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Chapter 6

Hydroxymethylation Influences on Intestinal Epithelial Cells in Health and Disease

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Abstract

Epigenetics describes modifications that affect gene expression that are not encoded within the DNA sequence. DNA methylation is the longest appreciated epigenetic modification and has been accepted to play a critical role in maintaining euchromatin and silencing genes. Recently, a separate and distinct covalent modification has been recognized; hydroxymethylation, which has been associated with increased gene expression as opposed to gene silencing. However, traditional methods to study DNA methylation also recognized hydroxymethylation and did not distinguish between these two distinct DNA covalent modifications. Furthermore, TET enzymes have been identified to play a critical role in active hydroxymethylation of previously methylated cytosine residues and may further result in conversion to cytosine. TET1 plays a critical role in intestinal epithelial differentiation and development, and this is also correlated with increased hydroxymethylation in terminally differentiated epithelial cells. Colon cancer, which arises from the colonic epithelium, exhibits decreased hydroxymethylation and altered gene expression.

Keywords: hydroxymethylation, intestinal epithelium, TET1

1. Introduction

Every cell in the human (and all mammalian) body contains the exact same genetic makeup with the exception of the gametes, which are haploid. However, despite containing the same DNA and genetic information there are a vast number of different cell types that perform functions essential to life. The difference in cell types is due to the genes that are selectively expressed, and those that are silenced combined with genes that are primed for activation in response to a stimulus. In this regard, the field of epigenetics is the study of mitotically

heritable changes/modifications to the genome that alters gene expression without changing the DNA sequence. There are three main types of epigenetic changes; specifically, noncoding RNAs, covalent modifications or methylation to DNA, and histone modifications. In this chapter, we will focus primarily on covalent modifications to DNA: DNA methylation and hydroxymethylation and the role this process plays in colonic epithelial cells. The intestinal epithelium has a rapid cellular turnover with the lifespan of a colonocyte being about 4–5 days [1]. Within the colonic epithelium, there are several cell types, including colonocytes, goblet cells, and endocrine cells, all of which arise and differentiate from a common intestinal stem cell located at the base of the crypts [1–3]. In this regard, the colonic epithelium is a unique system in which cellular differentiation and epigenetic alterations can be studied. Furthermore, two disease processes, colon cancer and ulcerative colitis integrally involve colonic epithelial cells. Alterations in DNA methylation and hydroxymethylation profiles are likely to play an important role in these processes. At the end of the chapter, we will discuss altered DNA hydroxymethylation in these disease states.

2. DNA methylation

DNA methylation was first discovered in 1948 in the calf thymus [4]. In the mid-1970s, two papers hypothesized that cytosine methylation could be a *de novo* process, could be inherited through somatic mutation through an enzymatic process, and result in the silencing of genes [5–7]. Methylation occurs at the 5C position of cytosine in regions of DNA that are rich in cytosine-guanine (CpG) dinucleotide. About 98% of the genome is deficient in CpG, with enrichment of CpGs clustered into "CpG islands," which are typically located near the centromere and within/near promoters. Furthermore, we are now recognizing that CpGs exist within introns and exons of genes and these residues may also be methylated [8, 9]. DNA methylation plays a critical role in imprinting, X-chromosome inactivation in females and silencing of transposons, and maintaining chromosomal stability [9–11]. Given the critical role that methylation plays in imprinting and X-chromosome inactivation and the pathology that results from alterations in these processes, DNA methylation was thought to be a static process and only reprogrammed during gametogenesis and embryogenesis.

DNA methylation has been thought to play an important role in gene silencing. When CpG islands within a gene promoter are methylated, the gene is silenced. This occurs through binding of methyl binding proteins to methylated cytosine which results in histone recruitment and heterochromatin, which is tightly condensed, thereby making the promoter inaccessible to transcription factors. However, recently, with improved technology and genetic sequencing, we have recognized that 5-mc may occur within a gene in introns and/or exons. Furthermore, 5-mc in intragenic regions may result in alternate splicing or transcripts [9, 12].

