mechanisms involved in assuring the faithful inheritance of the pattern of methylation during cell division. An important related question is whether the pattern of methylation is static or dynamic in somatic differentiated cells. If it is dynamic, the question is whether changes in methylation are only pathological or whether the pattern of methylation can change in response to physiological signals. If the pattern of methylation is dynamic, there must be mechanisms that guide the specificity of the process and protect the pattern of methylation from being ectopically disrupted.

4.2. DNMT1 methylates selectively hemimethylated sites

A simple explanation for why patterns of methylation are inherited during cell division is that

DNMT1, the enzyme responsible for replicating the DNA methylation patterns copies accurately the pattern of methylation of the template (2). A mechanistic explanation for this property of DNMT1 is its reported selectivity towards hemimethylated DNA (26). Replication of a sequence containing a methylated CpG dinucleotide will generate a hemimethylated substrate whereas replication of a sequence bearing a nonmethylated CpG will generate a non methylated substrate. Since DNMT1 prefers a hemimethylated substrate, only CpG sites that are complementary to methylated CpG sites in the parental strand are methylated, thus the pattern of methylation is accurately copied.

4.3. The DNA methylation pattern in somatic cells is not static

It is not clear yet whether methylation *in vivo* is guided exclusively by the state of methylation of the template. An additional question is what is the fidelity of DNMT1 catalyzed replication of the methylation pattern. *De novo* methylation of specific sequences was reported in somatic cells (27) and increased DNA methylation of certain genes was observed in the colon during cellular transformation or aging (28). It is possible that these documented changes in methylation are pathological and result from specific defects in either DNMT1 regulation, other *de novo* DNMTs, demethylases or other proteins interacting with these genes. Nevertheless, they reveal that the DNA methylation is potentially not static and that in addition to the template directed replication of the methylation pattern, mechanisms that alter the methylation pattern exist in somatic cells

4.4. The possible repair function of *de novo* DNMTs and demethylases

What are the mechanisms that might alter the DNA methylation pattern in somatic cells? It is possible that the DNMT1 is responsible for some of these changes and that the errors in DNA methylation result from either skipping of methylatable sites during replication or ectopic *de novo* methylation. DNMT1 can *de novo* methylate DNA *in vitro* albeit less efficiently than hemimethylated DNA and it is possible that it does so i*n vivo*. In addition, it was shown that a region of DNA that is heavily methylated can target *de novo* methylation in *cis* by DNMT1 (29). This process can lead to spreading of DNA methylation from either an aberrantly methylated sites or a cognate methylated region (30).

Mechanism that monitor errors in methylation by DNMT1 and correct them to protect the cell from a heritable drift in the DNA methylation pattern must exist. Recent data suggest that in addition to DNMT1 two other DNA methyltransferases that do not discriminate between hemimethylated and non methylated DNA exist, DNMT3a and DNMT3b (31). These DNMTs which are essential for *de novo* methylation during development might also be utilized to replenish aberrantly lost methylated sites during replication. In pathological conditions this activity might result in aberrant *de novo* methylation such as the *de novo* methylation of tumor suppressor genes in cancer cells.

In addition to this recent discovery of *de novo* DNMTs, we have recently shown that mammalian cells bear a demethylase activity (32, 33). Similar to the *de novo* methyltransferases, demethylases might be utilized to ensure that ectopic methylation events are removed. Since ectopic methylation can result in silencing of active genes, it is probably critical for the cell to be diligent in protecting itself from aberrant methylation.

4.5. Specificity of de novo DNMTs and demethylase(s)

De novo DNMTs and the cloned demethylase do not show a distinct sequence preference *in vitro.* It is therefore intriguing how they are able to differentiate an aberrant from a correct pattern of methylation *in vivo*. Based on our recent data we would like to propose a possible model. We have recently shown that histone acetylation can direct the demethylase to demethylate a sequence that is packaged in acetylated nucleosomes (Cervoni and Szyf, unpublished). Based on this data we propose that the state of acetylation of the nucleosomes determine whether its DNA is demethylated once its ectopically methylated. Similarly, it is possible that the *de novo* methylases prefer deacetylated nucleosomes. It is unknown however whether DNMT3a or DNMT3b target deacetylated nucleosomes or whether they are associated with histone deacetylases (HDACs) but DNMT1 was shown to associate with both HDAC1 (34) and HDAC2 (35).

