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The role of 5-hydroxymethylcytosine in human cancer

Gerd P. Pfeifer, **Wenying Xiong**, **Maria A. Hahn**, and **Seung-Gi Jin**
Beckman Research Institute, City of Hope Medical Center, Duarte, CA, USA

Abstract

The patterns of DNA methylation in human cancer cells are highly abnormal and often involve the acquisition of DNA hypermethylation at hundreds or thousands of CpG islands that are usually unmethylated in normal tissues. The recent discovery of 5-hydroxymethylcytosine (5hmC) as an enzymatic oxidation product of 5-methylcytosine (5mC) has led to models and experimental data in which the hypermethylation and 5mC oxidation pathways may become connected. Key discoveries in this setting include the findings that several genes coding for proteins involved in the 5mC oxidation reaction are mutated in human tumors and that there is a broad loss of 5hmC across many types of cancer. In this review, we will summarize current knowledge and discuss models of the potential roles of 5hmC in human cancer biology.

Keywords

5-hydroxymethylcytosine; 5-methylcytosine; DNA methylation; cancer epigenetics; TET protein

Introduction

For many decades, 5-methylcytosine (5mC) has been recognized as the only confirmed mammalian modified DNA base. This base is created in a post-replicative enzymatic process by which DNA methyltransferases incorporate a methyl group into DNA at the 5-position of the cytosine ring. The reaction occurs predominantly at CpG dinucleotide sequences allowing for semi-conservative copying of this short palindromic, methylated sequence (Riggs, 1975). Methylation patterns are generally quite stable at their CpG target sequences but under certain conditions, an unmethylated CpG site can become de novo methylated by a DNA methyltransferase, mostly involving DNMT3A or DNMT3B, or a methylated sequence can become demethylated, loosing a methyl group on either one or on both DNA strands (Figure 1). The demethylation process may occur in a passive manner by which methylated CpG sites are replicated in absence of the maintenance DNA methyltransferase DNMT1. Complete removal of methylation on both DNA strands in the passive demethylation mode requires at least two cell divisions, and if methylation is to be completely lost in most cells of a population, many replication cycles are required. However, a more rapid, so-called active DNA methylation pathway also exists and occurs genome-wide at certain stages of embryo or germ cell development (Reik, et al., 2001). During active DNA demethylation, the 5mC bases on one or both DNA strands are

Corresponding author: Gerd P. Pfeifer, Beckman Research Institute, City of Hope Medical Center, 1500 East Duarte Road, Duarte, CA 91010, USA, Phone: 1-626-301-8853, gpfeifer@coh.org.

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converted to cytosine in the absence of DNA replication. Mechanisms for active DNA demethylation have remained mysterious and controversial for a long time (Ooi and Bestor, 2008, Wu and Zhang, 2010). However, today the most convincing pathway for active demethylation is thought to be the one involving 5mC oxidation (Kohli and Zhang, 2013, Pfeifer, et al., 2013). In 2009, the existence of a second modified cytosine base, 5-hydroxymethylcytosine (5hmC), was demonstrated in cells of the nervous system and in embryonic stem cells (Kriaucionis and Heintz, 2009, Tahiliani, et al., 2009). The levels of 5hmC were orders of magnitude higher than those that could have arisen from oxidative damage to 5mC alone. An enzyme converting 5mC to 5hmC was identified as a protein encoded by the Ten-eleven translocation 1 (*TET1*) gene (Tahiliani, et al., 2009), a gene previously implicated in a chromosomal translocation in leukemia patients (Ono, et al., 2002, Lorsbach, et al., 2003). The Tet1 protein was identified based on its homology to a trypanosomal protein, JBP1, known to oxidize thymine to 5-hydroxymethyluracil (Yu, et al., 2007). Soon after the first report on TET1, two additional 5mC oxidases, TET2 and TET3, were also described and characterized (Ito, et al., 2010). These enzymes are 2-oxoglutarate- and Fe(II)-dependent dioxygenases that have catalytic domains similar to other oxidases of that same category including, for example, many histone lysine demethylases (Hou and Yu, 2010). The TET proteins are capable of not only producing 5hmC in DNA but can drive the 5mC oxidation process further to produce 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) as reaction products (Figure 2) (He, et al., 2011, Ito, et al., 2011). This stepwise oxidation suggested that the oxidation of 5mC to 5caC produces a DNA base that could be decarboxylated to form cytosine, thus allowing for complete conversion of 5mC back to C in an oxidation-decarboxylation cycle (Figure 2). However, such an enzymatic decarboxylase activity has so far not been identified and 5caC is chemically quite stable (Munzel, et al., 2011). As a biochemical pathway, decarboxylation of 5caC would seem most logical inasmuch as such a mechanism avoids breakage of the sugar-phosphate backbone. Instead, however, cells use a multi-step base excision repair mechanism to remove 5caC from DNA (He, et al., 2011). The 5caC base is an excellent substrate for thymine DNA glycosylase (TDG) (He, et al., 2011, Maiti and Drohat, 2011, Zhang, et al., 2012), an enzyme previously implicated in removal of thymine (as a deaminated 5mC base) from G/T mispairs at CpG sequences (Neddermann, et al., 1996).

