

# Linking Immunity, Epigenetics, and Cancer in Inflammatory Bowel Disease

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**Abstract:** Most of what is known about the pathogenesis of inflammatory bowel disease (IBD) pertains to complex interplay between host genetics, immunity, and environmental factors. Epigenetic modifications play pivotal roles in intestinal immunity and mucosal homeostasis as well as mediating gene-environment interactions. In this article, we provide a historical account of epigenetic research either directly related or pertinent to the pathogenesis and management of IBD. We further collate emerging evidence supporting roles for epigenetic mechanisms in relevant aspects of IBD biology, including deregulated immunity, host-pathogen recognition and mucosal integrity. Finally, we highlight key epigenetic mechanisms that link chronic inflammation to specific IBD comorbidities, including colitis-associated cancer and discuss their potential utility as novel biomarkers or pharmacologic targets in IBD therapy.

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**Key Words:** DNA methylation, histone modification, micro RNAs, microbiota, colitis-associated cancer, Crohn's disease, ulcerative colitis, cytokines, host defense, gene-environment interactions, innate immunity, adaptive immunity, animal models

Inflammatory bowel disease (IBD) is comprised of 2 major disorders: Crohn's disease (CD) and ulcerative colitis (UC). IBD results from a continuum of complex interactions between host-derived and external elements that involve various aspects of the intestinal microbiota, the immune system, the genetic composition/susceptibility of the host, and specific environmental factors (e.g., diet, smoking, stress, and hygiene).<sup>1–3</sup> Epigenetics is one of the most rapidly expanding fields in biology, interacting with genetic and environmental factors in affecting the immune system (Fig. 1). Epigenetics may be defined as changes in phenotype that persist through mitosis and even meiosis, but occur independently of changes to the underlying DNA sequence. Consequently, epigenetics is generally understood to be the study of mechanisms that control gene expression in a potentially heritable way.<sup>4</sup> Complex epigenetic states are orchestrated by several converging and rein-

forcing signals, including transcription factors, noncoding RNAs, DNA methylation, and histone modifications.<sup>5</sup> Great progress has been made in the description of epigenetic modifications in human health and disease. These advances have provided new insights into the role of epigenetic modifications in cancer, neurodevelopmental disorders, neurodegenerative and neurological diseases, and in autoimmune diseases.<sup>4</sup> Epigenetic alterations are likely to be found in other human diseases. It was proposed, more than a decade ago, that inherited and/or acquired epigenetic marks may be of etiological and pathogenic importance in IBD.<sup>6</sup> Many laboratories have worked on the problem, and some excellent reviews have appeared during the last 2 years that cover the emerging role of epigenetics in IBD.<sup>7–14</sup> The fundamental principles of epigenetic modifications and their molecular machineries relevant to IBD have recently been described in detail.<sup>4,7,8</sup> We therefore only summarize key features and new insights of relevant epigenetic modifications. We provide an overview of the epigenetic control of inflammation and, in particular, the epigenetic regulation of the differentiation and function of key effector cells of the innate and adaptive immune system. The article also focuses on recent advances in the understanding of the epigenetic control in IBD, which includes DNA methylation, histone modifications, and interplay with intestinal microbiota. Recent advances on the role of epigenetics in colitis-associated cancer (CAC) will also be discussed. Finally, we outline clinical and therapeutic implications and providing discourse on the challenges, perspectives, and outstanding questions for the emerging role of epigenetics in IBD.

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## CONCEPT AND PRINCIPLES OF EPIGENETICS

The term “epigenetics” was formally defined by Waddington<sup>15</sup> as “the causal interactions between genes and their products, which bring the phenotype into being.” Waddington used the metaphor of an “epigenetic landscape” to articulate the concept

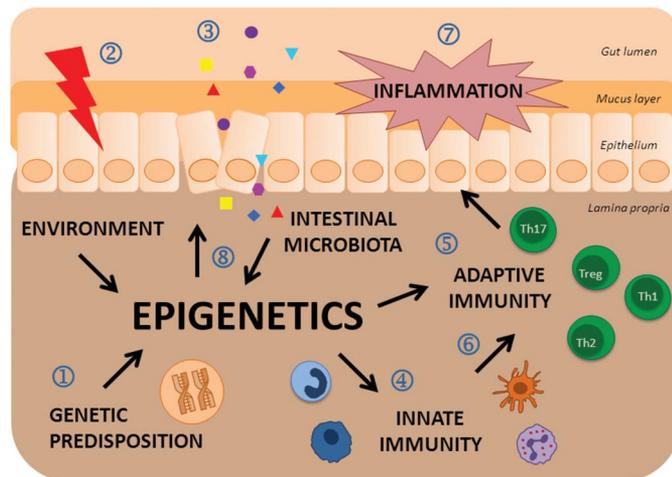


FIGURE 1. Roles for epigenetics in IBD pathogenesis. Epigenetics may interact with genetic factors (1), environmental factors like nutrition, smoking, stress or hygiene (2), and the intestinal microbiome (3) in affecting the immune system. Phagocytic cells within the lamina propria (e.g., monocytes/macrophages, DCs, neutrophils) and epithelial cell barrier represent the central components of the intestinal innate immune system (4). Antigens reaching the lamina propria activate innate immune cells followed by a response of T lymphocytes (Th, T-helper cells; Treg, T-regulatory cells), which represent the key cell population of the adaptive immune system (5). The innate and adaptive immunity arms are tightly cross-regulated serving to uphold intestinal homeostasis and thus to control the complex commensal–host crosstalk (6). The subsequent immune response has consequences on the initiation, resolution, and/or progression of intestinal inflammation (7). Bacteria can also regulate the epithelial gene expression through epigenetic mechanisms (8).

of molecular mechanisms that can reinterpret the invariant genetic code into a multitude of different phenotypic outcomes.<sup>16</sup> The modern concept of epigenetics refers to the heritable marking of DNA that leads to the alteration of gene expression independently of genetic information carried by the primary DNA sequence.

One of the earliest challenges was to elucidate how epigenetic mechanisms extract contrasting phenotypic identities from the same genetic code? It was evident that the DNA sequence must carry another layer of heritable information, although the corresponding molecular principles were not understood at the time. Epigenetic marking, or modification, of DNA is now universally accepted as the underlying mechanistic basis to this phenomenon. Epigenetic modifications possess the fundamental properties required to variably influence phenotype in a transmissible manner. They are heritable (i.e., mitotically stable) allowing their retention through successive cell divisions and have the capacity to directly, or indirectly, alter the transcriptional status of the underlying DNA sequence. Most importantly of all, perhaps, epigenetic modifications are fully reversible, allowing their erasure and subsequent reestablishment on passage through the parental germlines (although as we discuss below, incomplete “resetting” of the epigenome could explain the intergenerational inheritance of some adverse phenotypes). DNA

methylation, histone posttranslational modifications, and nucleosome positioning are the best-characterized epigenetic modifications. However, during the past decade, roles of chromatin remodeling complexes, the much-vaunted polycomb group proteins<sup>17</sup> and micro RNAs (miRNAs)<sup>18</sup> among others, have risen to prominence as critical modifiers of epigenetic modifications. Collectively, epigenetic modifications and their respective modifiers co-ordinate a multitude of molecular functions including gene transcription,<sup>19</sup> DNA-protein interactions,<sup>20</sup> protein translation,<sup>21</sup> and silencing of endogenous retrotransposons.<sup>22,23</sup> Through these functions, epigenetic modifications act deterministically on key developmental processes including growth, cellular fate and differentiation, immunity, X-chromosome inactivation, and genomic imprinting.

### EPIGENETIC SYSTEMS AND DISEASE MODELS

The overriding significance of epigenetics in the maintenance of normal biological functions is highlighted by the fact that human diseases often develop when epigenetic marks are incorrectly established, or are established at inappropriate times or locations. For example, monozygotic twins carry the same genetic code but, nonetheless, display discordant DNA methylation and histone modification profiles, which may alter the penetrance of, or susceptibility to, cancer and autoimmunity.<sup>24–26</sup> The inherent plasticity and reversibility of epigenetic modifications, although highly desirable in the cellular context, also renders them vulnerable to alteration by environmental stimuli. Accordingly, epigenetic modifications are potential mediators of gene-environment interactions underlying complex multifactorial diseases, including IBD.<sup>27</sup> Environmental factors known, or suspected, to have epigenetic effects include nutrition, stress, chemical exposure, pharmaceutical agents, and inflammation (reviewed in Ref. 28). Data from both humans and animal models suggest that environmental factors may elicit phenotypes associated with specific epigenetic modifications that can be inherited transgenerationally.<sup>29–34</sup> Epigenetic modifications may therefore exert a profound influence on the pathogenesis of IBD by connecting host gene function to known environmental risk factors including intestinal microbiota.