The combined action of demethylases and *de novo* DNMTs is hypothesized to guarantee that active genes do not undergo ectopic demethylation and that inactive genes remain methylated. The same set of principles might be in place when either physiological or pathological cues result in a change in the state of acetylation and activity of specific genes. These changes in acetylation might be then translated into specific changes in DNA methylation.

De novo methylation of inactive genes that are ectopically introduced into the cell or genes that are normally unmethylated is slow and inefficient (36). On the other hand, *de novo* methylation of sequences that are normally methylated in a given cell type and are erroneously demethylated by a pharmacological intervention with a DNMT inhibitor is relatively efficient (37). For example, the first exon of p16 gene is normally methylated in some cancer cells. Treatment with either an antisense inhibitor of DNMT or a pharmacological inhibitor 5-azacytidne results in activation and demethylation of the first exon. Once the DNMT inhibitor is removed, the p16 gene is silenced and the first exon is *de novo* remethylated within a number of days (37). The *de novo* remethylation of specific CpG islands is consistent with the hypothesis that the rate of methylation is determined by information which is independent from the state of methylation of the template. This data supports the model proposed above that *de novo* methylation repairs mistaken deviations in the pattern of methylation an that it is guided by the state of the activity of the gene to correctly recapitulate the methylation pattern.

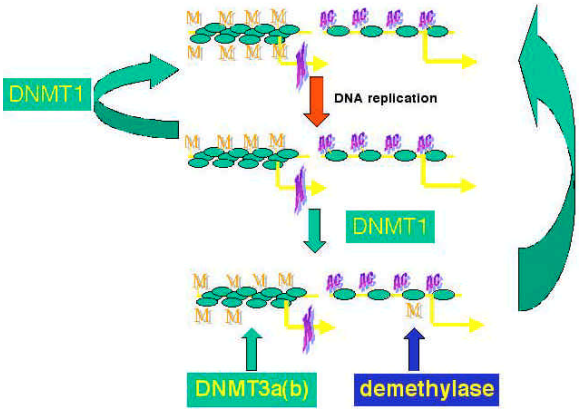

Figure 1. Maintenance of DNA methylation patterns. The chromatin is compartmentalized to domains that are tightly packed (ovals indicate nucleosomes) inactive hypoacetylated and hypermethylated (M indicates DNA methylation) and domains that are active, hyperacetylated (Ac indicates histone acetylation) and hypomethylated (top panel). Following replication of DNA (central panel) both the chromatin structure and DNA methylation are replicated (top panel). The accepted model is that DNMT1 accurately replicates the DNA methylation pattern since it can introduce a methyl group to a cytosine in the nascent strand only if the cytosine on the complementary parental strand is methylated. An alternative possibility is that DNMT1 is not accurate in its replication of the methylation pattern and ectopic methyl groups are either lost or added as indicated in the bottom panel. It is proposed that demethylase remove ectopic methyl groups whereas *de novo* DNMTs (DNMT3a and b) repair the lost methyl groups. These activities are directed by the chromatin structure. Demethylase interact with acetylated nucleosomes whereas *de novo* DNMTs prefer tightly packed chromatin. The outcome of the combined action of DNMT1 demethylase and *de novo* DNMTs is preservation of the DNA methylation pattern and its correlation to the chromatin structure (top panel).