It should be pointed out that several other pathways for DNA demethylation had been proposed earlier (Ooi and Bestor, 2008). These include, for example, a cytidine-deaminase-initiated process in which the deaminated base, i.e. thymine in case of 5mC deamination or 5-hydroxymethyluracil in case of 5hmC deamination, is removed by a DNA repair mechanism that might involve DNA glycosylases such as MBD4, TDG, or SMUG1 (Rai, et al., 2008, Guo, et al., 2011). Interestingly, plants do not contain TET homologues (Liu, et al., 2013b) and depend on a 5mC DNA glycosylase pathway mediated by ROS1, DME, or DML2/3 for active DNA demethylation (Zhu, 2009). These enzymes have no orthologues in mammalian cells.

Genome-wide DNA ‘demethylation’ in fertilized oocytes was long thought to result in unmethylated cytosines within hours after fertilization (Mayer, et al., 2000). However, in 2011 it was shown that this ‘demethylation’ in fact reflects genome-wide oxidation of 5mC

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catalyzed by Tet3 (Gu, et al., 2011, Iqbal, et al., 2011, Wossidlo, et al., 2011). The further oxidation products, 5fC and 5caC, can also be found in zygotes and in early cleavage-stage embryos (Inoue, et al., 2011). However, 5hmC itself seems fairly stable persisting throughout the first mitosis and first few cell divisions in early embryos (Iqbal, et al., 2011). Therefore, it is unlikely that a Tet3/Tdg pathway is operating at the whole genome level in zygotes or at other developmental stages. It seems intuitive that DNA breakage at millions of CpG sites during the Tdg-initiated base excision repair process would pose an enormous risk to genome integrity. For these reasons, it is more likely that Tet/Tdg-induced DNA demethylation operates gene-specifically at certain developmental or tissue differentiation stages rather than genome-wide. The other major wave of genome-wide DNA demethylation in mammals occurs in primordial germ cells. Whereas current evidence suggests that this demethylation is mostly passive and occurs during DNA replication (Seisenberger, et al., 2012, Kagiwada, et al., 2013), a specific role of Tet-mediated 5mC oxidation is also likely (Hackett, et al., 2013, Vincent, et al., 2013).

Clearly, there still is much to be learned about whether 5hmC represents a stable DNA base or rather is merely an intermediate in DNA demethylation, as outlined in Figure 2. If 5hmC were a stable epigenetic mark, it might be recognized by specific proteins, the so-called readers of the mark. Initial work showed that several proteins of the MBD family of methyl-CpG binding proteins are unable to interact with 5hmC suggesting that 5hmC may have a repelling effect on proteins that normally recognize 5mC (Jin, et al., 2010). However, MeCP2 and MBD3 are noticeable exceptions; these two MBD family proteins are able to bind to 5hmC (Yildirim, et al., 2011, Mellen, et al., 2012) although the data on MBD3 is controversial (Hashimoto, et al., 2012, Baubec, et al., 2013).

Soon after the discovery of 5hmC, its levels in various tissues and cell types have been characterized extensively (Kriaucionis and Heintz, 2009, Globisch, et al., 2010, Münzel, et al., 2010, Szwagierczak, et al., 2010) and its location in the genome has been mapped by various enrichment-based approaches or by single base resolution mapping techniques (Jin, et al., 2011b, Pastor, et al., 2011, Song, et al., 2011, Williams, et al., 2011, Nestor, et al., 2012, Yu, et al., 2012, Hahn, et al., 2013, Lister, et al., 2013, Thomson, et al., 2013b). The 5hmC base is most abundant in neuronal cells of the central nervous system but occurs in almost every tissue type at significant levels. These mapping techniques have found that 5hmC is present at promoters, enhancers and along gene bodies at various levels, also depending on the cell type (Laird, et al., 2013). When present in gene bodies, 5hmC levels are correlated positively with the level of expression of the 5hmC-marked genes (Jin, et al., 2011b, Song, et al., 2011). The mechanistic basis of this 5hmC-associated gene activation is still unknown.

