Chapter 4
Epigenetic Regulation of Intestinal Fibrosis

Chao Li and John F. Kuemmerle

Abstract Genome-wide association studies have identified over 200 risk loci associated with Inflammatory Bowel Diseases (IBD), Crohn's disease and Ulcerative colitis. These genetic factors, however, account for only a small proportion of genetic inheritability of disease. Our understanding of the pathogenesis of IBD has evolved and currently is thought to occur through the interaction between the host genome and their intestinal microbiome and metabolome with the innate and adaptive immune responses. Genetic risk alone, however, predicts only 25% of disease indicating that other factors including the intestinal environment can shape the epigenome and also independently confer heritable risk to patients. Epigenetic modifications regulate gene expression and protein production and play critical roles in shaping the intestinal immune response, mucosal homeostasis, and the wound-healing process. Analysis of the genetic risk in patients with Crohn's disease combined with epigenetic marks reveals regulatory mechanisms that affect gene expression and disease phenotype. This chapter will focus on what is known about the alteration in the epigenome in Crohn's disease and the mechanisms by which epigenetic risk factors determine development of fibrosis in Crohn's disease. Studies of the epigenome have highlighted new therapeutic targets for therapeutic intervention of the development and progression of fibrosis.

Keywords Fibrosis · Epigenetics · Inflammatory bowel diseases · Mesenchymal cells · Crohn's disease

C. Li
Department of Medicine, Virginia Commonwealth University, Richmond, VA, USA
e-mail: chao.li@vcuhealth.org

J. F. Kuemmerle (✉)
Department of Medicine, Virginia Commonwealth University, Richmond, VA, USA

Department of Physiology and Biophysics, VCU Program in Enteric Neuromuscular Sciences, Medical College of Virginia Campus, Virginia Commonwealth University, Richmond, VA, USA
e-mail: john.kuemmerle@vcuhealth.org

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4.1 Introduction

In 1942, C.H. Waddington coined the term epigenetics to explain how gene modulation regulates development. Since then research in epigenetics has progressed and such has our understanding of the epigenetic regulation of normal physiology and disease. Disease pathogenesis results from the heritable risk that accrues from alterations in DNA sequence, risk polymorphisms, and from alterations in the epigenome that control gene expression when exposed to environmental change. Epigenetic control of gene expression is exerted through modification of DNA regulatory elements or enhancers that induce transition of condensed heterochromatin, where gene accessibility is limited, to euchromatin, where genes are accessible for transcription regulated by histone modification and DNA methylation status. Gene expression is also controlled by small non-coding RNAs, microRNAs, which post-transcriptionally regulate gene expression. Crohn's disease is a polygenetic disorder with >200 risk loci identified by GWAS. However, understanding the risk of disease development or expression of a specific phenotype of Crohn's disease in a patient is not predicted or understood completely by genetic risk. Study of the epigenetic changes associated with development of intestinal fibrosis in Crohn's disease and fibrosis in other organs, including the lungs, heart, liver, and kidneys reveals patterns common to all. This review will focus on what is known about the mechanisms by which epigenetic risk factors determine the development of intestinal fibrosis in Crohn's disease and compare that to what we know from other fibrotic diseases.

4.2 Genetics

Inflammatory bowel diseases (IBD), Crohn's disease and ulcerative colitis, are polygenic diseases for which >200 risk loci have been identified to date [1, 2]. The most significant genetic associations are with the intracellular bacterial sensor, NOD2, autophagic responses, ATG16L1 and IRGM, and with IL-23R. Taken together this has been interpreted as showing how genetic architecture of Crohn's disease involves both defective innate and adaptive immune responses to intestinal microbiota [1]. To date a deeper analysis of GWAS data has not fully revealed a genomic basis that accounts for individual Crohn's disease phenotypes: inflammatory, fibrostenotic or penetrating [2, 3]. An approach using multi-locus genetic risk scores has improved the genetic risk assessment of IBD but also indicates that in addition to established risk variants other independent variables modulate disease progression [4, 5]. Ethnic variation of associated risk loci does not account for ethnic variations in disease location or behavior or phenotype in Crohn's disease [6, 7]. Purely genetic animal models of Crohn's disease are prone to underestimate the interactions between risk loci, "epistasis" [8]. Epistatic components need to be integrated into large-scale biostatistical models by estimating the contribution of non-genetic factors, termed missing heritability, which can be accounted for by epigenetics [9, 10].