Importantly, cytosine methylation (5-mc) is preserved during mitosis through DNA methyltransferase 1 (DNMT), which recognizes hemi-methylated DNA during the S-phase of mitosis and copies this pattern to the daughter strand [13–15]. This mechanism is essential to maintain tissue homogeneity and cell lineage in terminally differentiated cells, and there are many publications that have established DNA methylation profiles specific to certain tissues

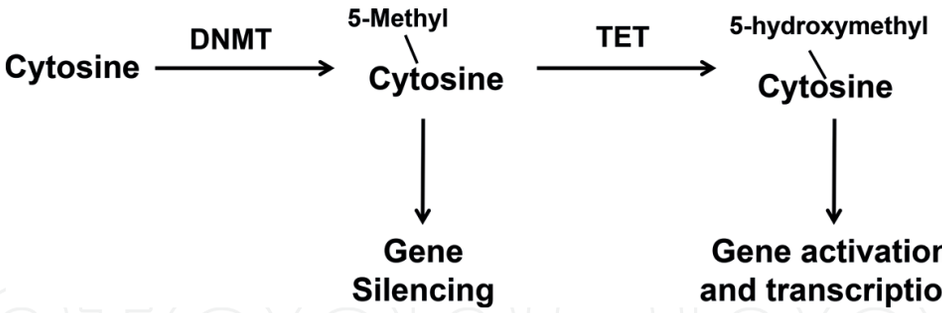

Figure 1. Cytosine may be methylated by DNA methyltransferases (DNMT) resulting in 5-methylcytosine (5-mc) which is associated with gene silencing when located in the promoter. 5-mc may be reduced by ten eleven ten (TET) enzymes resulting in 5-hydroxymethylcytosine (5-hmc) which is associated with euchromatin and gene transcription/activation.

and additionally tissues or cells with similar functions have similar methylation profiles [16, 17]. Maintenance of DNA methylation is an essential function and a recent study on human embryonic stem cells demonstrated lethality when DNMT1 was deleted. Specifically, when DNMT1 was conditionally deleted after initial development, the cells rapidly lost methylation and underwent cell death [18]. In addition to DNTM1, which copies DNA methylation marks during cell division, DNMT 3a and 3b are able to methylate DNA *de novo* (**Figure 1**). In mouse models, deletion of DNMT1 or DNMT3b is embryonically lethal and deletion of DNMT3a results in postnatal lethality [19, 20].

3. Hydroxymethylation

Hydroxymethylation of cytosine was described shortly after DNA methylation in 1950. At that time hydroxymethylated cytosine was described in a bacteriophage and was suggested to be a mechanism by which the virus evaded DNA degradation by the host [21, 22]. Several studies in the 1970s described hydroxymethylated cytosine in mammals but it was not until 2009 when high levels of hydroxymethylation were found in Purkinje cells [23]. Further studies revealed that there were detectable levels of 5-hmc across all tissue types with cells of the central nervous system containing the highest percent of hydroxymethylated cytosine residues [23].

Importantly, bisulfite sequencing that has traditionally been used to identify methylated cytosine bases only distinguishes covalently modified cytosine from unmodified cytosine, therefore methylated and hydroxymethylated cytosine were recognized as the same. With advances in technology and new interest in hydroxymethylation as having a potentially separate function from methylated cytosine, we are now able to distinguish one from the other at the single nucleotide level [7, 24]. Importantly, while 5-mc rich regions are associated with heterochromatin and gene silencing, 5-hmc is associated with euchromatin and genes with rich in 5-hmc are accessible to transcription factors. Methylated cytosines may undergo oxidation by the ten eleven ten (TET) enzymes resulting in 5-hydroxymethylcytosine (**Figure 1**). Further oxidation of 5-hmc by TET enzymes leads to 5-formylcytosine and 5-carboxylcytosine which are then excised and replaced by a new cytosine residue. In this regard, TET enzymes