It was also noted soon that 5hmC is strongly depleted in human cancers of many different histological origins (Jin, et al., 2011a, Yang, et al., 2013a). A few specific types of human cancer are characterized by mutations in one of the *TET* genes, *TET2*, but some tumor types can also carry mutations in the pathway producing the 2-oxo-glutarate cofactor, most commonly represented by mutations in isocitrate dehydrogenase 1 and 2 (IDH1 and IDH2). However, the majority of human cancers do not contain *TET1*, *TET2*, *TET3*, *IDH1* or *IDH2*

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mutations and yet, they also show a dramatic loss of 5hmC when compared to corresponding normal tissue.

In this review, we will summarize current knowledge of the role of 5hmC in human cancer and speculate about possible mechanisms of its depletion in tumors as well as the interplay between aberrations in 5hmC pathways and alteration of 5mC patterns in human cancers.

Aberrant DNA methylation patterns in human cancer

It has been known for several decades that DNA methylation patterns in tumors differ drastically from those found in their normal tissue counterparts. Whereas DNA hypomethylation at a global, genome-wide level was recognized and described early on (Romanov and Vanyushin, 1981, Feinberg and Vogelstein, 1983b, Feinberg and Vogelstein, 1983a, Gama-Sosa, et al., 1983), the aberrant hypermethylation of CpG-rich DNA regions, the so-called CpG islands, was observed subsequently (Baylin, et al., 1986) and is now a major area of research in cancer epigenetics (Baylin and Jones, 2011). Hypermethylation of CpG islands is found in a variety of malignancies and is a pervasive change in tumors often affecting hundreds or even a few thousand independent CpG islands across the genome (Costello, et al., 2000, Rauch, et al., 2008). Methylation of specific CpG islands is of interest for development of disease biomarkers and for predicting treatment responses or survival of cancer patients (Laird, 2003, Ushijima, 2005). However, we are still very much in the dark when it comes to understanding the mechanistic pathways that leads to these methylation changes. A common observation is that a large fraction of the genes that become methylated in tumors are targets of Polycomb repression complexes in normal tissues or in embryonic stem cells. These genes most often include homeobox genes and other developmental transcription factors (Rauch, et al., 2006, Ohm, et al., 2007, Rauch, et al., 2007, Schlesinger, et al., 2007, Widschwendter, et al., 2007, Gal-Yam, et al., 2008, Hahn, et al., 2008). Such genes are not expressed or are expressed only at very low levels in normal somatic tissues and often are characterized by bivalent chromatin architecture that includes both active (H3K4me3) and repressive (H3K27me3) histone marks. Therefore, methylation of these Polycomb target genes at CpG dinucleotides along their promoters does not lead to a fundamental ‘downregulation’ of gene expression (Sproul and Meehan, 2013). Rather, DNA methylation is considered as a silencing event that is more permanent than that imposed by repressive histone modifications and is almost irreversible once it has occurred (although this may not hold true in light of Tet-induced DNA demethylation suggesting that DNA methylation is possibly more dynamic than previously thought). Current ideas about the role of CpG island hypermethylation in cancer include models in which the methylation events serve to silence differentiation-associated genes thus persistently locking the tumor cell population into an undifferentiated state (Wu, et al., 2010, Sproul, et al., 2012, Kalari, et al., 2013, Timp and Feinberg, 2013, Nejman, et al., 2014). In that sense, DNA hypermethylation can be considered as a pathway that reduces cellular plasticity of gene expression.

However, despite of decades of research, the mechanistic basis for CpG island methylation in cancer has remained unclear. The methylation state of CpG dinucleotides can be seen as a steady state level situation in which methylation and loss of methylation are balanced (Figure 1). In this scenario, hypermethylation can be viewed as a shift in the balance and can

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be promoted by increased methylation or by a failure of demethylation. Overexpression of DNA methyltransferases can be observed in tumors, but is thought to be mostly a consequence of enhanced cell division in the tumor cell population. Such overexpression also does not explain why certain CpG islands become hypermethylated and others never undergo this change. Interest in DNA demethylation processes, which have remained controversial for a long time (Ooi and Bestor, 2008, Wu and Zhang, 2010), has been revitalized by the discovery of an active, oxidation-dependent pathway in which 5mC is converted to 5hmC (Tahiliani, et al., 2009). Since this mechanism may have a potentially important role in shifting the methylation balance in tumors towards demethylation (when 5mC oxidation is over-active) or towards hypermethylation (when 5mC oxidation is impaired), there has been considerable interest in analyzing 5mC oxidation pathways in human cancer.