Epigenetics may not only play a role in IBD pathogenesis, but may also determine the outcome of specific disease sequelae such as CAC<sup>35,36</sup> discussed in detail in a subsequent section of this article. Nonetheless, the strong link between epigenetics, growth deregulation, and cancer is exemplified here by reference to the phenomenon of “genomic imprinting,” which, to date, remains one of the most informative epigenetic paradigms of human disease.<sup>37,38</sup> The existence of imprinting came from seminal nuclear transplantation studies in mice, showing that reconstituted diploid embryos derived from 2 paternal or 2 maternal pronuclei are not viable.<sup>39,40</sup> Therefore, despite carrying identical genetic information and residing within the same nucleus, the parental genomes are functionally nonequivalent because of differential marking by epigenetic modifications. An explanation for this form of non-complementation was subsequently attributed to the existence of imprinted genes, which are differentially expressed or silenced in

a parent-of-origin–dependent manner.<sup>41</sup> This form of monoallelic expression is governed by the acquisition of differential epigenetic marks in the gametes, which are maintained and appropriately interpreted by the transcriptional machinery in somatic cells. Imprinted genes play pivotal roles in mammalian development with particular emphasis on the regulation of prenatal growth, postnatal behavior and metabolism.<sup>42–47</sup> However, the unusual expression of imprinted genes from only 1 of the 2 parental alleles effectively renders them functionally haploid. Consequently, imprinted genes are especially vulnerable to a form of misregulation known as “loss of imprinting,” which manifests either as the inappropriate reactivation of the normally silent allele, causing “biallelic” overexpression, or silencing of the active allele, leading to loss of function. Because imprinted gene products are exquisitely sensitive to changes in dosage, their altered expression through loss of imprinting can lead to a number of growth disorders in humans, including cancer.<sup>48–53</sup> Evidence for parent-of-origin effects on familial IBD transmission has emerged, suggesting a potential involvement of imprinting.<sup>54,55</sup> Moreover, imprinting effects on the IBD susceptibility loci, *NOD2*, *PRDM1*, and *IL12B* were recently reported, although the influence of ethnicity was noted.<sup>56</sup> Genomic imprinting is not further discussed here, but its inclusion serves as a powerful illustration of how epigenetics can elicit diametrically opposite functionalities on 2 identical DNA sequences, even within the context of a single nucleus.

## DNA METHYLATION

The most widely studied epigenetic modification is DNA methylation that occurs in mammals by the covalent attachment of a methyl group to the 5' carbon of the cytosine residue within symmetrical cytosine-guanine (CpG) dinucleotides.<sup>57</sup> This symmetry, coupled with the preference of the principal DNA methyltransferase, DNMT1, for hemimethylated cytosines as a substrate,<sup>58</sup> provides a mechanistic basis by which methylation patterns are stably inherited through successive cell generations. Specific attention is given below to the role of DNMTs. Methylation occurs at approximately 70% of CpG dinucleotides in the genome overall but shows regional differences in its distribution. CpG dinucleotides are significantly under-represented in vertebrate genomes due to “CpG suppression,”<sup>59</sup> a phenomenon in which the prevalence of CpGs is constrained because of their inherent propensity to undergo point mutation by means of the spontaneous deamination of 5-methylcytosine to thymine. Accordingly, in humans and mice, CpG dinucleotides are the least frequent dinucleotide, comprising less than 1% of all dinucleotide permutations. Exceptions to this rule are CpG-islands (CGI), CpG-dense regions typically, but not exclusively, associated with gene promoters. Unlike other regions of the genome where CpGs are mostly hypermethylated, CGIs are generally protected from methylation. Aberrant de novo methylation of CGI promoters, including those of tumor suppressor genes (TSGs), is a hallmark of many human diseases including cancer (reviewed in Refs. 60,61) and is one of the features discussed below in relation to CAC. However, there is growing appreciation that less CpG-dense, “non-CGI”

promoter regions are more often affected by cell lineage-specific differences in methylation, which directly correlate with their transcriptional status, than canonical CGI promoters.<sup>62,63</sup> Similarly, lineage- and cancer-specific methylation patterns are also much more prevalent at “CGI shores,” i.e., regions of lower CpG density that typically flank canonical CGI sequences.<sup>64,65</sup>

DNA methylation is strongly correlated with gene silencing and as such is widely believed to participate directly in transcriptional repression. Mechanisms by which this might occur are not fully understood, but are likely to involve the recruitment of methyl-CpG binding domain proteins and histone deacetylases (HDACs), which together orchestrate transcriptional repression through induction of heterochromatin.<sup>60,66</sup> Methylation-dependent repression is also thought to play a critical role in preventing the inappropriate transcription of genomic repeat elements including retrotransposons, the failure of which can lead to genome instability.<sup>22,23</sup> DNA methylation is therefore essential for normal cellular functioning through the correct regulation of gene expression and maintenance of genome structural integrity. Whether DNA methylation is definitively instrumental in transcriptional repression remains an area of intense debate, because many researchers hold the view that DNA methylation is a consequence rather than a cause of repression. Epigenomic data, showing that de novo methylation predominantly targets repressed CGI promoters, argue that methylation may, at least in some contexts, be a consequence and not the cause of repression.<sup>67–69</sup>

Much attention has been devoted to the identity of the *trans*-factors that initiate and maintain CpG methylation patterns both as biomarkers of disease activity and as potential therapeutic targets. An extensive body of literature assigns this role to the DNMTs. On the premise of protein sequence homology, the DNMT family was initially thought to consist of 5 members: DNMT1, DNMT2, DNMT3A, DNMT3B, and DNMT3L;<sup>70</sup> however, recognition post hoc that DNMT2 methylates RNA,<sup>71</sup> sets it apart from the other DNMTs. Furthermore, DNMT3L lacks intrinsic 5-cytosine-methyl-transferase activity, instead serving as an accessory factor for DNMT3A function.<sup>71–74</sup> Current literature broadly subdivides 5-cytosine-methyltransferase activity into “maintenance” and “de novo” modalities, the former predominantly regulated by DNMT1, reflecting its substrate preference for hemimethylated DNA,<sup>58</sup> and the latter mostly attributed to DNMT3A/3B (and DNMT3L) which methylate either hemi- or un-methylated DNA.<sup>75–77</sup> Neither of these subdivisions is absolute because DNMT1 may be additionally required for the de novo activities of DNMT3A/3B in some contexts.<sup>78,79</sup> Furthermore, the ability of DNMT1 to maintain the correct fidelity of genome methylation may depend on the presence of DNMT3A/3B.<sup>80,81</sup> The pivotal *in vivo* functions of the DNMTs have been formally demonstrated in mouse transgenic and knockout studies. Mice with genetic *Dnmt1* deficiency, or transgenic overexpression, show embryonic lethality coupled with respective loss or gain of genome methylation.<sup>82,83</sup> Similarly, *Dnmt3a* or *Dnmt3b* genetic deficiency led to postnatal and embryonic lethality, respectively, whereas *Dnmt3a/3b* compound deficiency additionally blocked

normal *de novo* methylation of the genome during postimplantation development.<sup>84</sup> Together, these studies highlight the essential requirement for correct genome methylation levels in normal growth, development, and physiological functioning. Altered DNA methylation and DNMT expression/regulation were among the first epigenetic changes to be reported in IBD,<sup>85–90</sup> particularly in relation to CAC.<sup>91–93</sup> Additional discussion of this topic is provided in later section of this article.