Examination of genetic risk loci by pathway analysis or gene ontogeny identifies groups of polymorphisms likely to play a critical role in pathogenesis of fibrostenosis. TGF-β is a key cytokine that is central to the development of fibrosis. The TGF-β pathway includes identified risk variants in Smad3 and Smad7, variants in the cytokine-activated Jak-Tyk2-STAT3 pathway. Each of these play a role in the regulation of TGF-β expression and function. Polymorphisms in Suppressor of cytokine signaling 3 (SOCS3), the negative regulator of cytokine induced activation of the Jak-Tyk2-STAT3 pathway are also seen in subjects with IBD [11, 12]. Mesenchymal cells: fibroblasts, myofibroblasts and smooth muscle, play a central role in the development of fibrosis as the key cell types are activated and produce TGF-β1 and excess extracellular matrix including collagen and fibronectin [13]. The functional outcomes of mutations in these key GWAS risk loci that mechanistically result in TGF-β1-dependent fibrosis are distinct from the outcomes of mutations leading to initial and sustained inflammation in epithelial and immune cells in the intestine. In the case of TGF-β signaling Smad7 is increased in epithelial and immune cells and inhibits Treg responses. In contrast Smad7 is diminished in affected intestinal subepithelial myofibroblasts and muscle cells allowing sustained TGF-β1 signaling and excess extracellular matrix production, leading to the stricture formation [14–16].

Other loci have been identified that confer risk of fibrostenotic disease that involve other pathways leading to fibrosis in the intestine. The 5T5T polymorphism at the matrix metalloprotein-3 (MMP3) gene increased the risk of developing fibrostenotic complications [17]. The MMPs and tissue metalloproteinases (TIMPs) are key regulators of the balance between extracellular matrix deposition and degradation. Homozygosity for rs1363670 G-allele near IL-12B is an independent risk factor for development of fibrostenosis and for a shorter time to critical stricture formation in the ileum [18]. Other risk alleles have been identified in patients with penetrating disease. It is worth noting that the Montreal classification of Crohn's disease is hierarchical. Patients may express a penetrating phenotype that is the result of underlying fibrostenosis. Thus, studying Montreal Class B2 fibrostenotic Crohn's disease, distinct from B1 inflammatory and B3 penetrating phenotypes is of crucial importance in understanding risk loci and susceptibility of a particular phenotype [19].

4.3 Epigenetics

The identified genetic factors and susceptibility loci account for only 13.6% of disease variability and no more than 25% of the genetic risk in Crohn's disease [1, 7]. Epigenetic processes translate environmental events associated with genetic risk into regulation of chromatin state, shapes the expression of genes, and thereby the activity of specific cell types that participate in disease pathophysiology. Epigenetic mechanisms are emerging as key mediators of the effects of both genetics and the environment on gene expression and disease [20]. Epigenetic modifications represent a fundamental regulatory mechanism that has a profound influence on a

multitude of different phenotypic outcomes in a chromatin-templated environment for both normal and pathological development. In addition to a set of inherited epigenetic marks, there are non-heritable epigenetic marks that are more dynamic and change in response to environmental stimuli [21]. In Crohn's disease interaction of the environment, including the intestinal microbiome and metabolome, with the susceptible patient's genome and immune system, jointly shape the epigenome. These non-genetic effects that alter gene expression and function are implied by the results of multi-locus genetic risk analyses and represent the missing heritability in GWAS [4, 5].