Loss of 5hmC in human cancer

Oxidation of 5mC by TET family proteins produces substantial levels of 5hmC, which can easily be detected and quantitated in mammalian tissues (Globisch, et al., 2010). However, the further oxidation products, 5fC and 5caC, occur at rather low levels only and are close to undetectable altogether in many tissues (Globisch, et al., 2010). This could mean that either the TET-mediated oxidation process in most situations does not proceed further beyond 5hmC or, alternatively, the 5fC and 5caC bases are effectively removed from the genome. There is evidence that the latter situation may apply, at least in certain cell types or developmental stages (He, et al., 2011). We also know that 5hmC is a fairly stable DNA base and no excision repair activity has been described that would effectively remove 5hmC from DNA.

Several recent studies have reported that the levels of 5hmC are substantially reduced in human cancers (Haffner, et al., 2011, Jin, et al., 2011a, Kudo, et al., 2012, Lian, et al., 2012, Orr, et al., 2012, Gambichler, et al., 2013, Liu, et al., 2013a, Yang, et al., 2013a). In these studies, different methodologies have been used to quantitate 5hmC levels in tumor versus normal tissues. These methods include antibody-based immunofluorescence and immunohistochemistry approaches, immuno-dot blots, and liquid chromatography coupled with mass spectrometry. A clear tumor-specific loss of 5hmC was seen using all these different methodologies. Figure 3 shows an example where loss of 5hmC is seen in a melanoma tumor in comparison to adjacent normal skin sections using immunohistochemistry staining. Studies focusing on solid tumors have observed a loss of 5hmC ranging from ~50% to over 90% in tumors of almost all major histological types, including lung cancer, breast cancer, colorectal cancer, prostate cancer, liver cancer, glioma, ovarian cancer, melanoma, and several other tumor types (Haffner, et al., 2011, Jin, et al., 2011a, Kudo, et al., 2012, Lian, et al., 2012, Orr, et al., 2012, Gambichler, et al., 2013, Liu, et al., 2013a, Yang, et al., 2013a). A reduction of 5hmC also has been seen in certain cell types that are highly proliferative and carry stem cell character such as the crypt cells of the small intestine (Haffner, et al., 2011, Jin, et al., 2011a), basal cells of stratified epithelia (Haffner, et al., 2011) and proliferative neural progenitor cells in mouse embryo brain (Hahn, et al., 2013).

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The mechanistic basis for loss of 5hmC in cancer is currently unclear. One simple, and probably incomplete explanation is that tumor cells proliferate more rapidly than corresponding normal cells of the same tissue and cannot maintain 5hmC. In this situation, a 5hmC base when present on one DNA strand, would not undergo copying of the methylation and hydroxymethylation state by the maintenance DNA methyltransferase, DNMT1 in combination with a TET protein. In fact, in vitro experiments have shown that DNMT1 is incapable of methylating an unmethylated CpG site on the strand opposite to a hydroxymethylated CpG site (Valinluck and Sowers, 2007, Hashimoto, et al., 2012, Otani, et al., 2013). Also, the maintenance methylation cofactor UHFR1, which aids DNMT1 in the methylation reactions following DNA replication, does not have a good affinity to 5hmC-containing CpG sites (Otani, et al., 2013). However, the lack of maintenance of 5mC in proliferating cells at CpG sites containing 5hmC cannot be the only factor leading to loss of 5hmC in tumors. Also, we do not see a similar loss of total 5mC as we see for 5hmC in tumors, the loss of 5hmC always being much greater (Jin, et al., 2011a). Interestingly, changes in 5hmC occur early as a consequence of exposure of mammalian liver to a non-genotoxic carcinogen (Thomson, et al., 2012, Thomson, et al., 2013a) but it is unclear if other known carcinogens can impair 5hmC formation or maintenance.

Another possible explanation for the loss of 5hmC in cancer is an impairment of TET activity or function. The level of *TET* expression at the RNA level seems to be reduced in some but not all cancers examined (Jin, et al., 2011a, Lian, et al., 2012, Yang, et al., 2013a). Recent studies indicated that expression of TET family members is controlled by microRNAs which can be dysregulated leading to changes in TET expression in cancer (Cheng, et al., 2013, Fu, et al., 2013, Song, et al., 2013a, Song, et al., 2013b). Another possibility, discussed in more detail below, is that *TET* genes or components of co-factor pathways, i.e. IDH1/IDH2, are mutated in cancer leading to loss of function for 5mC oxidation. However, these mutations are found in only a few select tumor types including myeloid malignancies, gliomas and a few others, whereas the global reduction of 5hmC is seen universally in almost every tumor type analyzed.