## HISTONE MODIFICATION

Genomic DNA in eukaryotic cells is packed together with special proteins, termed histones, to form chromatin. The fundamental unit of chromatin is the nucleosome. Individual nucleosomes each comprised a complex of 8 core histone proteins, 2 molecules each of the histones H2A, H2B, H3, and H4, encompassing 146 bases pairs of genomic DNA. The histone octamer forms the structural basis of the nucleosome on which the DNA strand is wrapped around. Core histone proteins are tightly folded, however residues in their protruding amino-terminal regions, or “tails,” can be altered covalently by numerous posttranslational modifications (reviewed in Ref. 94) of which acetylation and methylation are the most pertinent to this review article. These modifications are important for determining the accessibility of the DNA to the transcription machinery and for DNA replication, recombination, chromatin condensation, and mRNA splicing. Accordingly, histone modifications are major contributors to epigenetic patterns of inheritance and play key roles in determining the transcriptional state of the genome. Unlike DNA (cytosine) methylation, which is mainly associated with transcriptional repression, histone modifications are extremely diverse and can be associated either with active transcription or repression,<sup>95</sup> providing an additional dimension to epigenetic regulation of gene expression. Histone acetylation and methylation are the best-characterized posttranslational epigenetic modifications of histone tails. While acetylation is generally considered an active mark, histone methylation can promote either transcriptional activation or repression, depending on the level of methylation (mono [me1]-, di [me2]- or tri [me3]-methylation) and the specific residues (lysines and/or arginine) involved. For example, highly transcribed, open “euchromatin” regions of the genome are enriched in active marks including histone (H)3 lysine (K)4, H3K36 and H3K79 trimethylation, and acetylation (ac). Conversely, transcriptionally repressed, closed “heterochromatin” regions are typically enriched in repressive marks, H3K9me3, H3K27me3, and H4K20me3 and show reciprocal depletion in acetylation levels. Although it is still not entirely clear how these histone marks impact mechanistically on transcription, their presence is highly predictive for gene expression or silencing (reviewed in Ref. 96). A multitude of different histone modifications, each with different activating or repressive effects, can be present simultaneously leading to the widely advocated histone code hypothesis.<sup>97,98</sup> It was proposed that histone modifications, in addition to their individual functions, manifest a complex array of interdependencies allowing for dozens of different epigenetic landscapes to be

established, thereby fine tuning higher order chromatin structural organization, gene expression, and repression.<sup>97,98</sup> Therefore, a single mark, acting alone, is unlikely to dictate significant transcriptional effects. Rather, as recent data further attest, the combined effects of all modifications enriched within specific gene regions instruct the assembly of many different chromatin states, each with unique transcriptional and/or other functional outcomes.<sup>99</sup>

Current literature cites the existence of numerous enzymes that catalyze either the addition or the removal of histone posttranslational modifications. For example, histone acetylation, which is strongly correlated with regions of active transcription, is regulated by both histone acetyltransferases (HATs) and HDACs that respectively add or remove acetyl groups nonspecifically at a number of residues in histone tails. Accordingly, HAT and HDAC activities correlate with decreased and increased transcription, respectively. Mechanisms by which the acetylation/deacetylation of histones mediate transcription have been sought but are not well understood. However, one possibility is that acetylation neutralizes positively charged lysines, destabilizing the interaction with negatively charged DNA resulting in a more open chromatin structure accessible to transcription factors and other coactivator proteins.<sup>100</sup> The process of histone methylation is much more specifically regulated than histone acetylation. Histone methyltransferases (HMTs) and the more recently discovered “demethylases” are the enzymes mediating this process, targeting specific residues within individual histone core proteins. Recent advances in knowledge in this area and their broad relevance to human disease have sparked renewed interest in epigenetic modifiers as potential therapeutic targets. Small molecule inhibitors have been designed against the key enzymes, thus far discussed here, including HMTs, HATs, HDACs, and DNMTs.<sup>101,102</sup> The tremendous therapeutic potential of such inhibitors and their possible application in IBD treatment is briefly discussed here under “Diagnostic and Therapeutic Implications.”

## NUCLEOSOME POSITIONING AND CHROMATIN REMODELING

Although the packaging of DNA by nucleosomes occurs in a highly regulated and ordered fashion, the overall structure of the nucleosomal array is not fixed, but rather shows a highly variable distribution along the DNA strand. One of the main influences on this irregular pattern is the binding of other “nonhistone” proteins, such as transcription factors and accessory proteins, to the DNA leading to the local displacement of nucleosomes. Accordingly, the precise positioning of nucleosomes can be altered in a highly regulated manner to elicit functional changes in gene expression (reviewed in Ref. 103). For example, nucleosomes act as a physical barrier to transcriptional initiation by preventing access of transcription factors to their consensus binding sites in the DNA sequence. Nucleosomes can also regulate transcript elongation by impeding the progression of bound RNA polymerases through the structural gene. Even relatively minor nucleosome displacements can bring about, or reflect, changes in RNA polymerase II

activity. The 5' and 3' regions of genes are generally devoid of nucleosomes, thus providing space for the assembly and disassembly of multifactor transcription complexes.<sup>104</sup> The eviction of a nucleosome from the region surrounding the transcription start site of genes correlates with active transcription. Conversely, occupation of the transcription start site by a nucleosome is the characteristic feature of repressed genes.<sup>105</sup> Besides playing a direct role in transcription, nucleosome positioning has also been described to influence local DNA methylation patterns.<sup>105</sup> In particular, nucleosome enrichment across gene exons and depletion within introns is remarkably similar to the distribution of DNA methylation. The findings support the view that nucleosome occupancy plays a key role in shaping the genomic methylation landscape. The functional significance of this relationship is unclear but it could act to define intron–exon boundaries or participate in mRNA splicing. Changes in nucleosome positioning are regulated by chromatin remodeling complexes, which are large multiprotein assemblies that alter the composition or organization of nucleosome core proteins. Current literature recognizes 5 families of chromatin remodeling complexes: SWI/SNF, ISWI, NuRD/Mi-2/CHD, INO80, and SWR1 based on conservation of their principal catalytic domains (reviewed in Ref. 106). These complexes regulate transcription by inducing adenosine triphosphate hydrolysis-dependent protein conformational changes, which, in turn, provoke reconfiguration of nucleosome positioning. Each of these families possesses both unique and partially overlapping functional specializations. SWI/SNF proteins are key regulators of gene expression,<sup>107</sup> whereas ISWI family members participate in chromatin condensation and transcriptional repression.<sup>108</sup> NuRD/Mi-2/CHD family proteins are functionally diverse. Some family members facilitate lateral sliding and eviction of nucleosomes, thus acting as transcriptional activators, while others promote repression by means of intrinsic HDAC and/or methyl-CpG binding domain activity.<sup>109,110</sup> Finally, proteins of the INO80 group participate in a wide array of chromatin-related functions including transcription, DNA replication, and repair.<sup>111–113</sup>

## RNA INTERFERENCE

Noncoding RNAs comprise endogenous small single-stranded miRNAs that are present at lower levels than mRNA. The miRNAs regulate gene expression at the posttranscriptional level and thereby numerous biological processes. The miRNAs bind to untranslated mRNAs and inhibit mRNA translation (partial sequence complementarity) or cause mRNA degradation (complete sequence complementarity). To date, 1872 precursor and 2578 mature miRNA sequences have been described in humans (<http://mirbase.org>, accessed February 3, 2014). Each miRNA may show complementarity with many different mRNAs, and each mRNA may be targeted by many different miRNAs. The miRNA-mediated gene regulation is critical for normal cellular processes such as cell cycle, differentiation, proliferation, apoptosis, and, most importantly in the context of this review article, innate,

and adaptive immune functions.<sup>114</sup> The expression of miRNAs themselves in the immune system can be regulated at different steps of their biogenesis by immunogenic stimuli.<sup>115</sup> Altered miRNA expression has been associated with many diseases including IBD.<sup>14</sup>

## EPIGENETIC REGULATION OF INNATE AND ADAPTIVE IMMUNITY

Phagocytic cells within the lamina propria (e.g., macrophages, dendritic cells [DCs], and neutrophils) and epithelial cell barrier represent the central components of the intestinal innate immune system.<sup>116</sup> Antigens reaching the lamina propria activate innate immune cells followed by a response of T-lymphocytes, which are the prime effector cells of adaptive immunity. The innate and adaptive immunity arms are tightly cross regulated serving to uphold intestinal homeostasis, thereby controlling complex commensal–host crosstalk through the recruitment, maintenance, and regulation of effector functions of various intestinal immune cells (Fig. 1).<sup>117</sup> Macrophages represent the most abundant mononuclear cell population of the intestine. Macrophages play an important role in intestinal antigen presentation to other immune cells in the lamina propria and in sustaining intestinal immune homeostasis.<sup>118</sup> It has been suggested that blood monocytes are the exclusive source of macrophages in inflamed intestinal mucosa with both peripheral monocytes and their derivatives playing important roles in the pathophysiology of IBD.<sup>119</sup> In addition, several DC subtypes form a further central part of the functional mucosal barrier of the intestine and play an important role in IBD pathogenesis.<sup>120</sup> Recent advances have highlighted a fundamental role of DCs in intestinal innate immune homeostasis.<sup>121</sup> DCs initiate immune responses during microbial invasion and inflammation through antigen presentation and also polarize subsequent adaptive immune responses.<sup>122</sup>

Emerging evidence suggests an important role for epigenetic mechanisms in modulating both the innate and adaptive immune systems. This includes the differentiation and function of monocytes/macrophages, DCs, neutrophils, and T-helper (Th) cell subsets. Importantly, inflammation may directly drive epigenetic reprogramming leading to aberrant immune responses. Chromatin-based events are potentially important in amplifying and perpetuating the inflammatory response as well as regulating inflammation-induced transcription, tolerance, and T-cell lineage commitment<sup>7</sup> (and references cited therein).