Epigenetics is defined as a "stably inherited phenotype that results from mechanisms other than changes in DNA sequence" [11]. Although initially an individual's epigenome was not thought to be heritable, there is now increasing evidence that epigenetic inheritance can persist for multiple generations [22]. Evidence from a number of lines of investigation demonstrate epigenetic heritability from cell to cell during mitosis, from generation to generation during meiosis, and include true transgenerational inheritance [23], which means transmittance of information from one generation to the next that affects the traits of offsprings without alteration of the sequence of DNA. Such mechanisms have been shown to include incomplete erasure of DNA methylation, parental effects, transmission of distinct RNA types (e.g. mRNA, non-coding RNA, miRNA), and persistence of subsets of histone marks [23]. Epimutations, epigenetic changes that are sustained in the germ line, can be transmitted in a true intergenerational fashion by surviving the developmental reprogramming that erases epigenomic changes present in the parent. This mechanism has been shown to be operative in animal models of liver fibrosis. Remodeling of DNA methylation and histone acetylation in offspring of mice harboring epigenetic changes altering TGF-β1 expression resulting in liver fibrosis is lower in male F_1 and F_2 generations through a process termed suppressive adaptation [24]. Humans with milder non-alcoholic fatty liver disease have hypomethylation of the anti-fibrogenic factor *PPAR-γ* promoter compared to patients with more severe fibrosis lending support to this notion. All these findings suggest transmission of an epigenetic suppressive adaptation that can help offspring better adapt to future hepatic insults that might result in fibrosis. Suppressive adaptation, however, was not seen in the setting of renal fibrosis [24].

Even though all cells within the intestine or an organism share a common genome, gene expression in an individual cell type is regulated by the unique epigenetic events that affect that cell type and may be distinct from neighboring cell types. This can account for the sometimes contradictory epigenetic mechanisms that are identified as regulating gene expression in different cell types such as epithelial, immune and mesenchymal cells. Understanding which mechanisms regulate gene expression in a cell type that are critical to a disease process, e.g. mesenchymal cells and fibrosis, is difficult when based on an epigenetic analysis of DNA obtained from heterogeneous cell populations.

Epigenetic changes that regulate gene expression and function are grouped into four main types: DNA methylation, histone modifications, nucleosome positioning and small or non-coding RNAs. No information on nucleosome positioning as it

relates to fibrosis in Crohn's disease exists to date and therefore will not be discussed further here. The other processes are discussed in greater detail as it relates to the development of fibrosis in general and to what is known about the development of fibrosis in patients with Crohn's disease (Table 4.1).

Table 4.1 Genes that can be regulated by epigenetic mechanisms in the development of intestinal fibrosis

Gene	Expression level in intestine	Epigenetic mechanism			References
		Methylation	Histone modification	miRNA	
Smad3	↑	N/A	HDAC1	miR-21, miR-154, miR-29	[25–28]
Smad7	↓	DNMT1	N/A	miR-21, miR-17-5p	[27, 29]
SOCS3	↓	DNMT1	N/A	miR-19b	[11, 12, 30]
MMP	↑	Promoter	HDAC	miR-17, miR-18a, b & miR-19a, b,	[31, 32]
α-SMA	↑	CpG, DNMT1, DNMT3b	H3K4me1	N/A	[33, 34]
COL	↑	DNMT1, DNMT3b	H4 acetylation, Fli-1 acetylation, H3K4me1	miR-18a, b and miR-19a, b, miR-29	[34–40]
VMP1	↑	N/A	N/A	miR-21	[41]
TGF-β1	↑	Smad7 methylation, Smad4 hypermethylation	H3K4me3↑, H2A. Z↑, ↓H3K9me2 and H3K9me3 on the promoters of ECM genes	miR-21, miR-17 and miR-19a, b	[29, 42]
COX2	↓	Promoter hypermethylation or hypomethylation	↓Histone H3 and H4 acetylation, ↑ in H3K9me3, H3K27me3, and DNA methylation	Reported mostly in cancer research, miR-101, miR-26b, miR-146a, miR-16 and miR-122	[35, 43]
CXCL10	↑	N/A	H3	Unknown	[44]
TIMP1	↑	DNMT1	H3K4me1	miR-17, miR-29, miR-1293	[36]
Spry-1	↓	Promoter hypermethylation	HDAC↑ Spry-1 gene expression	miR-29, miR-21	[37]
PTEN	↓	DNMT1-induced hypermethylation	Its interaction with histone H1 to keep chromatin condensation	miR-21	[42]