also function in active demethylation [25, 26]. Additionally as indicated previously in this chapter, 5-methylcytosine associates with methyl binding proteins which promote heterochromatin formation, these methyl binding proteins are not able to recognize hydroxymethylated cytosine and this is another mechanism by which hydroxymethylation may serve to make genes more accessible. Finally, hydroxymethylation may facilitate passive demethylation as DNTM1 has low affinity for 5-hmc and during DNA replication lack of recognition of a previously methylated cytosine, now 5-hydroxymethylcytosine would not have the methyl mark copied to the daughter strand. Hydroxymethylation not only serves as an intermediate step in active demethylation, but is also fairly stable and present in relative abundance compared to 5-flucytosine and 5-carboxylcytosine; therefore, hydroxymethylation may serve an additional and unique function [21, 26–29].

Studies that evaluate global DNA methylation or hydroxymethylation and gene expression have inconsistent results. As our sequencing technology and ability to distinguish 5-hmc from 5-mc has improved, we are coming to understand that regions that are rich in 5-hmc may not be "activated" but rather be "poised for activation" through a delicate balance between 5-mc, 5-hmc and activating (H3K4me3) and repressive (H3K27me3) histone marks [21, 30]. Furthermore, studies in the central nervous system have indicated that changes in specific 5-hmc residues without change in overall methylation can have profound effects on gene expression [31]. Importantly the dynamics and abundance of 5-hmc is cell type specific and changes during development. In this regard, here we will examine the role of 5-hmc in intestinal epithelial cell development and differentiation.

4. Intestinal epithelial cells

The colonic epithelium is one of the largest cellular compartments with a very rapid turnover. The intestinal epithelium is a single cell thick barrier that serves not only as a barrier to protect the underlying lamina propria immune cells from the luminal antigens and bacteria, but these cells also play a role in metabolism, water and nutrient absorption, sensing the luminal environment for potential pathogens, and maintaining a mucus barrier. The colonic epithelium forms crypts and villi which increases the surface area and absorptive surface area. The intestinal epithelium is composed of four major cell types that arise from a common precursor cell. Enterocytes/colonocytes arise from intestinal stem cells which divide to become rapidly dividing transition zone cells, these cells divide up to six more times and migrate up to the tips of the villi during their differentiation. The enterocytes/colonocytes at the tips of the villi play a role in maintaining tight junctions as well as metabolic and absorptive functions, and have a short lifespan of 4–5 days after which they are sloughed off of the surface and mew cells must replace them. The intestinal epithelium also consists of mucin-producing goblet cells, endocrine cells and Paneth cells, which migrate down to the base of the crypts and play a role in bacterial sensing and are relatively long-lived. Over the past decade, we have come to understand that all of these cell types in the intestinal epithelium arise for a common progenitor cell that resides in the transitional zone between the crypts and villi at the +5 position and are referred to as $LGR5^{+}$ cells (intestinal stem cells) [1, 2].

Culture of LGR^{+}5 cells in a 3D matrigel (substituting for the extracellular membrane) supplemented with growth factors: WNT and Noggin to allow for stem cell expansion, R-spondin which maintains stem cell populations, and EGF to promote cell proliferation results in the development of a 3D structure with distinct crypt-like and villus-like structure with a central lumen referred to as organoids. Organoids have successfully been generated from both human and mouse LGR5 cells. These organoids contain all of the cell types present in the intestinal epithelium with crypt-like domains containing Paneth cells and LGR5+ stem cells, villi with villin-positive cells and also enteroendocrine and goblet cells scattered throughout the organoid [3]. Organoids have successfully been generated from isolated LGR5+ cells supporting this notion of LGR5 cells as a pluripotent progenitor for the intestinal/colonic epithelium [3, 32].