Epigenetic modification of chromatin plays an important role in macrophage polarization and function. However, current literature on the epigenetic regulation of macrophage polarization and functional consequences for macrophage gene expression and phenotype is limited. Epigenetic analyses of macrophage polarization at various tissue sites during the initial inflammatory activation, immune homeostasis and the resolution of inflammation have primarily focused on histone acetylation and methylation, with limited analysis of nucleosome remodeling<sup>123</sup> (and references cited therein). Nevertheless, evidence supports the hypothesis that epigenetic changes fundamentally reprogram macrophages to exhibit altered gene expression in response to

environmental stimuli. Briefly, defined combinations of active and repressive histone marks regulate the chromatin states of inflammatory cytokine gene loci relevant for polarized M1/M2 macrophage phenotypes and thus transcription rates in response to acute stimulation and polarizing stimuli. This also includes the epigenetic regulation of key inflammatory cytokine genes, which allows a fine-tuned rapid and effective immune response and cytokine production, respectively. Classically activated M1 macrophages produce proinflammatory cytokines, which mediate resistance to pathogens and contribute to tissue destruction, whereas alternatively activated M2 macrophages produce anti-inflammatory cytokines, which promote tissue repair and remodeling.<sup>124</sup> Furthermore, epigenetic mechanisms might also explain the gene-specific signature of tolerant macrophages following the state of acute immune activation. However, the identity of epigenetic mechanisms regulating macrophage tolerance in response to environmental cues requires further clarification.<sup>123</sup> A better understanding of the epigenetic mechanisms that mediate repression of inflammatory cytokine gene expression in (human) macrophages represents an important area for future research and for potential new therapeutic approaches. Given that intestinal macrophages derive from peripheral blood monocytes, it will also be important to analyze epigenetic marking of monocytes. Recent studies have begun to explore the epigenetic control of monocyte differentiation and function. Importantly, this includes persistent enhanced effector immune functions of monocytes after a primary infection or vaccination and subsequent protection and resistance of the host against a secondary (re-)infection independent of adaptive immunity. These adaptive features of innate immunity have recently been described as trained immunity.<sup>125</sup> For example, a NOD2-mediated epigenetic change at the level of an active histone mark (H3K4me3) is the mechanism through which mycobacterial components (bacille Calmette-Guérin) enhance innate immune responses.<sup>126</sup> The modified methylation status of cytokine promoters after bacille Calmette-Guérin vaccination in human monocytes and the blockade of the *in vitro* training effects with methyltransferase inhibitors suggests that the innate immune response in humans can be reprogrammed epigenetically. Thus, epigenetic reprogramming has potential preventive and therapeutic purposes in inflammation and autoimmunity. One example is systemic lupus erythematosus, where monocytes play a central pathophysiological role and have been described as having aberrant behavior in a number of assays. Furthermore, cytokines can induce changes in the epigenome of systemic lupus erythematosus monocytes and persistence of these changes might lead to heritable changes in gene expression, which drive many of these aberrant functions.<sup>127</sup> Altered monocyte-derived macrophage functions (spontaneous and lipopolysaccharide [LPS]-induced tumor necrosis factor alpha release) and modified DNA methylation in white blood cells have also been described in women treated with oral contraceptives.<sup>128</sup>

Furthermore, during the transition of human immature monocyte-derived DCs into activated (LPS-conditioned) and tolerized (transforming growth factor beta-conditioned) DCs, changes in the modification of histones (H3K4me3 and

H2K27me3) and alteration in these epigenetic marks may have a role in the resulting gene regulation of these cells (e.g., chemokines, cytokines, cell surface molecules, and transcription factors).<sup>129</sup> In addition, the chemotactic activity of monocyte-derived immature DCs and M1 macrophages can be altered by chromatin modulation in these cells. For example, simvastatin, a widely used statin that blocks cholesterol synthesis but also has pleiotropic immunomodulatory and anti-inflammatory properties, can induce repressive chromatin at the *chemokine (C-C motif) ligand 2 (CCL2)* promoter. CCL2, also known as monocyte chemotactic protein 1 (MCP1), recruits monocytes, memory T cells, and DCs to sites of injury and inflammation. The reduced gene expression and secretion of CCL2 in monocyte-derived cells was accompanied by enrichment of repressive marks, H3K27me3/H3K9me3 and depletion of active marks, H3ac and H3K4me3 at the CCL2 promoter.<sup>130</sup> Downregulation of CCL2 in these cells may affect their chemotactic activity leading to reduced recruitment of monocyte-derived DCs and proinflammatory M1 macrophages to sites of tissue injury and/or inflammation. It has been argued that immunoparalysis/immunosuppression observed after sepsis is mediated by DC dysfunction arising from the perpetuation of an aberrant gene expression program.<sup>131</sup> In this regard, it has been reported that chronic repression of *Il12*, which encodes a key host defense cytokine, in DCs from postseptic mice correlates with promoter enrichment of bivalent (i.e., active and repressive) marks H3K4me3 and H3K27me2.<sup>132</sup> Although epigenomic studies on DCs are limited, some of these suggest a role for histone modification and DNA methylation in DC differentiation and function. Furthermore, epigenetic perturbations mediated by HDAC inhibitors are likely to modify the function of DCs from immunostimulatory to immunomodulatory. Regulation of H3K9me2/me3 marks by cell type-specific HMTs or histone demethylases is essential for the development and differentiation of DCs.<sup>133</sup> Furthermore, several reports indicate the involvement of DNA methylation in regulating DC-specific gene expression programs. The majority of DNA methylation changes seem to arise early during hematopoietic lineage commitment, and only a few are acquired during terminal differentiation<sup>133</sup> (and references cited therein).

Beside monocytes/macrophages and DCs, neutrophils also play a critical role in the maintenance of intestinal immune homeostasis. They are critical for bacterial clearance, release cytokines and antimicrobial proteins, and phagocytize invading microbes that translocate across the intestinal epithelial cell (IEC) layer. During the inflammatory response, neutrophils also contribute to the recruitment of other immune cells and facilitate mucosal healing by releasing mediators necessary for the resolution of inflammation.<sup>134</sup> Neutrophil migration into the infectious site is markedly impaired in severe sepsis and associated with depletion of active H3ac marks at the *C-X-C chemokine receptor type 2 (CXCR2)* promoter in neutrophils.<sup>135</sup> *CXCR2* encodes a receptor for interleukin 8 (IL8) and mediates neutrophil migration to sites of inflammation. Another example of epigenetic control of neutrophil differentiation and function arises from the observation that

neutrophils from mice that lack the transcription factor Jun dimerization protein 2 (Jdp2), which regulates histone modification, displayed impaired bactericidal function, apoptosis, and surface expression of lymphocyte antigen 6 complex, locus G (Ly6G).<sup>136</sup>

Accumulated evidence shows that epigenetic mechanisms are key determinants of CD4<sup>+</sup> Th cell differentiation and function. Th cells exist in a variety of epigenetic states that determine their function, phenotype, and capacity for persistence. These polarization states include Th1, Th2, Th17, and T-regulatory (Treg) cells. Briefly, Th1, Th2, and Th17 cells are important for eradicating intracellular pathogens, helminthes, and extracellular bacteria/fungi, respectively. Th1 and Th17 cells are also involved in many types of autoimmune diseases, whereas Th2 cells contribute to allergic responses. Treg cells are critical in maintaining self-tolerance and in modulating immune responses to infections.<sup>137</sup> The state of chromatin and DNA methylation at lineage-restricted cytokine and transcription factor genes, as well as their regulatory elements in Th cells, both reflects and affects their function in transcription<sup>138</sup> (and references cited therein). Different profiles of DNA methylation, active and repressive histone marks, RNA interference, and methyl-CpG binding domain proteins are associated with active and accessible, inactive but poised, as well as silenced gene loci in Th cells. Consequently, these distinguishing epigenetic marks ensure that transcription of Th1-type cytokines (e.g., IFN $\gamma$ ) and Th2-type cytokines (e.g., IL4, IL5, IL13) is restricted to the appropriate lineage.<sup>137–140</sup>