(continued)

Table 4.1 (continued)

Gene	Expression level in intestine	Epigenetic mechanism			References
		Methylation	Histone modification	miRNA	
PPAR-α	↓	Promoter hypermethylation	N/A	miR-21, miR-10b, miR-33a	[25, 45, 46]
STAT3	↑	DNMT1	HDACs, SET1, LSD1, EZH2	miR-21, miR-17, miR-29, miR-98	[26, 27, 37, 38, 42, 47]
Thy-1	↑	DNMT1, hypermethylation	H3, HDAC inhibitor	N/A	[43, 44]
IL-27	↓	N/A	N/A	N/A	[48]
NOD2	↓	DNA methylation	H3K4Me2 and H4Ac, H3K27Me3 histone modifications	miR-29, miR-192	[48]
TNF-α	↑	DNA demethylation	Histone acetylation, H3K9 and H3K4 methylation	miR-23a, miR-155, miR-346	[48]
SMT1	↓	DNA methylation	N/A	N/A	[48]
IL-19	↓	N/A	N/A	N/A	[48]

↑ = upregulation, ↓ = downregulation, N/A = study not done

Epigenome-wide association studies (EWAS) have been performed in patients with IBD and provide an analysis of differentially methylated sites or regions in different tissues from the IBD patients within different populations [45]. These findings are difficult to reproduce or understand how they confer risk of disease due to several confounding factors including the selection of different patient populations (adult vs pediatric), the selection of different tissue resources (PBMCs, EBV transformed B cell lines, or colonic biopsies or intestinal mucosal), and selection of different locations (ileum, jejunum, colon, or rectum) [45, 49]. Further EWAS using large populations of IBD patients, who are deeply phenotyped will be needed to integrate what we know from GWAS into a more complete understanding of the pathogenesis of specific IBD phenotypes. More importantly, the combination of advanced, large parallel/next generation sequencing approaches will convert research findings into a translational platform that informs personalized precision medicine.

4.4 Epigenetics and Fibrosis

Fibrosis is characterized by an integrated cascade of cellular and molecular mechanisms that results in excess extracellular matrix production, which is initiated by tissue injury in any organ and lead to destruction of normal tissue structure and

finally organ failure. Although there are a variety of cell types that participate in fibrogenesis, it is the mesenchymal cells that generate the TGF-β-mediated production of collagen-rich scar tissue. Fibrotic disease-related death accounts for ~45% of all deaths in the developed countries. Accumulation of emerging evidence indicates that epigenetic mechanisms play a role in promoting a heritable pro-fibrotic phenotype in mesenchymal cells including fibroblasts, myofibroblasts and muscle cells, which are actively involved in development of fibrosis.

4.5 DNA Methylation

The addition of a methyl group to the 5′ carbon of the cytosine residue by replacement of the hydrogen in position 5 (5MeC) in the context of cytosine-guanine (CpG) dinucleotides that are clustered in CpG islands is the most widely studied epigenetic modification. Sixty to eighty percent of the CpG dinucleotides present in the human genome are methylated [49] and show regional differences in its distribution. About 30,000 CpG islands are present in the human genome, typically extend for 300–3000 base pairs, and are located close to or within 40% of gene promoters. Methylation typically, but not always, represses gene expression by either interfering with the binding of transcription factors to their DNA binding sites or by recruiting methyl-CpG-binding proteins that attract histone and chromatin modifying enzymes. DNA methyltransferases (DNMT)-1 and DNMT-3a and 3b are the primary enzymes responsible for methylation of CpG islands [50]. DNMT-1 is a maintenance methyltransferase whereas DNMT-3a and 3b are de novo methyltransferases. Methylation can be reversed by either active or passive demethylation. The ten-eleven translocation methylcytosine dioxygenase (TET) family of enzymes catalyze active demethylation via oxidation of cytosines forming the 5-hydroxymethyl-2′-deoxycytine (5HMeC) which attracts DNA excision and repair machinery thereby restoring DNA to its demethylated state [51]. This suggests that oxidation is part of a demethylation pathway of DNA. Passive demethylation occurs when maintenance methylation is absent and progressive dilution of 5meC occurs during DNA replication [52].