One interesting observation is the recent recognition that optimal function of TET enzymes depends on the presence of ascorbic acid (vitamin C), which seems to be important for maintaining the TET oxidase cofactor Fe(II) in its reduced state (Blaschke, et al., 2013, Chen, et al., 2013, Minor, et al., 2013). Ascorbic acid is often lacking in cell culture media leading to very low levels of 5hmC in cultured proliferating cell lines. However, when ascorbic acid is added to these cell cultures, levels of 5hmC are strongly increased and return to baseline level after withdrawal of ascorbic acid from cell culture media (Blaschke, et al., 2013). This raises the question whether lack of ascorbic acid in cancer cells in vivo might impede the function of TET proteins leading to strongly decreased levels of 5hmC in tumors. Much further research is required to determine the exact mechanisms of 5hmC depletion in cancer.

Loss of 5hmC as a biomarker for cancer

The loss of 5hmC in cancer is observed almost universally across a spectrum of human malignant tumors. This raises the possibility that this loss could be exploited for developing

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a cancer biomarker. Some interesting data are already available suggesting that this type of approach may be feasible. For example, in several studies of human benign, pre-malignant and malignant melanocytic lesions, it was shown that benign nevi, dysplastic nevi and Spitz nevi show normal levels of 5hmC, but 5hmC is drastically reduced in malignant melanoma specimens (Lian, et al., 2012, Gambichler, et al., 2013, Larson, et al., 2014, Uchiyama, et al., 2014). A standard biomarker used in diagnostic pathology examinations to score for presence or absence of malignant cells in tissue biopsies is the proliferation marker Ki67, a nuclear antigen that is present in actively dividing cells. However, staining with Ki67 antibody can lead to false negative results with a rather high degree of discordance for staining results in different laboratories (Polley, et al., 2013). Therefore, an additional biomarker for cancer would be useful. In previous studies, we have observed that signals for Ki67 staining and for 5hmC staining are mutually exclusive (Jin, et al., 2011a) with Ki67-positive cells almost never being positive for 5hmC. This finding again emphasizes the fact that cell proliferation is incompatible with maintaining normal levels of 5hmC. However, another interesting observation we made was that not only the Ki67-positive cells in a tumor are lacking 5hmC, but in fact most cells of a tumor, including the Ki67-negative ones, have lost 5hmC (Jin, et al., 2011a). This finding indicates that cells with a past history of proliferation that make up the bulk of the tumor mass, but are currently not cycling, have undergone 5hmC loss. This fact may be of interest to pathologists because a tumor may be present in a biopsy, but is currently dormant having few proliferating, Ki67-positive cells. Such tumors should easily be detectable by anti-5hmC staining.

Relationship between 5hmC loss and DNA hypermethylation in cancer

The general reduction of 5hmC in human cancer tissues raises the question as to whether this reduction bears any relationship to the DNA methylation (5mC) changes so commonly observed in tumors. As mentioned earlier, the extent of loss of 5hmC does not parallel the magnitude of loss of 5mC, so DNA hypomethylation in tumors does not seem to have much to do with the 5mC oxidation pathway. However, loss of 5hmC may directly impinge upon DNA hypermethylation in cancer. There are examples for specific cancer types, in which *TET* or *IDH* genes are mutated; these will be discussed below. However, for most solid tumors, which contain wildtype *TET* or *IDH* genes, this possible connection has not yet been investigated. For example, one could imagine that specific CpG islands that undergo gain of 5mC in tumors are never occupied or modified by TET proteins. In that sense, TET-mediated oxidation of 5mC can be seen as a protective mechanism that safeguards CpG islands from occasional misdirected CpG methylation. Once such a spurious methylation event has occurred, the aberrant 5mC is oxidized by a TET protein and the resulting oxidized base is either diluted by passive methylation loss during DNA replication, or the 5hmC is further oxidized and is then removed by TDG-dependent base excision repair. Thus, TET oxidation of 5mC at CpG islands may serve as a methylation repair pathway as previously proposed (Jin, et al., 2011b, Pfeifer, et al., 2011, Williams, et al., 2011, Williams, et al., 2012). This mechanism may be defective at CpG islands that undergo cancer-associated hypermethylation, such as those also occupied by Polycomb complexes. On the other hand, those CpG islands that never become methylated in cancer, including those associated with many housekeeping genes, may have particularly active TET-mediated

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oxidation reactions taking place on aberrantly introduced methylated CpG sites. Further research is required to elucidate these points.