Th1/Th2 development is also epigenetically regulated, involving chromatin modifications of the *IFN $\gamma$*  gene locus and the epigenetic control of the Th2 cytokine locus.<sup>141</sup> Less is known about the epigenetic processes controlling Th17-cell differentiation and transcription of lineage defining Th17 cytokines (e.g., IL17, IL21, IL22, and IL26). However, recent developments describe epigenetic mechanisms that can explain the constrained plasticity of Th17 cells by the epigenetic status of genes encoding for the master transcriptional regulators of polarization and canonical cytokines.<sup>137,142,143</sup> The discovery that forkhead box P3 (FoxP3) is the transcription factor that specifies the Treg cell lineage has facilitated recent progress in understanding Treg cell biology.<sup>144</sup> Treg cells do not express a lineage-defining cytokine; on the contrary, production of most inflammatory cytokines is repressed.<sup>143</sup> Constitutive FoxP3 expression is required to maintain the immunosuppressive activity of Treg cells.<sup>137</sup> FoxP3 both activates and represses target genes by epigenetically regulating histone modifications through recruitment of several histone-modifying proteins.<sup>145</sup> For example, genes activated by FoxP3 show enrichment of active marks, H3K4me3, H3K9/14ac, and H4K16ac. Conversely, genes repressed by FoxP3 show enrichment of the repressive mark, H3K27me3.<sup>146,147</sup> Treg cells, and also conventional CD4<sup>+</sup> T cells, show lineage-specific methylation of regions overlapping methylation-sensitive enhancers within the vicinity of immunologically relevant genes. These findings argue that DNA methylation plays key role in establishing and maintaining cell type-specific gene expression by restricting the lineage activity of cell type-specific enhancers.<sup>148</sup>

In summary, epigenetic regulation of key cytokines and transcription factors specific to cells of the innate and adaptive immune systems plays an important role in the development, differentiation, phenotype and function of monocyte/macrophages, DCs, neutrophils, and Th cells (Fig. 1). The dynamic interactions between the genome and epigenome have important implications for a better understanding of human inflammatory diseases (including IBD) and their pathophysiology, thereby enabling the development of gene-specific therapeutic approaches.

## EPIGENETIC CONTROL AND INTESTINAL MICROBIOTA

Intestinal microbiota profoundly affect host immune composition under physiologic conditions and are likely the most important environmental factor in IBD as targets of the inflammatory response.<sup>149</sup> New evidence indicates that intestinal bacteria can regulate epithelial gene expression and the intestinal immune response through epigenetic mechanisms (Fig. 1). Similarly, bacterial and host (self-DNA) epigenetics can also directly affect host genetics to trigger inflammatory processes.<sup>12</sup> Several mechanisms have been proposed to link epigenetic modifications with inflammation, involving the innate immune response against microbiota.

Butyrate is an endogenous metabolite formed during fermentation of dietary fibers by the intestinal microbiota and is a potent inhibitor of histone deacetylase (HDAC) activity. Butyrate-dependent HDAC inhibition has been shown to upregulate expression of the pattern recognition receptor protein NOD2 by increasing histone acetylation.<sup>150</sup> Likewise, sodium butyrate increases the production of intestinal alkaline phosphatase, an endogenous protein responsible for detoxification of bacterial LPS.<sup>151</sup> A number of other studies have similarly inferred that bacteria induce histone modifications, thereby modulating the inflammatory response and key cellular processes of the epithelium in the gastrointestinal tract (Fig. 1).<sup>152–160</sup> Additionally, commensal probiotic bacteria demonstrably modulate the IL23/Th17 axis, which has parallel significance to the IL12/Th1 axis in regulating IBD pathogenesis.<sup>3</sup> Conversely, *Bifidobacterium breve* and *Lactobacillus rhamnosus* (LGG) may exert their anti-inflammatory effects in the gut, at least in part, by modulating IL23 and IL17 endogenous synthesis through inhibition of histone acetylation and enhancement of DNA methylation.<sup>161</sup> However, Th1 mucosal immunity is characterized by overproduction of IFN $\gamma$ , and it has been further shown that levels of *IFN $\gamma$*  promoter methylation in peripheral T cells correlate with immune response to microbial components and expression/secretion of IFN $\gamma$  in patients with UC.<sup>162</sup>

Mucosal DNA methylation can also react to changes in commensal microbiota.<sup>11</sup> Oral treatment with a genetically modified strain of *Lactobacillus acidophilus* deficient in lipoteichoic acid effectively ameliorated inflammation-induced colitis and restored intestinal homeostasis in experimental models.<sup>163</sup> Lipoteichoic acid is a major immunostimulatory cell wall component of Gram-positive bacteria, which can specifically bind to toll-like receptors (TLRs) on host cells. *L. acidophilus* bacteria also protect mice from CAC presumably by reversal of cancer-related DNA methylation

within promoters of genes that normally block or restrain intestinal cancer progression.<sup>164</sup> Bacterial gene products may dampen detrimental gut inflammation and protect against inflammatory conditions, including IBD and CAC, acting not only through immune cell modulation, but also through direct interactions with the gut epithelium (Fig. 1).<sup>165</sup> For example, the pattern recognition receptor, TLR4 senses the presence of LPS from Gram-negative bacteria, and it has been shown that *TLR4* transcription is epigenetically repressed in IECs to prevent excessive inflammatory responses to commensal bacteria.<sup>166</sup> Furthermore, *Tlr4* methylation levels are significantly lower in IECs of the large (but not small) intestine of germ-free (GF) mice than in those of conventional mice. These findings argue that commensal bacteria contribute to the maintenance of intestinal symbiosis by controlling the epigenetic modification of the host gene.<sup>167</sup> Contrariwise, the mucosal microbiome composition is significantly altered in *Tlr2*-deficient mice compared with wild-type mice but similarly associated with epigenomic and transcriptomic modifications.<sup>168</sup> Immune-related gene expression is significantly altered by *Tlr2*-deficiency and correlates with DNA methylation changes. Bacterial presence also results in epigenetic modifications in gingival epithelia and bacteria-induced expression of epithelial antimicrobial molecules human  $\beta$ -defensin 2 (hBD2) and CC chemokine ligand 20 (CCL20).<sup>169</sup> Challenge of neonatal GF mice with conventional microbiota normalized *Cxcl16* promoter hypermethylation to levels typically observed in specific pathogen-free mice.<sup>170</sup> CXCL16 is expressed at high levels by human epithelial cells and plays an important role in invariant natural killer T (iNKT) cell recruitment during inflammation. The iNKT cells probably play an important role in the pathogenesis of UC.<sup>1</sup> *Cxcl16* protein levels in serum and mRNA expression in the colon are significantly higher in GF mice compared with specific pathogen-free mice. Hence, numbers of iNKT cells have been shown to be persistently increased in the colonic lamina propria of GF mice compared with specific pathogen-free mice, resulting in increased morbidity in oxazolone-induced colitis, a murine model of UC.<sup>170</sup> The findings argue that microbial exposure drives epigenetic mechanisms that determine both *Cxcl16* gene expression and consequent iNKT cell recruitment in the colon. Interestingly, colonization of adult GF mice with conventional microbiota did not protect against mucosal iNKT accumulation and related pathology.<sup>170</sup> These results indicate that age-sensitive contact with commensal microbes is critical for colitis susceptibility. Consequently, colonic mucosal epigenetic maturation continues through early postnatal development in mice and may contribute to the age-associated increase in colitis susceptibility.<sup>171</sup> In this regard, a marked increase in sensitivity to dextran sulfate sodium (DSS)-induced colitis was observed in mice after prenatal maternal exposure to epigenetically active (methyl-donor [MD]) diet.<sup>172</sup> This phenotype was associated with changes in mucosal DNA methylation, gene expression, and the intestinal microbiome. Interestingly, the same MD diet regimen did not alter colitis susceptibility in young adult mice when administered postnatally. These results provide evidence that prenatal epigenetic reprogramming of mucosal immunology through maternal dietary factors, but not postnatal diet,

creates a persistent effect on the enteric microbiome by inducing longstanding modification during early development relevant to mammalian colitis. Developmental dietary intervention induced alteration of normal colonic mucosa-associated microbiota shifts may have contributed to the observed increase in colitis susceptibility in the MD supplemented offspring.<sup>173</sup> Thus, prenatal nutritional programming can modulate the mammalian host to harbor a colitogenic microbiome.<sup>174</sup>

These studies provide important insights into microbe-specific immunity through epigenetic regulation. They highlight the intimate interrelationship between expression of immune-related genes and immunity pathways in the host with compositional and functional differences of the microbiome. Based on this promising data, future research should aim to further elucidate potential epigenetic mechanisms underlying the interplay between the microbiome and host immunity.