4.6. Summary

In summary, I suggest that in addition to DNMT1 the replication of the DNA methylation pattern during cell division is dependent on demethylase(s) and *de novo* DNMTs that guard the methylation pattern from drifting. The repair methylation enzymes are guided by the state of acetylation of chromatin which is also inherited during replication. According to this hypothesis, an incorrect pattern of methylation is established only if the epigenomic information embodied in the chromatin structure is lost or changed. The demethylase and *de novo* methylases might also participate in a controlled physiological change in the DNA methylation pattern in somatic cells.

An alternative hypothesis which is consistent with the data discussed above is that the preference of DNMT1 to hemimethylated sequences is not paramount *in vivo*. That is, DNMT1 is not directed in its methylation by the pattern of methylation of the paternal strand. The proper inheritance of the replication pattern is an outcome of the combined action of DNMTs and demethylases which are guided by the chromatin structure (see Figure 1 for a model).

5. REGULATION OF THE DNA METHYLATION MACHINERY BY CELL GROWTH SIGNALS

5.1. Multiple mechanisms coordinate DNA methylation with the cell cycle

Since maintaining the DNA methylation pattern is of prime interest to the cell, multiple mechanisms have evolved to ensure that cell division does not occur in the absence of DNA methylation. One mode of coordinating DNA synthesis with DNA methylation is by regulating the expression of the DNA methylation enzymes with the cell cycle.

5.2 . Regulation of DNMT1 levels by mitogenic signals

Two modes of regulation have been described for DNMT1. First, the *dnmt1* gene is regulated by the important protooncogenic mitogenic Ras-Jun signaling pathway (38, 39) as well as the T antigen-Rb oncogenic pathway (40). Second, the abundance of *dnmt1* mRNA is reduced to non detectable levels at the Go phase of the cell cycle and is dramatically induced upon entrance into the S-phase of the cell cycle (41). This is accomplished by a posttranscriptional mechanism and involves interaction with an RNA binding protein that recognizes a conserved element in the 3' UTR of the *dnmt1* RNA and destabilizes the mRNA (Detich et al., unpublished). The abundance of this protein is highly increased at Go. This protein is a good candidate to play a critical role in coordination of DNA methylation and DNA synthesis and might play an important role in the control of the cell cycle as will be discussed below.

5.3. Cell cycle regulation of *de novo* methylases and demethylase/Mbd2 and Mbd3

Recent data indicates that the expression of *dnmt3b* also varies during the cell cycle and peaks during S but it peaks earlier than *dnmt1*(42). *Dnmt3a* on the other hand is expressed consistently at low levels throughout the cell cycle (42). Similarly both *Mdb2/demethylase* and *Mdb3* (MDB3 is homologous to MDB2/demethylase and our preliminary data indicates that it also has a demethylase activity) mRNA levels vary with the cell cycle with two peaks, one before S and an additional peak upon entrance into G2 (Detich et al., unpublished data). The fact that the *de novo* DNMT *dnmt3b* and the *demethylase* mRNAs oscillate with the cell cycle but in a different pattern than *dnmt1* is consistent with the idea that they play distinct roles at different junctures during cell cycle. This is consistent with a repair function for demethylase and the *de novo* DNMTs in contrast to the maintenance methylation function of DNMT1. Alternatively, it is possible that the *de novo* DNMTs and demethylase are responsible for either methylation or demethylation of specific DNA sequences and that these activities are limited to distinct time points in the cell cycle. This idea is supported by the observation that inactivation of the *dnmt3b* gene by homologous recombination results in loss of methylation of centromeric minor satellite repeats DNA sequences specifically (31). These two hypotheses are not mutually exclusive and *de*

novo DNMTs might be involved in both specific DNA methylation as well as repair. It is not yet clear whether there are sequences that are specifically demethylated at distinct points in the cell cycle.