Recently, studies have evaluated global methylation and hydroxymethylation in the colonic epithelium. These studies showed relatively similar levels of methylation in the crypts and in association with rapidly dividing (Ki67+) cells in the transition zone; whereas, there was an enrichment of hydroxymethylation at the tips of the villi and a decreased prevalence of hydroxymethylation in the crypts in Ki67-positive cells. These data suggest that hydroxymethylation is gained during differentiation and preferentially expressed in non-dividing, terminally differentiated cells [33].

Alterations in hydroxymethylation profiles in colonic epithelial cells can also be recapitulated *in vitro*. When T84 colonic epithelial cell line is cultured at low density *in vitro*, the cells lack polarity, rapidly divide, and express low levels of global hydroxymethylation. However, as these cells divide and come into contact with other cells they differentiate and polarize to form a monolayer. This process is associated with an increase in hydroxymethylation specifically in promoters of genes involved in maintaining tight junctions, regulation of actin and endocytosis. Furthermore, enrichment in hydroxymethylation co-localizes with binding sites for colonic epithelial-specific transcription factors including HNF4A, RXRA and CDX2 with relatively little change in the hydroxymethylation status of GATA6 (**Figure 2A**). Importantly, this increase in hydroxymethylation also positively correlated with gene expression in a dose-dependent manner with the genes with the highest concentration of hydroxymethylation being the most highly expressed [34].

In mouse models, it is possible to separate the colonocytes at the villous from LGR5^{+} cells using a combination of cell scraping and LGR5 isolation by FACS when LGR5 expression is linked to a fluorescent indicator. This study also confirms that not only is there a difference in the abundance of hydroxymethylation in the LGR5^{+} cells compared to the terminally differentiated enterocytes, but also that hydroxymethylation correlates with highly expressed or inducible cells and changes during differentiation. This study demonstrated that there were over 10,000 differentially hydroxymethylated regions between LGR5^{+} cells and colonocytes. Hydroxymethylation in LGR5^{+} cells localized to promoters of intestinal stem cell markers, such as LGR5, and gene ontology analysis revealed that the functions of the preferentially hydroxymethylated genes were involved in developmental processes, cell differentiation and other stem cell functions. In contrast, in the LGR5^{-} colonocytes that were terminally differentiated, hydroxymethylation was enhanced in the promoters and within genes that control metabolic processes, nutrient transport and other enterocyte functions, and genes with enriched hydroxymethylation also exhibited higher expression at the RNA level [33].