## EPIGENETIC MODIFICATIONS IN IBD

To date, relatively little is known about the role of epigenetics in IBD. Initial DNA methylation studies have been directed predominantly towards IBD-related cancer (see Epigenetics in Colitis-associated Cancer section below). More recent genome-wide DNA methylation studies used peripheral blood or intestinal biopsy specimens of patients with IBD and healthy controls to identify methylation differences in genes regulating several pathways associated with IBD.<sup>85–90</sup> A significant number of differentially methylated loci contained genes linked directly to the immune response, host response to bacteria, and IL23/Th17 and IL12/Th1 pathways. Additionally, genes identified as being differentially methylated in epigenome-wide methylation-association studies have also been identified as susceptibility genes in genome-wide association studies including *TNF*, *NOD2*, *IL19*, *IL27*, *CARD9*, *ICAM3*, and *IL8RB*.<sup>88,89</sup> Furthermore, mucosal genome-wide methylation profiling showed evidence of differential methylation between (1) active CD and controls, (2) active UC and controls, (3) inactive UC and controls, and (4) inactive CD and inactive UC. Interestingly, differences were not found in methylation profiles between (1) quiescent CD and controls and (2) active CD and active UC.<sup>89</sup> Future epigenome-wide methylation association studies are widely anticipated to confirm the diagnostic and prognostic potential of DNA methylome profiling in IBD.<sup>175</sup> Importantly, the function of epigenetic marks and their role in IBD pathogenesis in individual cell types remains to be comprehensively defined.

Histone modifications are less extensively studied in IBD than DNA methylation. However, patterns of histone acetylation in the colon of rats with colitis and humans with CD have been described.<sup>176</sup> Relative enrichment of the active marks, H4K8/K12ac and H4K5/K16ac, was observed in the inflamed mucosa and in Peyer's patches, respectively in comparison with nondiseased tissue. A gene-specific and temporal-specific pattern of histone modifications on the activated gene for collagen type I, the most abundant component of the fibrotic extracellular matrix,

can be induced by fibrosis relevant cytokines (IL1 $\beta$ , transforming growth factor beta, and tumor necrosis factor alpha) suggesting that epigenetic factors regulate fibrotic gene transcription relevant to IBD with a fibrostenosing phenotype.<sup>177</sup> Nevertheless, current understanding of the role played by histone modifications in IBD derives mostly from experimental or clinical trials of HDAC inhibitors, which are discussed below.

Studies in animal models have shown that intestinal miRNAs regulate gut homeostasis.<sup>178</sup> Other studies have sought miRNA expression profiles in the peripheral blood and gut biopsy specimens of healthy controls versus adult/pediatric patients with active and inactive IBD. The miRNA expression profiles were found to be dysregulated at both the tissue level and in peripheral blood of UC and CD patients. In addition, studies have shown that IBD-related glucocorticoid therapy can modify the expression profile of different miRNAs; however, the data are conflicting.<sup>179</sup> A number of comprehensive reviews have been published on the potential value of miRNAs in IBD during recent years to which we refer the reader.<sup>8,13,180</sup> Specific miRNA profiles and identification of associated targets might provide additional insight into IBD pathogenesis and help to predict IBD susceptibility, progression, relapse, and response to therapy. However, miRNA profiling in IBD is in its infancy and additional studies are required to identify reliable and consistent IBD-associated miRNA profiles and to attribute-associated functions.

## EPIGENETICS IN COLITIS-ASSOCIATED CANCER

A direct link between chronic inflammation and cancer susceptibility is well established; however, causal molecular mechanisms remain poorly understood. Advances in knowledge have nonetheless been forged through the study of several gastrointestinal cancers and have uncovered evidence to support inflammation-dependent transformation of epithelial cells as a mechanism of neoplasia. For example, studies of the *Helicobacter pylori*-associated gastric cancer paradigm<sup>181</sup> have illuminated key genetic and epigenetic pathways underlying inflammation-induced cancer.<sup>182–185</sup> Focusing here on cancer susceptibility in IBD, we highlight recurrent themes of classical cancer genetics and cytokine-dependent perturbation of gastrointestinal epigenetic inheritance, which resonate strongly through emerging research in this field.

Individuals with IBD show a significantly increased risk of acquiring CAC for which the most prominent risk factors are the localization, duration, and severity of gastrointestinal inflammation. CAC remains a significant complication in the long-term management of IBD; thus, the elucidation of molecular mechanisms underlying CAC is of high priority. As discussed above, accumulated evidence points to a role for inflammation in the pathogenesis of numerous cancers. Therefore, CAC provides an ideal human disease system through which fundamental concepts of inflammation-related carcinogenesis can be explored and widely disseminated. Similar to the pathogenesis of IBD per se, the development of CAC is highly heterogeneous and is likely to be driven by the converging effects of host genetic and epigenetic factors, as well as the influence of environmental factors, such as the intestinal microbiome (reviewed in Refs. 186,187).

As discussed above, DNA methylation and histone modification together establish stable and heritable patterns of gene expression in normal cells. However, alterations in these key epigenetic modifications, leading to global deregulation of gene expression, are frequently observed in both inflammation and cancer, as well as in inflammation-related cancer.<sup>92,188</sup> Aberrant DNA methylation is one of the earliest molecular changes in cancer and may correlate with the early stages of both IBD and CAC.<sup>187</sup> In cancer cells, normally hypermethylated sequences become globally hypomethylated, which can lead to genomic instability, a characteristic feature of colorectal cancers (CRC).<sup>189</sup> Conversely, normally unmethylated CGI promoters, including those at some TSG loci, become aberrantly hypermethylated leading to their repression. Inactivation of TSGs through promoter hypermethylation provides a persuasive epigenetic mechanism of cancer pathogenesis.<sup>60</sup> Hypermethylated TSGs have been suggested as potential epigenetic biomarkers of cancer risk on which new approaches to clinical screening might be based.<sup>190</sup> For example, specific gene methylation patterns are highly predictive of precancerous pathology in the gastrointestinal tract and can signify the presence of inflammation and dysplasia, as well as cancer. These data strongly implicate a role for epigenetic alterations in inflammation-dependent carcinogenesis.

The available literature supporting roles for epigenetic modification in CAC remains significantly underdeveloped. Moreover, the DNA methylation studies that have recently emerged have often made contradictory findings, although these have largely been in respect of the CpG-island hypermethylator phenotype (CIMP) phenomenon. CIMP is a well-described epigenetic phenomenon in a subset of sporadic CRC involving the coordinate methylation-dependent silencing of TSG loci. However, “CIMP-positive” CRC cases frequently display molecular correlates of microsatellite instability and secondary genetic mutations, which complicates interpretation of CIMP as a driver of CRC. Nonetheless, CIMP has been widely advocated as an epigenetic marker of CRC risk, progression, and survival,<sup>191</sup> prompting investigators to search for “CIMP” signatures as both a mechanism and marker of CAC. Age-related increases in CGI methylation levels are also known to occur in the colonic epithelium and may act as confounding factors in this regard. One study reported that age-related CGI methylation may show accelerated progression in IBD because of increased cell turnover in the context of inflammation.<sup>91</sup> The findings were argued in evidence of an epigenetic mechanism for cancer susceptibility in UC. Nevertheless, in a subsequent study, the same investigators analyzed methylation levels of 11 genes in UC-associated cancers, UC-associated dysplasias, and sporadic CRC cases to determine if accelerated age-related methylation in dysplastic UC translates into subsequent cancer. They reported that CIMP was infrequent in UC-associated cancers compared with sporadic CRC, thus arguing against a major role for DNA methylation (CIMP) as a driver of CAC.<sup>192</sup>