Mutations in the 5mC oxidation pathway in human tumors

The *TET1* gene was initially identified as a translocation partner in a leukemia giving it its name, ten-eleven-translocation 1 (*TET1*) (Lorsbach, et al., 2003). In 2009, it was first reported that the *TET2* gene is mutated in patients with myeloproliferative diseases including myelodysplastic syndrome (MDS), acute myeloid leukemia (AML) and chronic myelomonocytic leukemia (CMML) (Abdel-Wahab, et al., 2009, Delhommeau, et al., 2009, Jankowska, et al., 2009, Kosmider, et al., 2009, Langemeijer, et al., 2009). These *TET2* mutations have been most commonly found along exons 3a and 10, the largest exons of the gene (Delhommeau, et al., 2009). It is likely that *TET2* mutations represent a loss of function phenotype. For example, Kosmider et al reported that about half of the patients with *TET2* mutations had two defective *TET2* gene copies (Kosmider, et al., 2009). Thus, *TET2* may play a tumor suppressive role. Several laboratories have confirmed such a functional role of Tet2 by creating *Tet2* knockout mice in which important *Tet2* exons have been deleted (Ko, et al., 2011, Li, et al., 2011, Moran-Crusio, et al., 2011, Quivoron, et al., 2011). *Tet*2-deficient mice show phenotypes similar to those seen in human CMML patients that have *TET2* mutations (Moran-Crusio, et al., 2011). Hematopoietic stem cells derived from *Tet2* knockout mice have a proliferative advantage. As expected, the levels of 5hmC are reduced in bone marrow and spleen cells of *Tet2*-deficient mice (Ko, et al., 2011, Quivoron, et al., 2011). Since TET2 functions as a 5mC oxidase, it was of interest to examine if *TET2* mutations in patients have any effect on the levels and gene-specific patterns of 5mC in cancer cells of these same patients. In one study, *TET2* mutation in AML were linked to genome-wide DNA hypermethylation (Figueroa, et al., 2010). However, other studies reported that bone marrow samples from patients with mutated *TET2* show DNA hypomethylation (Ko, et al., 2010, Perez, et al., 2012). It is important to note that such correlative studies may not be straightforward at all because myeloid malignancies not only are characterized by mutations in *TET2* but also frequently carry mutations in other epigenetic modifier genes including *EZH2*, *IDH1*, *IDH2*, *MLL*, *DNMT3A*, *ASXL1*, and others (Abdel-Wahab and Levine, 2013). Since mutations in several of these factors may directly or indirectly impinge upon DNA methylation patterns, the situation is very complex.

Mutations in TET cofactor pathways

2-oxoglutarate is a cofactor for iron-dependent dioxygenase enzymes including certain lysine demethylases and 5mC oxidases. 2-oxoglutarate is formed enzymatically in the tricarboxylic acid (TCA) cycle by the enzyme isocitrate dehydrogenase (IDH). IDH1 and IDH2 are metabolic enzymes that are mutated in some cancer genomes and thus represent examples where metabolism and cancer are linked directly through mutational events. *IDH1* mutations are commonly observed in human brain tumors, specifically in grade II and III gliomas, in which they are present in up to 70 % of the patient population (Yan, et al., 2009). *IDH1* or *IDH2* are also mutated in myeloid leukemias and a few other malignancies (Figueroa, et al., 2010, Yen, et al., 2010, Pansuriya, et al., 2011). Tumor-specific *IDH1* mutations are almost exclusively seen at arginine 132 suggesting a gain of function rather

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than loss of function phenotype in tumors. Interestingly, the common IDH1 mutant protein, in which arginine is substituted by histidine at position 132, is capable of producing 2-hydroxyglutarate (2HG) rather than the complete oxidation product 2-oxoglutarate (Dang, et al., 2009). Providing a possible mechanistic explanation for the selection of such specific IDH1 mutations in cancer, it was found that 2HG acts as a competitive inhibitor of 2-oxoglutarate-dependent enzymatic activities including TET proteins and lysine demethylases (Xu, et al., 2011).

Several studies have examined whether human cancers that carry IDH1 mutations have aberrant DNA methylation patterns. Gliomas with IDH1 mutations are clearly associated with widespread hypermethylation of CpG islands (Noushmehr, et al., 2010), the so-called CpG island methylator (CIMP) phenotype (Toyota, et al., 1999). Furthermore, introduction of DNA encoding a codon 132 mutant IDH1 protein into human astrocytes or generation of knock-in mice in which a wildtype copy of the *Idh1* gene is replaced with a mutant copy, produces a DNA hypermethylation phenotype (Sasaki, et al., 2012, Turcan, et al., 2012). The mechanism of IDH1-mutant-induced DNA hypermethylation is not yet clear. In studies of human grade II/III gliomas, we did not observe a substantial difference in levels of 5hmC between IDH1/2 mutant and wildtype brain tumors (Jin, et al., 2011a). The codon 132 IDH1 mutant, through its enzymatic reaction product 2HG, will not only affect TET enzymes that produce 5hmC, but 2HG also is known to inhibit many lysine demethylases (Xu, et al., 2011). Because of the strong interplay between histone modifications and DNA methylation, inhibition of lysine demethylases may impact upon DNA methylation patterns at CpG islands indirectly leading to a CpG island hypermethylation phenotype.