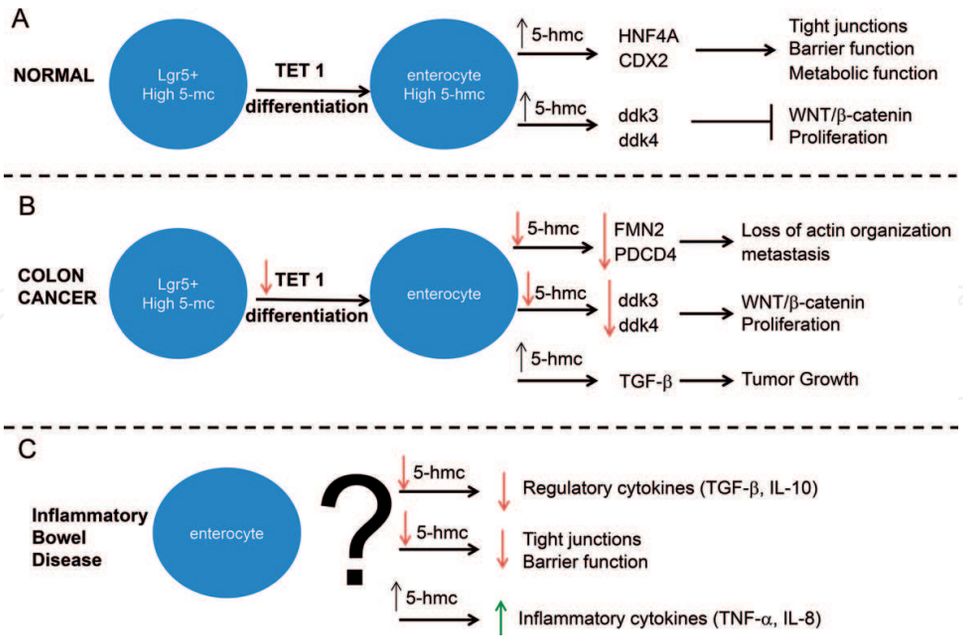

Figure 2. Overview of the role of hydroxymethylation in (A) normal intestinal epithelium, (B) colon cancer and (C) proposed role in inflammatory bowel disease. (A) TET1 activity results in hydroxymethylation of specific cytosine residues as cells rapidly divide and migrate. This results in downregulation of the WNT signaling pathway (reduced proliferation) and upregulation of genes associated with the function of these terminally differentiated cells. (B) In colon cancer, there is decreased TET1 activity and therefore decreased overall hydroxymethylation which leads to a loss of organization, and increased cellular proliferation and inappropriate growth. (C) In IBD, we propose that aberrant hydroxymethylation may result in a decrease in hydroxymethylation of genes associated with barrier function, and downregulatory cytokines and/or increased hydroxymethylation (and therefore increased expression) of pro-inflammatory cytokines.

TET1, which actively can convert methylated cytosine to hydroxymethylated cytosine in epithelial cells, is critical for intestinal epithelial cell maintenance and differentiation. In this regard, TET1-deficient mice exhibit colonic shortening, with shorter villi, with fewer $LGR5^+$ cells. These mice are smaller than their littermate controls and ultimately deletion of Tet1 is lethal. Furthermore, $LGR5^+$ cells derived from these TET1-deficient mice are also unable to form organoids, which confirms the role of TET1 and hydroxymethylation as a critical step in epithelial cell differentiation and maintenance [33, 35].

5. Hydroxymethylation in colon cancer

As we have discussed above, hydroxymethylation is critical in the differentiation of colonic epithelial cells, and there is a change specifically in the distribution of hydroxymethylation as cells divide and differentiate from the LGR5 cells in the crypts (hydroxymethylation in genes involved in proliferation and stem cell functions) and differentiated colonocytes at the tips of the villi (metabolic functions and tight junction). Colonic adenocarcinoma arises from the colonic epithelial cells and is the number two cause of cancer-related deaths in the United States. Traditionally, cancer progresses through four stages from adenomatous-type polyps to adenomas, to invasive disease and then metastasis. It has long been recognized that there

are aberrant methylation profiles in cancers including colon cancer, and the thought was that increased methylation of tumor suppressor genes would result in silencing of these genes and lead to malignant transformation and growth. However, until recently, bisulfite sequencing could not differentiate between methylation (associated with gene silencing when in the promoter) and hydroxymethylation (associated with gene activation).