5.4. Targeting of DNMT1 to the replication fork; concurrent methylation and replication

An additional mechanism that guarantees that the pattern of methylation is transferred to the newly replicated DNA is the physical linking of the processes of DNA methylation and replication. This is accomplished by targeting of DNMT1 to the replication fork through a special fork targeting domain in its N-terminus (43). In addition, DNMT1 associates with PCNA a protein that binds a number of proteins in the replication fork and is responsible for the elongation of the nascent DNA strands and the processivity of DNA replication (44). The fact that the DNMT1 protein is moved along the fork with the DNA replication machinery results in concurrent methylation and replication of DNA (45).

It has been also proposed that DNMT1 is present in the replication fork only at distinct times in the cell cycle. Since particular groups of genes replicate at specific time points in the cell cycle, limiting the presence of DNMT1 in the replication fork to specific time points determines which sequences are methylated. For example, it was proposed that at early S, DNMT1 was not found in the replication fork and that this could explain why CpG islands that replicated early were not methylated (46). One emerging idea is that DNMT1 is targeted to specific regions of the genome during replication by its association with distinct multiprotein complexes (35). This can be an additional mechanism regulating the inheritance of specific DNA methylation patterns during replication.

In summary, DNMT1 is transferred during S phase to the replication fork and is associated with replication proteins such as PCNA. As a consequence, methylation occurs concurrently with replication. This is an important mechanism that guarantees that the pattern of methylation of DNA is preserved in newly synthesized DNA and maintained through cell division.

6. DNMT1 CONTROLS CELL GROWTH PARAMETERS

6.1. 1nhibition of DNMT1 in cancer cell lines arrests DNA replication and cell growth

A number of recent observations raise the possibility that in addition to the expected regulation of DNMT1 by cell growth signals, DNMT1 controls cell growth (9). Inhibition of DNMT1 in transformed cell lines by either antisense to *DNMT1* (6, 47, 48), hairpin inhibitors of DNMT1 (5) or 5-azaCdR (8) inhibits cell growth. It is still unclear whether non transformed cells react in a similar manner. We have recently shown that novel hairpin direct inhibitors of DNMT1 and antisense inhibitors of DNMT1 inhibit the firing of origins of replication of DNA suggesting that DNMT1 might be directly required for DNA replication (7). This activity of DNMT1 inhibitors has been the basis for the proposal that DNMT1 inhibitors might be anticancer agents (49, 50). DNMT1 antisense inhibitors are now in phase II clinical trials.

6.2. Possible involvement of methylation of tumor suppressor genes

Identifying the mechanism behind the cell growth inhibition caused by DNMT1 inhibitors can guide us in our approach to the question of how DNMT1 is coordinated with the cell cycle. A simple and reasonable model is that inhibition of DNMT1 results in a passive loss of methylation during replication. This model is consistent with the biochemical function of DNMT1. Since certain tumor suppressor genes are silenced by methylation in tumor cells, hypomethylation of these genes results in their activation which can cause cell arrest (8). For example, in the bladder carcinoma cell line T24, the 5' region of the tumor suppressor p16 is methylated and the gene is silenced. Upon treatment with 5-azaCdR, the gene is demethylated and activated (8). As predicted by this mechanism, a similar change in cell cycle kinetics does not occur in non transformed cells since they do not bear methylated tumor suppressor genes (8). If this mechanism is true, then the observed changes in cell cycle kinetics by DNMT1 inhibitors reflect a pathological condition in tumor cells and do not illustrate a normal regulatory pathway by DNMT1.

6.3. DNMT1 regulates expresion of tumor suppressor genes by a mechanism that does not involve DNA methylation

Some recent data suggests that the simple mechanism described above might not be explaining the entire picture. First, surprisingly it was observed that the main tumor suppressor gene that is induced in A549 cells upon treatment with antisense and direct inhibitor of DNMT1 is p21, a gene that is not methylated in A549 cells (51). Second, the p21promoter-CAT reporter construct which was completely unmethylated, since the plasmid was harbored in *E, coli* which did not have a CpG DNMT, was also induced by inhibition of DNMT1. Taken together these data suggest that DNMT1 might control the expression of critical cell cycle genes by a mechanism that does not involve DNA methylation (51).