Conversely, other studies have unequivocally shown hypermethylation of loci previously linked to cancer pathogenesis, *CDHI*, *GDNF*, *HPPI*, and *MYOD1*, in active UC compared with quiescent UC.<sup>92</sup> Other studies have replicated the latter finding of *CDHI* hypermethylation in both dysplastic and tumor tissue taken

from subjects with UC, compared with nondysplastic tissues.<sup>93,193</sup> Furthermore, aberrant CGI promoter hypermethylation of the TSGs, *P53*, *P14<sup>AR</sup>*, *P16<sup>INK4a</sup>*, *P21<sup>CIP1</sup>*, and *MLH1* in UC lacking neoplasia has been collectively reported by several studies.<sup>194,195</sup> The concept was further developed by Dhir et al who investigated DNA methylation levels within canonical WNT/beta-catenin signaling pathway components downstream of the intestinal TSG, *adenomatous polyposis coli (APC)*. Deregulation of WNT/beta-catenin signaling in association with APC genetic inactivation is a key mechanism of sporadic CRC pathogenesis; however, somatic mutations within this pathway are not commonly reported in CAC. They instead found that methylation-dependent inactivation of several WNT pathway genes is a frequent, early event in both IBD and IBD-related cancer,<sup>196</sup> providing additional evidence to support a potential epigenetic route to CAC. Another potentially important mechanism relates to the CGI hypermethylation and inactivation of the *suppressor of cytokine signaling 3 (SOCS3)* locus leading to hyperactivation of the oncogenic IL6/STAT3 pathway in UC and CAC.<sup>197</sup>

Although progress has undoubtedly been made, the candidate gene approach favored by many of the above-described studies provides a limited view of potentially widespread epigenetic deregulation in CAC. Consequently, the full extent of epigenetic alterations that may precede the emergence of CAC remains poorly understood. Genome-wide approaches to comprehensively determine DNA methylation (and histone modification) patterns will be critical to an improved understanding of the epigenetic principles underlying CAC and their active deployment in IBD disease management. Studies of this genre have yet to emerge en masse; however, a recent microarray-based genome-wide analysis revealed that extensive CGI hypermethylation is much less prevalent in CAC compared with sporadic CRC cases. Consistent with the findings of earlier candidate gene approaches,<sup>192</sup> Olaru et al<sup>198</sup> reported the existence of a CIMP-positive CAC subset, showing key similarities to sporadic CRC in age of cancer onset and extensive cancer-specific CGI hypermethylation. However, the authors attributed the acquisition of CIMP in this subset of CAC to the effects of aging rather than inflammation per se, further concluding that CGI methylation plays a limited role in CAC pathogenesis.

The conclusions of Olaru et al are supported by epigenomic evidence that CGI hypermethylation in human malignancies (including colon cancer) may, in general, be less extensive than initially hypothesized.<sup>62</sup> Furthermore, revisions to the much-vaunted model of CGI methylation-dependent cancer pathogenesis have been proposed. For example, although CGI hypermethylation generally correlates with repression of TSGs, the assumption that methylation per se is instrumental in their repression may not always be correct. On the contrary, recent studies show that cancer-related de novo methylation is attracted predominantly to CGI promoters that are already repressed in normal tissues. In these instances, methylation does not directly participate in repression and therefore is not necessarily a driver of cancer.<sup>67–69</sup> With the above caveats noted, future research should address whether extensive CGI hypermethylation might still inactivate rare but nonetheless pivotal protective genes in CAC. Alternatively, there is

growing recognition of an important role for less CpG-dense, non-CGI promoters, and “CGI shores” as targets of lineage-specific differential methylation in cancer.<sup>65</sup> Mapping the methylation profiles of these regions on a genome-wide scale has the potential to unravel many of the unresolved epigenetic questions surrounding CAC pathogenesis and should be an immediate priority of IBD research.

Definitive mechanisms to explain the inflammation-associated perturbation of gastrointestinal epigenetic inheritance in CAC and in other gut inflammatory cancers have not been firmly established in vivo. However, fundamental roles for specific proinflammatory cytokines and the reactive oxygen/nitrogen species induced as a consequence of their bioactivity have begun to crystallize. Among the best available evidence, the proinflammatory cytokine, IL1 $\beta$ , a key determinant of inflammation-related cancer risk in humans, was shown to stimulate DNMT1 activity in a nitric oxide (NO)-dependent manner through upregulation of inducible nitric oxide synthase (*iNOS*).<sup>199</sup> Similarly, IL6, acting through DNMT1, was shown to promote hypermethylation of genes associated with tumor suppression, adhesion, and apoptosis, thereby enhancing the oncogenic phenotype of colon cancer cells. These results argue that cytokine-dependent, DNMT-mediated gene silencing is a key mechanism of inflammation-related colon tumorigenesis.<sup>200</sup> This concept is further supported by evidence that exogenous IFN $\gamma$  exposure can elicit genome hypermethylation in concert with increased expression of the de novo methyltransferase, DNMT3B, in human colonic epithelial cells.<sup>201</sup>

Oxidative stress, which is a key component of inflammatory reactions, was elegantly shown to provoke recruitment of a repressive complex containing DNMT1, DNMT3B, HMT proteins of the polycomb group and HDAC to CGI promoters in human colon cancer cells. The investigators further showed that DNMT1, polycomb, and HDAC proteins were similarly enriched on CGI promoters in a mouse model of colitis.<sup>202</sup> Therefore, oxidative stress can promote recruitment of a repressive complex that may explain the de novo hypermethylation and loss of gene function that accompanies the progression of inflammation-related neoplasias, including CAC. A recent study showing that IL6 promotes increased DNMT1 expression leading to epigenetic inactivation of the STAT3-negative regulator, *SOCS3*, in human CAC provides direct clinical relevance for many of the above-discussed mechanisms (summarized in Fig. 2).<sup>203</sup> A number of small molecule inhibitors of DNMTs are currently being evaluated in clinical trials, the outcomes of which could engender novel therapeutic approaches to the challenging complication of CAC in patients with IBD.

## DIAGNOSTIC AND THERAPEUTIC IMPLICATIONS

Epigenetic research could provide IBD biomarkers to (1) confirm diagnosis and disease phenotype; (2) predict the course of the disease and relapses; (3) evaluate the response to therapy; and (4) prediction and detection of CAC. This includes DNA

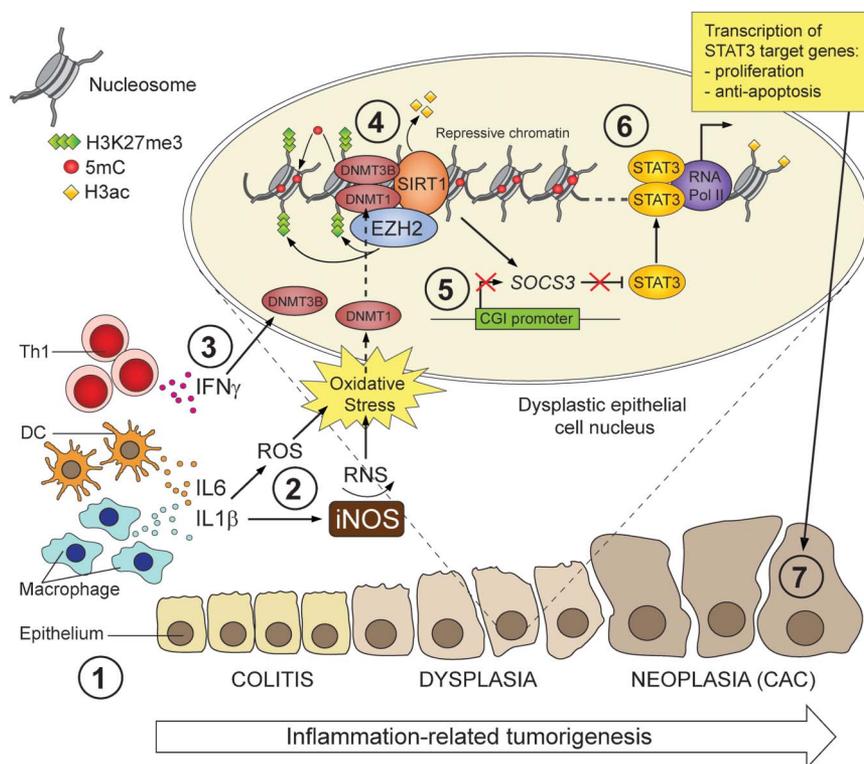


FIGURE 2. Proposed model of cytokine-mediated epigenetic deregulation of mucosal homeostasis in CAC development. Chronic inflammation of the intestinal epithelium occurs during IBD-related colitis, involving recruitment of innate (DC and macrophage) and adaptive (Th1) immune cells and associated release of proinflammatory cytokines such as IL6, IL1 $\beta$ , and IFN $\gamma$  (1). IL1 $\beta$  and IL6 release generate reactive oxygen species, although IL1 $\beta$  also directly activates iNOS leading to generation of RNS. Reactive oxygen/nitrogen species (ROS/RNS) trigger oxidative stress in epithelial cells leading to increased DNMT1 expression and activity (2). IFN $\gamma$  release from Th1 cells promotes increased expression and activity of DNMT3B in epithelial cells (3). DNMT1 and DNMT3B form a repressive complex with the polycomb group HMT protein, enhancer of zeste homolog 2 (EZH2) and the HDAC enzyme, sirtuin-1 (SIRT1), which is recruited to CGI promoters. DNMT1/3B drive de novo DNA hypermethylation (5-methylcytosine; 5 mC), EZH2 promotes repressive H3K27me3 marks, and SIRT1 drives histone deacetylation (removal of active H3ac marks), which collectively lead to the establishment of repressive chromatin and silencing of CGI promoters (4 and 5). As a consequence, the CGI promoter of SOCS3 (negative regulator of STAT3) becomes repressed (5), resulting in hyperactivation of the oncogenic transcription factor STAT3 and transcription (RNA Pol II) of its target genes associated with proliferation and apoptosis resistance (6). These molecular events collectively contribute to inflammation-related tumorigenesis in CAC (7).