Two other genes encode proteins of the TCA cycle that are mutated in a subset of human cancers: fumarate hydratase (FH) and succinate dehydrogenase (SDH). Their substrates, fumarate and succinate respectively, accumulate under conditions were FH or SDH are mutated (Yang, et al., 2013b). It has been shown that both fumarate and succinate are competitive inhibitors of 2-oxoglutarate-dependent dioxygenases including TET enzymes (Xiao, et al., 2012). In gastrointestinal stromal tumors, SDH-mutated tumors showed an order of magnitude higher DNA hypermethylation relative to the same category of tumors but with *KIT* gene mutations (Killian, et al., 2013). Similarly, *SDH* mutations were linked to a DNA hypermethylation phenotype in paraganglioma (Letouze, et al., 2013). Mutations in FH and SDH were linked to lower levels of 5hmC (Mason and Hornick, 2013, Castro-Vega, et al., 2014). These data indicate that altered epigenomic patterns are common consequences of metabolic pathway aberrations. The distinct role of 5mC oxidation versus other dioxygenase (eg. lysine oxidation) pathways in producing the altered DNA methylation patterns in these types of cancer warrants further investigation.

Perspective

During the past five years, we have witnessed many exciting new findings regarding modified cytosines in mammalian DNA spurred by the discovery of 5hmC and TET proteins as 5mC oxidases in 2009 (Kriaucionis and Heintz, 2009, Tahiliani, et al., 2009). There is evidence suggesting that the 5mC oxidation pathway is important for human cancer biology. Mouse knockout models for *Tet2*, a gene mutated in certain human hematological

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malignancies, have proven a cancer-causing role for dysfunctionality of the 5mC oxidation pathway. However, depletion of 5hmC is a universal occurrence in human cancer, not just those with *TET2* or *IDH1* gene mutations. More research efforts are required to understand the mechanisms of 5hmC loss in tumors and the biological consequences of this loss for tumor progression.

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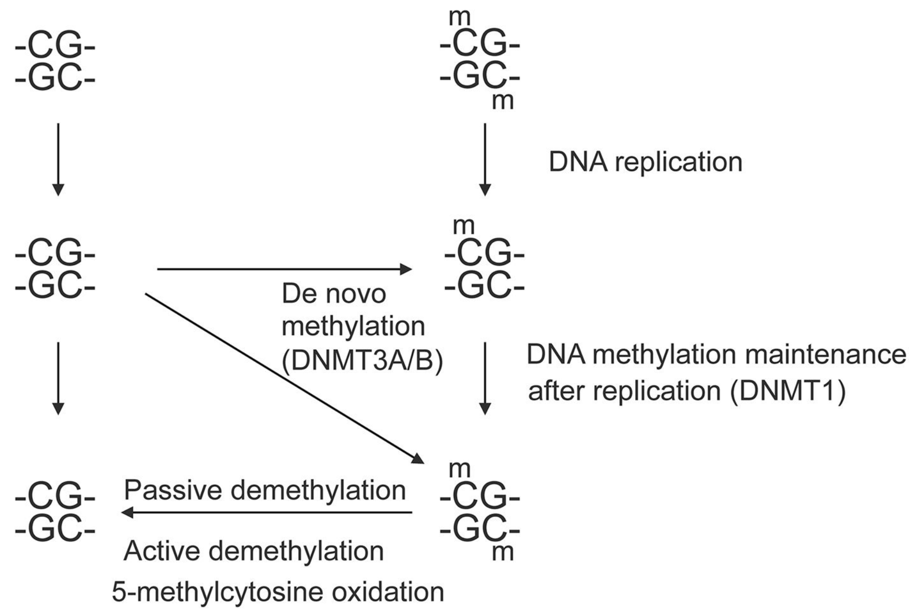

Figure 1. Stability and propagation of DNA methylation patterns

A balance between DNA methylation and demethylation determines steady state levels of methylation at CpG sites.