6.4 The DNMT1 protein can associate with other proteins that are involved in regulating gene expression

The concept that DNMT1 can control gene expression independent of DNA methylation seems to be counterintuitive. How could an enzyme that has a clear catalytic function repress gene expression? A preliminary answer to this question might be found by inspecting the physical structure of the DNMT1 protein. It has been previously suggested that only the 500 amino acids long carboxy termnus tail bears functional domains that are required for methyl transferase activity (52). Other domains were previously proposed to have other regulatory roles (52). For example, a region in the N-terminus was shown to direct DNMT1 to the replication fork (53) and to bind the replication fork protein PCNA (44). While an obvious explanation for the additional domains of DNMT1 is that is that they are required for targeting DNA

methylation, it is also possible that these functions have other regulatory functions in the cell cycle (9, 54).

Perhaps the strongest data supporting a direct transcriptional regulatory role for DNMT1 are three recent findings showing that DNMT1 interacts with HDAC1 (histone deacetylase I) (34), indirectly with HDAC II through a novel protein that interacts with DNMT1 DNMP1 (35), and with Rb and E2F (55). These different interactions could result in direct suppression of gene expression by histone deacetylation. Thus, inhibition of DNMT1 might be reducing the suppressor effects of DNMT1, resulting in rapid induction of certain tumor suppressor genes.

The idea that histone deacetylation plays a critical role in regulation of tumor suppressor genes expression is supported by the observation that inhibitors of histone deacetylase induce selectively the expression of tumor suppressors such as p21 similar to DNMT1 inhibitors (56, 57). E2F1 and Rb are important regulators of expression of many cell cycle genes. The fact that DNMT1 associates with E2F1 and Rb is consistent with the hypothesis that DNMT1 is involved in regulation of cell cycle genes.

6.5. Model: DNMT1 is a candidate S phase specific transcriptional suppressor of tumor suppressor genes

Taken together the tight regulation of DNMT1 expression by the cell cycle and its ability to associate with repressor complexes, we propose the following regulatory role for DNMT1. Both tumor suppressor genes and genes required for DNA synthesis and replication are considered housekeeping genes which bear very similar transcriptional regulatory elements such as Sp1 elements and E2F1 binding sequences. Nevertheless, these genes are not active during the entire cell cycle but are differentially expressed at different phases of the cell cycle. How can this be accomplished? Factors must exist that target a subgroup of housekeeping genes to be suppressed during specific phases of the cell cycle.

DNMT1 is specifically expressed during the S phase of the cell cycle (41). We propose that DNMT1 binds the promoters of tumor suppressor genes and silences them, thus allowing DNA replication and the cell cycle to progress. Promoters of DNA synthetic genes are perhaps not bound by DNMT1 and are transactivated by Sp1, E2F1 and other ubiquitous transcription factors that interact with housekeeping genes. One interesting question is whether DNMT1 interacts directly with DNA or whether it associates with other proteins that interact with DNA such as Rb or E2F (55). Our data suggests that DNMT1 can directly and specifically interact with nonmethylated CpG containing DNA (5, 58). Thus, we propose that DNMT1 is a S phase specific repressor of a subset of CG rich genes. DNMT1 inhibitors therefore activate tumor suppressor genes by a mechanism that does not involve DNA methylation.

6.6. DNMT1 might play a regulatory role in the DNA replication fork

As discussed above, DNMT1 is a resident of the replication fork and its presence there is coordinated by a specific interaction with PCNA (44). It is possible that the presence of DNMT1 in the replication fork plays a regulatory role in addition to its enzymatic function. The fact that inhibition of DNMT1 inhibits DNA replication (7) might support a direct regulatory role for DNMT1 in the replication fork. It is not clear yet what is the possible mechanism of such a regulatory function?