4.6 DNA Methylation and Fibrosis

DNA methylation status has been examined in a number of disease processes that result in tissue fibrosis including systemic sclerosis, pulmonary and cardiac fibrosis, hepatic fibrosis, and intestinal fibrosis in Crohn's disease [21, 48, 53–58]. Hypermethylation of specific genes as well as global changes in DNA methylation have been identified in these organ systems. Two genomic studies in patients with idiopathic pulmonary fibrosis (IPF) demonstrated extensive DNA methylation changes in the control of IPF gene expression [35, 59, 60]. CpG island methylation changes are present in genes linked to a fibro-proliferative phenotype in IPF and to

myeloproliferative diseases, via miR-17–92 cluster that include increased DNMT-1-mediated feedback affecting DNA methylation and microRNA expression [30, 33]. Notably altered CpG island methylation in the α-smooth muscle actin (α-SMA) promoter is present in pulmonary fibroblasts and myofibroblasts of patients with IPF [34]. A core set of genes known to be related to fibrosis, including several collagens, were differentially methylated in patients with progressive renal fibrosis compared to controls [41]. Recently, in a rat model of hypoxia-induced cardiac fibrosis, global hypermethylation of gene expression was observed along with upregulation of both DNMT-1 and DMNT-3b that resulted in increased collagen and α-SMA [42]. Whether DNA methylation is fundamental to transcriptional repression still remains elusive with some researchers arguing that DNA methylation is a consequence rather than a cause of gene repression [34, 41, 61]. Aberrant DNA methylation is a classic hallmark of cancer and many other diseases, which is composed of loss of DNA methylation and hypermethylation of specific gene promoters. Moreover, whether cyclical demethylation and remethylation processes plays a role in intestinal fibrosis is unknown and awaits clarification with further investigations. Importantly, Watson et al. showed that hypoxia-induced cardiac myofibroblasts could be reversed back to a fibroblast by both silencing of HIF-1α and exposure to 5′-Aza′C, a non-specific DNMT inhibitor [42]. Our recent work has implicated methylation as a regulator of *Smad7* and *Socs3* gene silencing in human myofibroblasts and smooth muscle cells from affected ileum as expression was restored after treatment with the demethylating agent 5′-Aza′C or knockdown of DNMT-1 [27]. These findings indicated the important role of DNA methylation in mesenchymal cell function in the development of intestinal fibrosis in Crohn's disease.

Genome-wide methylation profiling in patients with IBD has identified numerous sites that are differentially methylated between cases and controls [53]. The most highly statistically significant include genes controlling altered immune activation, responses to luminal bacteria and regulation of the Th17 pathway [48]. A significant enrichment in DNA methylation was seen within 50 kb of several Crohn's disease GWAS risk loci including IL-27, IL-19, tumor necrosis factor (TNF), Soluble latent membrane-type 1 (SMT1) and NOD2. In this study by Nimmo and colleagues, methylation status was predictive of disease activity [48]. In pediatric Crohn's disease, Adams et al. provided evidence that 4 of the most differentially methylated regions resided in proximity to the vacuole membrane protein-1 (VMP1) GWAS locus [29]. VMP1 is a putative transmembrane protein that has been reported to be involved in different biological events including autophagy, cell adhesion, and membrane translocation [62]. The microRNA (miR)-21 gene lies within the VMP1 gene. They share a common transcription start site and promoter region but pri-miR-21 possesses its own unique promoter thus VMP-1 and pri-miR-21 can be differentially transcribed. Primary miRNA (pri-miRNA) with about 100 nucleotides are transcribed from miRNA genes in the nucleus by RNA polymerase II and further processed into pre-miRNA by a microprocessor complex. Our own recent work has demonstrated that the increased transcription of pri-miR-21 in muscle cells and myofibroblasts of patients with fibrostenotic Crohn's disease