Since hydroxymethylation has now been appreciated in mammalian cells and it is now recognized that hydroxymethylation has a distinct role in gene regulation and expression, recent attention has been turned to the role of hydroxymethylation in cancer. A recent study evaluated global hydroxymethylation in colon cancer tissue compared to matched, adjacent normal tissue and demonstrated that hydroxymethylation was profoundly less in colon cancer tissue compared to normal [36]. Furthermore, Tet1, which as discussed is essential for active hydroxymethylation in intestinal epithelium cells, is decreased in colon cancer and occurs as an early event [37, 38]. Additionally, in other cancers such as breast cancer, decreased Tet1 expression is associated with more aggressive malignancy and metastatic disease [11]. Taken together, we may hypothesize that decreased Tet1 expression may result in altered/decreased hydroxymethylation. In support of this hypothesis, the role of Tet1 in colon cancer was evaluated in the colonic epithelial cell line derived from colon adenocarcinoma $CaCo_2$. $CaCo_2$ cells were transfected with inducible Tet1 under control of doxycycline. When these cells were treated with doxycycline for 96 hours, these cells had slower growth and there were 300 genes with altered expression, and 60% of the genes with increased expression in the Tet1-induced state had enriched hydroxymethylation and correlated with genes that had high levels of methylation in the wild type ($TET1^{low}$) cells. Gene ontology analysis showed significant enrichment in the WNT/β-catenin signaling pathway of the TET1-deregulated genes. To support this notion, the nuclear level of b-catenin was decreased when TET1 expression was induced, supporting the notion that TET1 expression leads to decreased WNT/β-catenin signaling. Further studies showed that DDK3 and DDK4 which are upstream regulator of the WNT pathway were upregulated in the TET1-induced cells and these genes expressed a higher level of hydroxymethylation when compared to the wild type ($Tet1^{low}$) cells (**Figure 2B**), this in turn provides a mechanistic explanation as to Tet1-induced downregulation of WNT through increased expression of WNT pathway inhibitors. Further studies were done using a xenograft system and TET1-induced CaCo2 cells implanted into a mouse resulted in smaller tumors than the wild-type CaCo2 xenografts [39].

Our group also evaluated hydroxymethylation in colon cancer compared to adjacent normal tissue in patients with colon cancer. While we did not find any specific pathways that exhibited decreased hydroxymethylation in colon cancer, we did identify numerous genes that lost hydroxymethylation and expression. Specifically, two of these genes include: FMN2 (formin 2) and PCDC4 (programmed cell death 4). FMN2 is involved in the organization of actin cytoskeleton and cell polarity; therefore, decreased expression of this gene may result in decreased contact inhibition and the disorganization, which is seen in colonic adenocarcinoma. Furthermore, PDCD4 is also downregulated and exhibits loss of hydroxymethylation in colon cancer. Loss of PDCD4 has been associated with colon adenocarcinoma and these tumors tend to be more aggressive [34, 40]. Additionally, there were also genes that gained hydroxymethylation and exhibited increased expression including BMP7 and TGFB1. Bone

morphogenetic protein 7 (BMP7) is a secreted protein of the TGF-β superfamily, and TGFB1 (transforming growth factor beta 1) is known to be increased in tumor cells and may induce the canonical WNT signaling (**Figure 2B**) [34]. Taken together there is strong evidence that alterations to gene-specific hydroxymethylation profiles may be an early step that occurs in colon cancer leading to progression of disease. Altered hydroxymethylation may serve as a biomarker or predictor of disease severity and/or targets for future therapy.

6. Hydroxymethylation in IBD

Inflammatory bowel disease (IBD) affects more than 3 million adults (estimated 1.3% of adults) in the United States [41]. Importantly, the incidence of IBD has been increasing over the past 10 years especially in developed and developing countries. Furthermore, environmental influences appear to influence IBD with the two major phenotypes, ulcerative colitis and Crohn's disease, demonstrating opposite correlation with smoking. Specifically, smoking is protective in ulcerative colitis and patients may experience flares with cessation of smoking; whereas in Crohn's disease, smoking worsens disease [42–44]. Recent studies including the GWAS have identified an important role for genetics as well as gene regulation and epigenetics in IBD [45, 46]. To date the GWAS have identified 163 loci that are associated with IBD; however, only approximately 10% of these loci are located in DNA coding regions suggesting an important role for gene regulation [47, 48]. Given that genetics account for only 10% of cases of IBD, the increased incidence in developing countries and the identified environmental influences, IBD is likely due to a gene by environment interaction, which also implicates epigenetics as a key driver in disease pathogenesis. Previous studies have implicated a role for alterations in DNA methylation in IBD; however, these studies showed discordant results in genes with "methylated" promoters and RNA expression. This may be due to the fact that these studies were performed prior to the development of the technology to distinguish methylated cytosine (silenced genes) from hydroxymethylated cytosine (activated or primed for activation). Furthermore, these studies were performed in intact tissues or mixed cell populations [49]. Since methylation plays a critical role in maintaining tissue homogeneity, and as described previously, hydroxymethylation is increased in terminally differentiated cells, methylation and hydroxymethylation are best evaluated in a single cell population. Since colonic epithelial cells are the most widely affected cell type in IBD, hydroxymethylation plays a critical role in colonic epithelial cell differentiation and function, and prior studies have suggested altered methylation profiles in IBD tissues, we may infer that there is a role for aberrant hydroxymethylation of genes, potentially involved in barrier function or cytokine production, that contributes to the pathogenesis of IBD (**Figure 2C**). Future studies to evaluate differential hydroxymethylation in epithelial cells from affected/unaffected mucosa from IBD patients may provide further insights into novel pathways or genes implicated in IBD pathophysiology. Additionally further understanding into the mechanisms by which hydroxymethylation profiles are altered either through changes in TET expression or environmental exposures that promote or inhibit hydroxymethylation we may identify novel targets for therapy.