One possibility that has been suggested before is that by binding PCNA, DNMT1 competes out the binding of p21 (44). P21 is induced by many growth inhibitory signals and is believed to form an inhibitory quaternary complex with PCNA CDKs and cyclins (59). In addition, p21 can directly inhibit the activity of PCNA in the replication fork and therefore DNA synthesis (60). This mechanism predicts that inhibitors of DNMT1 will free PCNA to interact with p21 and as a consequence arrest DNA replication. In accordance with this hypothesis we have recently demonstrated that hairpin-based direct inhibitors of DNMT1 that repress initiation of DNA replication also inhibit the interaction between PCNA and the N terminus of DNMT1 (58).

6.7. DNMT1 stimulates entry into the S phase of the cell cycle: a model

The two putative methylation independent mechanisms of action of DNMT1 suggested above provide us with a working model on how DNMT1 regulates cell cycle events. Induction of DNMT1 upon entrance to S results in displacement of p21 from its interaction with PCNA and as a result it enables initiation of replication. DNMT1 also acts as a transcriptional repressor of tumor suppressor genes such as p21 that block the entrance into the S phase of the cell cycle. Both activities of DNMT1 result in stimulation of cell division. This model proposes that there is a bilateral relationship between the cell cycle and DNMT1. Not only DNMT1 is regulated by cell cycle control signals as expected, but unexpectedly DNMT1 might control cell cycle events. If DNMT1 plays an important role in controlling the cell cycle as proposed here, then the signals and proteins that coordinate the expression of DNMT1 with entrance to S might play an important role in cell cycle control (see Figure 2 for a model).

The model proposed here requires a number of future experiments to validate it and leaves a number of questions that should be answered. First, it has to be demonstrated *in vivo* that DNMT1 interacts and represses tumor suppressor genes. Second, it has to be demonstrated that DNMT1 can specifically bind CpG elements in tumor suppressor gene promoters in the absence of methylation. Third, it has to be tested whether DNMT1 interacts with certain promoters directly or indirectly by binding other transcription factors such as Rb and E2F1. Fourth, the elements that target certain promoters to be suppressed by DNMT1 have to be clarified and the correlation between these elements and those that are responsible for induction by TSA (an HDAC inhibitor) and other HDAC inhibitors should be established. It is possible that a certain combination of factors is common to promoters induced by either inhibition of DNMT1 or TSA.

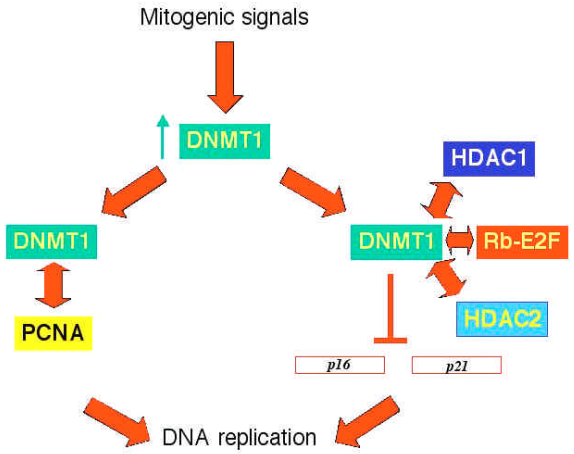

Figure 2. Bilateral relationship between DNMT1 and the cell cycle. Mitogenic signals induce the expression of DNMT1. DNMT1 can associate with either Rb-E2F, HDAC1 or indirectly with HDAC2. These interactions can result in suppression of expression of tumor suppressor genes, stimulating entrance into the S phase of the cell cycle. An additional involvement of DNMT1 that might be important for DNA replication is its association with PCNA in the replication fork.

7. THE IMPLICATIONS OF THE CELL CYCLE REGULATORY ACTIVITY OF DNMT1 FOR UNDERSTANDING ITS INVOLVEMENT IN CELLULAR TRANSFORMATION

7.1. Methylation of tumor suppressor genes in cancer cells

It is clear that if DNMT1 regulates cell cycle events by mechanisms that do not involve DNA methylation, it has important implications on our understanding of how DNMT1 might be involved in cellular transformation. Since DNMT1 is a multifunctional protein, it is not yet clear whether the methyl transferase activity or its other regulatory domains are involved in cellular transformation.