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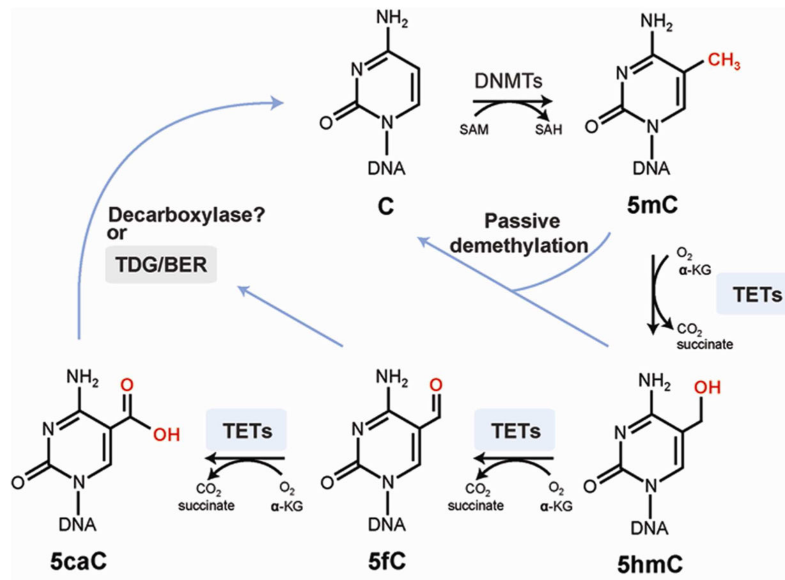

Figure 2. Oxidation of 5-methylcytosine in a DNA demethylation pathway

Enzymes and reaction intermediates are shown schematically.

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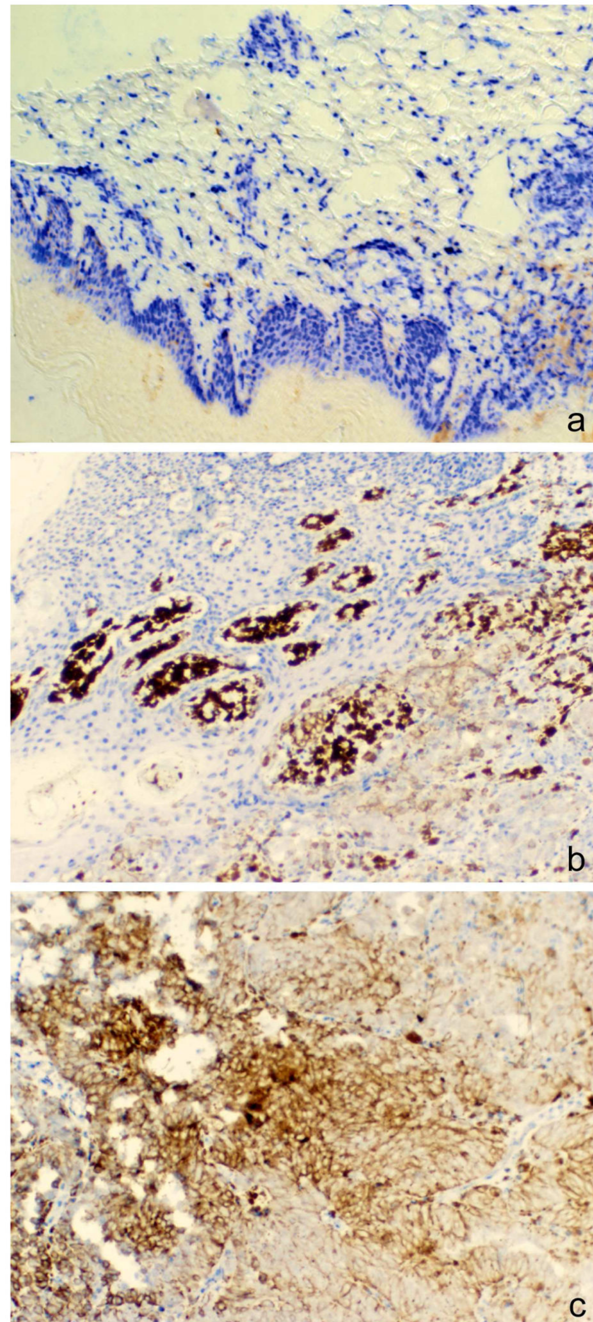

Figure 3. Reduced staining of 5-hydroxymethylcytosine in a melanoma biopsy

Different sections of the same biopsy showing an area of normal skin (A), normal skin adjacent to a melanoma (B; tumor in lower right quadrant) and a melanoma tumor section (C) were stained with anti-5hmC antibody and AP-blue dye (Blue Alkaline Phosphatase Substrate Kit, Vector Laboratories).