methylation analyses<sup>92,204–209</sup> and miRNA profiling<sup>210–216</sup> in fecal samples, mucosal biopsy specimens, and/or peripheral blood/serum. It has been proposed that routine DNA methylation profiling in colonic tissue from patients with IBD might serve as a potential biomarker of disease, intestinal inflammation, disease phenotype, and/or the early detection of CAC. Several marker genes have been advocated for this purpose including *methylated-in tumor-1* (*MINT1*),<sup>208</sup> *cyclooxygenase-2* (*COX2*),<sup>208</sup> *runt-related transcription factor 3* (*RUNX3*),<sup>208</sup> *signal transducer and activator of transcription 4* (*STAT4*),<sup>209</sup> *protease-activated receptor 2* (*PAR2*),<sup>206</sup> *slit protein homolog 2* (*SLIT2*),<sup>204</sup> *transmembrane protein with EGF-like and 2 follistatin-like domains 2* (*TMEFF2*),<sup>204</sup> *forkhead box protein E1* (*FOXE1*),<sup>205</sup> *synaptic nuclear envelope protein 1* (*SYNE1*),<sup>205</sup> and *multidrug resistance protein 1* (*MDR1*).<sup>207</sup>

Besides these potential diagnostic applications, further studies of IBD-associated epigenetics might lead to new therapeutic approaches. Chromatin regulators, as therapeutic

compounds that suppress proinflammatory cytokine gene induction and inflammation, offer potential for gene- and patient-specific immunomodulatory therapy that can even induce remission in patients with chronic IBD. Anti-inflammatory effects of HDAC inhibitors have been firmly established in several models of intestinal inflammation and colitis.<sup>217,218</sup> However, these compounds may confer additional protective benefits by promoting the survival and barrier function of IECs.<sup>219</sup> A range of small molecule inhibitors of chromatin-modifying enzymes are being efficacy-tested in clinical trials for use in non-IBD conditions.<sup>7</sup> However, the therapeutic effects of these compounds in IBD have not yet been confirmed. Targeting individual HAT/HMT/HDAC enzymes with high specificity has shown immense therapeutic promise in other human diseases and could form part of a new strategy in IBD therapy. Specificity remains a key priority here because some global inhibitors of HDACs and DNMTs may have off-target effects that promote both pro- and

anti-inflammatory sequelae and increase the risk of side effects and/or malignancy.<sup>7</sup>

In addition, several compounds targeting DNA methylation status have been demonstrated to have potential therapeutic effects in experimental colitis models and/or patients with IBD. This includes compounds that act as methyl donors, which increase global methylation levels or small compounds that affect methylation-dependent gene expression. Black raspberries, for example, are a natural food rich in protective antioxidants and anti-inflammatory compounds including folic acid. Dietary intake of black raspberries may suppress colonic ulceration by correcting promoter hypermethylation of homeostatic genes that systematically regulate inflammation in a model of DSS-induced colitis.<sup>220</sup> Likewise, inhibition of DNA methylation in DSS-induced colitis results in disease exacerbation, whereas folate supplementation to promote methylation partially ameliorates the severity of colitis.<sup>201</sup> Dietary folate did not significantly affect the intestinal microbiome and inflammation in DSS-induced colitis in another study.<sup>221</sup> However, prenatal and lactational exposure to a methyl-deficient maternal diet (folate, vitamin B12, and choline) was found to aggravate DSS-induced colitis in rats.<sup>222</sup> Dietary (pro-)oxidants can cause epigenetic changes in antioxidant defense and an upregulation of inflammatory processes in IECs as evidenced by altered promoter methylation of *superoxide dismutase (SOD) 2* and *glutathione peroxidase (GPx)*. Treatment of IECs with a demethylating agent or antioxidant normalized the activities of SOD2 and GPx and prevented inflammation.<sup>223</sup> Furthermore, IEC-specific deficiency of DNMT or HDAC activity leads to an altered mucosal inflammatory response and barrier function.<sup>224,225</sup>

Other therapeutic approaches aim to target gene expression mediated by DNA methylation more specifically. For example, the tylophorine analog W-8 was found to induce *FoxP3* promoter demethylation, thus upregulating *FoxP3* expression. This effect was sufficient to drive naive CD4<sup>+</sup> T-cell differentiation to correctly functional and immunosuppressive Treg cells with protective properties in murine experimental colitis.<sup>226</sup> Increased differentiation of Treg cells and inhibition of Th17 cells were also observed in DSS-induced colitis after treatment with a potent ligand of the aryl hydrocarbon receptor (AhR), which has been shown to modulate Treg- and Th17-cell differentiation.<sup>227</sup> Interestingly, analysis of mesenteric lymph nodes and lamina propria cells in allied colitis experiments revealed reciprocal hyper- and hypo-methylation effects on *FoxP3* and *Il17* promoters respectively, which ameliorated with AhR ligand treatment.

## CONCLUSIONS AND FUTURE PERSPECTIVES

Significant advances in knowledge of IBD pathogenesis have been achieved during the past decade. The literature reviewed here serves to illustrate that epigenetic mechanisms, underlying the immunological and oncogenic aspects of IBD, have already contributed extensively to this progress in understanding. The advent of epigenome-wide methylation association studies has been exploited to great effect both in cancer biology and in nonmalignant

complex diseases such as autoimmunity or diabetes (reviewed in Ref. 175) and its undoubted utility is becoming palpable in IBD research.<sup>85,88,90</sup> Further large scale studies of this type are imminently anticipated and will impact profoundly on our understanding of IBD by adding a second major dimension of disease heritability to complement, and perhaps reintegrate, the extensive genetic data garnered from genome-wide association studies. Better access to and reduced cost of massively parallel/next generation sequencing technologies will facilitate exploration beyond DNA methylation, prospectively allowing clinical translation of the much-vaunted histone code paradigm into novel approaches to the management of IBD and its associated comorbidities, including CAC.

Many of the earliest epigenetic studies in IBD research focused exclusively on CAC; however, it has become increasingly apparent that epigenetic mechanisms exert a pivotal influence on all facets of IBD pathogenesis. Heritable programs of gene transcription established by DNA methylation, histone modification, and RNA interference regulate the development and function of innate and adaptive immunity, pathogen recognition, and host microbiome interactions as well as mucosal homeostasis and integrity. Deregulated epigenetic inheritance within each of these key modalities has now been firmly established in both clinical IBD and experimental models. Mouse genetic models have proven indispensable in evaluating gene-specific effects and their functional consequences, yet the picture is far from complete. Convergent themes of transgenerational epigenetic inheritance and gene-environment interaction have emerged through rodent models of experimental colitis, suggesting that maternal diet may, through modification of the intestinal epigenome, influence the penetrance and/or severity of IBD-related inflammatory pathology. Further investigation of this exciting phenomenon is nonetheless required to determine the extent of its potential therapeutic value.

Finally studies of cytokine- and oxidative stress-dependent carcinogenesis have thrown light on epigenetic mechanisms linking deregulated immunity to IBD-related cancer. These findings will not only inform IBD management but may also have broad application in other human cancers for which inflammatory provenance has been firmly established. The inherent stability and plasticity of epigenetic modifications will be the key factors to their prospective translation as predictive biomarkers or as targets for pharmacologic intervention in IBD therapy.

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