A long list of data demonstrated that genes that were critical for maintaining a transformed state were methylated in many tumor cells (61). This list is increasing at a fast rate and a recent paper has shown that aberrant methylation of the p16 and/or O6-methyl- guanine-DNA methyltransferase promoters can be detected in DNA from sputum in 100% of patients with squamous cell lung carcinoma up to 3 years before clinical diagnosis (62). This data implies that there is a defect in the replication of the DNA methylation machinery in cancer cells. However, since as discussed above the final methylation pattern is probably a product of the balance of a number of reactions, methylation demethylation and nucleosome acetylation, it is not clear which of these functions is altered in cancer cells.

Since increased DNMT1 expression had been demonstrated in many cancer cells (63) and some tumor samples (64-66), DNMT1 overexpression was an obvious candidate to be responsible for hypermethylation of tumor suppressor genes. However, a number of data are inconsistent with this hypothesis. First, the hypermethylation observed in cancer cells is specific to either a class of CG rich promoters or unique tumor suppressor gene promoters. There is no global hypermethylation in cancer cells. It is difficult to understand how over expression of an enzyme that is responsible for the global replication of DNA methylation patterns results in specific hypermethylation of a distinct class of genes. In addition, there is no clear correlation between the cellular levels of DNMT1 and the level of methylation of tumor suppressor genes in human colorectal tumors (67).

One important line of data that supports the idea that over expression of DNMT1 can lead to methylation of tumor suppressor genes comes from cell lines that express high levels of ectopic DNMT1 (68). In these transfectant lines a number of CG rich promoters were shown to be hypermethylated. However, even in these lines *de novo* methylation is a slow process. It is possible that since transfectants that over express DNMT1 are transformed, methylation of CpG islands is a downstream consequence of DNA methylation independent changes in gene expression caused by DNMT1. In addition, a recent report has shown that the *p16* gene remains methylated in a human colon cancer line that lacks DNMT1 which was inactivated by homologous recombination (69). This is clearly inconsistent with the hypothesis that over expression of DNMT1 is responsible for hypermethylation of tumor suppressor genes such as *p16*.

An alternative hypothesis is that the discrete hypermethylation of specific genes like p16 results from a change in the level of expression in some cancer cells of a putative protein interacting with the regulatory regions of *p16*. Such a protein might be involved in controlling the accessibility of the gene to either DNMT1, one of the *de novo* DNMTs or demethylase. For exampe, *p16* is not methylated in normal cells but it is methylated in T24 blader carcinoma cell line. *p16* is artificially demethylated following treatment with a DNMT1 inhibitor (37). However, once the inhibitor is removed, *p16* is *de novo* methylated (37) suggesting that the T24 cell "remembers" that *p16* should be methylated. This is consistent with a specific factor recognizing *p16* that is either specifically absent or overproduced in these cells. Identifying factors that target genes like p16 for methylation and silencing would have important implications upon our understanding of how specific methylation events are generated in cancer cells and might reveal novel important therapeutic targets (see Figure 3 for model).

7.2. The cell cycle regulation of DNMT1 is disrupted in cancer

Early data suggested that DNMT1 is highly overexpressed in different cancer lines and tumors as discussed above. Whereas this data is disputed by some recent observations (70) others support it (71). However, more recent studies point towards disruption in cell cycle regulation of DNMT expression in cancer (42, 72, 73).