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7. Conclusion

Epigenetics, mitotically heritable changes/modifications to the genome that alter gene expression without changing the DNA sequence, plays a critical role in embryogenesis, normal development and disease. Specifically, DNA methylation has long been recognized as a critical epigenetic modification that is critical to normal function and tissue homogeneity. Alterations in methylation profiles have also been identified in and are thought to play a role in autoimmune diseases as well as malignancy. However, until recently technology did not allow for the differentiation between methylated cytosine (5-mc) and hydroxymethylated cytosine (5-hmc). Given that recent studies have indicated that hydroxymethylated promoters is associated with gene activation, it is important to further evaluate the role of hydroxymethylation in development and disease.

In this chapter, we have discussed the critical role of DNA hydroxymethylation in the differentiation of intestinal epithelial cells. Differential hydroxymethylation of specific gene promoters results in appropriate function of the LGR5 stem cells and the differentiated enterocytes. We have also discussed that higher levels of hydroxymethylation are associated with terminally differentiated, non-dividing cells in the normal tissue. Furthermore, we implicated a role for hydroxymethylation in the pathogenesis of colonic adenocarcinoma through increased WNT signaling, and also differential hydroxymethylation in specific genes that may act as tumor suppressors or growth factors in paired tissue samples. Finally, we have presented evidence that supports a potential role for altered hydroxymethylation in IBD.

Future studies evaluating hydroxymethylation at the single nucleotide level in a single cell population may be extremely valuable in identifying potential novel pathways or drug targets in disease. Importantly, as discussed, hydroxymethylation is a stable epigenetic modification, but is also dynamic in that hydroxymethylation may be gained or lost at a specific location. This is important because altered hydroxymethylation in a cell population may result from a gene x environment interaction and then be propagated as cells divide, thereby leading to disease pathogenesis. In regards to the intestinal epithelium, use of organoids may allow researchers to identify environmental exposures to the epithelium that may result in altered hydroxymethylation. Furthermore, since the intestinal epithelium can easily be isolated from tissue specimens, further studies may be performed on paired specimens to delineate normal and diseased tissue in patients with IBD and colonic adenocarcinoma.

Abbreviations

5-hmC	5-hydroxymethylcytosine
5-mC	5-methylcytosine
CDX2	Caudal type homeobox 2
CpG	Cytosine-guanine dinucleotides

ddk	Dickkopf WNT signaling pathway inhibitor
DNMT	DNA methyltransferase
FMN2	Formin-2
GATA6	GATA-binding protein 6
HNF4A	Hepatocyte nuclear factor 4 alpha
iSC	Intestinal stem cell
LGR5	Leucine-rich repeat-containing G-protein-coupled receptor 5
PDCD4	Programmed cell death 4
RXRA	Retinoid X receptor alpha
TET	Ten eleven ten enzyme

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