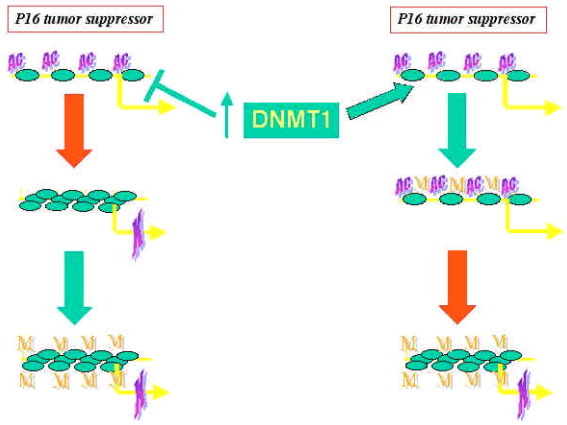

Figure 3. Inhibition of tumor suppressor gene expression by DNMT1. Ectopic expression of DNMT1 might result in suppression of tumor suppressor genes such as p16 by two mechanisms. First ectopic expression of DNMT1 might result in increased methylation of the p16 gene. Methylation of the p16 gene (M) induces changes in chromatin structure, loss of histone acetylation (Ac) and silencing of the gene (right panel). Alternatively, expression of DNMT1 results in suppression of gene expression by a methylation independent mechanism. The inactive chromatin targets *de novo* DNMTs and protects from demethylases resulting in ectopic methylation of the gene.

Thus, perhaps the main defect in DNMT1 in cancer cells is not its overall expression levels but rather its improper regulation during the cell cycle as has been previously proposed (54). Thus, it is possible that as a consequence of activation of different oncogenic gene expression programs, DNMT1 is induced at the wrong phase of the cell cycle. This could result in direct silencing of tumor suppressor genes and activation of DNA replication by removing p21 from the PCNA complex as discussed above.

I propose that the persistent silencing of tumor suppressor genes leads towards a change in their chromatin structure. This leads to *de novo* methylation and inhibition of repair demethylation as discussed above. The covalent modification of tumor suppressors leads to a permanent silencing of these genes and bolstering of the transformed phenotype.

A remaining question is what are the mechanisms through which oncogenic pathways disrupt the cell cycle regulation of DNMT1. One possibility is that activation of transcription of *dnmt1* by the Ras-Jun pathway overrides the destabilization of *dnmt1* mRNA during Go (38). As described above we have previously identified a 3' element in the *dnmt1* mRNA that destabilizes it at the Go phase of the cell cycle, perhaps by interacting with a Go specific RNA binding protein. This element might play an important role in transformation. Future studies of the proteins interacting with this element will allow us to address this possibility.

8. CONCLUSIONS

Whereas we are far from understanding how the DNA methylation pattern is inherited during replication, new data suggests that the process might be more complex than originally thought. In addition to DNMT1, the pattern of methylation is shaped by an interplay between *de novo* methylases and demethylases as well as proteins that modify chromatin and configure the chromatin structure. This new picture of the DNA methylation machinery points towards the exciting possibility that the DNA methylation machinery is dynamic and not a static fixture in somatic cells as previously thought. DNA methylation might respond to transient physiological signals in addition to developmental, environmental and pathological cues. Since changes in methylation are heritable, DNA methylation might be the main channel through which environmental and developmental cues can shape our inherited base of information and perhaps transmit it to our progeny.

One of the important challenges facing us is to understand how the different factors that regulate DNA methylation relate to each other and how these interactions are orchestrated to assure the inheritance of the DNA methylation pattern. It is proposed that this complex machinery guarantees that the DNA methylation pattern accurately reflects the epigenomic information embodied in the chromatin structure.

Since it is so critical to verify that the DNA methylation pattern is faithfully inherited, a bilateral relationship has been established between DNMT1 and the cell cycle. The cell cycle regulates DNMT1 and DNMT1 regulates the cell cycle. This guarantees that the processes of DNA methylation and replication are coupled. This process is possible since DNMT1 is a multifunctional protein which has in addition to its catalytic activity, a transcriptional repressor domain that might silence the expression of tumor suppressors and the ability to interact with PCNA in the replication fork. Future studies will help unravel these newly discovered functions of DNMT1 and the role that they might have in regulating the cell cycle.

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