results in the sustained TGF-β1 signaling that results in excess collagen and extracellular matrix production and fibrosis [62]. This process uniquely characterizes patients with Montreal Class B2 fibrostenotic Crohn's disease as distinct from patients with Montreal Class B1 inflammatory and Montreal Class B3 penetrating Crohn's disease [19, 62, 63]. These various studies have suggested numerous specific gene-specific methylation events occur in different organ systems as well as provided evidence for promising therapeutic targets through the modulation of epigenetic changes (Table 4.1).

4.7 Histone Modifications of DNA and Post-Translational Modifications of Proteins

Histones are also key players in epigenetic regulatory mechanisms. The four core histones, H2a, H2B, H3 and H4 associate as two H2A–H2B dimers and a H3–H4 tetramer and comprise the nucleosome [64]. Adjacent nucleosome octamers are separated by ~50 kb of DNA with the linker histone, H1 interposed between them. Histones are subject to post-translational modifications on their tail regions including phosphorylation, acetylation, methylation, ubiquitination, SUMOlyation, and ADPribosylation. These post-transcriptional modifications contribute to their ability to regulate the transcriptional state of genomic DNA. Generally euchromatin, open or lightly packed chromatin with accessible DNA and actively transcribed genes, and heterochromatin, condensed or tightly packed inaccessible chromatin, are distinguished by different levels of acetylation and/or methylation of specific histone residues and their position along the genome in promoter regions or intron/exon regions [65, 66]. Histone modifying enzymes catalyze the post-translational modification of histones and non-histone proteins. This large group of enzymes include histone acetyltransferases (e.g. p300/CBP) and histone deacetylases (e.g. HDACs), and lysine methyltransferases (e.g. LSD) [31]. Gene transcription regulated by histones is the cumulative influence of multiple histone modifications that result from the activity of histone modifying enzymes. Data from the ENCODE project has identified key histones and their modifications that have become the most highly studied for their ability to control accessibility of chromatin and thereby regulation of gene expression. Unlike DNA methylation, which typically results in transcriptional silencing, histone modifications exert divergent effects depending upon specific conditions and genes, thus adding another layer of complexity to epigenetic regulation of gene expression in fibrosis. While histone acetylation generally plays antifibrotic roles in many fibrotic diseases, histone methylation plays either transcriptional activation or repression depending on the number of methylations (mono, bi, or tri methylations) and the specific residues and their locations (lysine and/or arginine) [31, 32, 66, 67]. The functions of specific histone modifications have been studied in different diseases and are summarized in Table 4.2 [32, 67–79].

Table 4.2 Histone modifications that regulate gene expression with putative functions

Histone mark	Putative functions	Supplemental references
H3K4me1	Associated with active enhancer and other distal elements, but also closed or poised enhancer A marker of primed enhancers and gene expression during embryogenesis It marks regions of DNA methylation loss during normal ageing.	[32, 67, 68]
H3K4me2	Associated with active enhancer, transcription factor binding in genome-wide datasets. Mark of regulatory elements associated with promoters and enhancers.	[69]
H3K4me3	Active enhancer. Mark of regulatory elements primarily associated with promoters/transcription starts. Hall mark of active gene promoters. It promotes rapid gene activation.	[70, 71]
H3K9ac	Mark of regulatory elements with preference for promoters. Associated with gene activation. It can differentiate active enhancers from inactive ones.	[72]
H3K9me1	Preference for the 5′ end of genes, enriched at the transcriptional start site of active genes.	[70]
H3K9me3	Inactive chromatin, repressive mark associated with constitutive heterochromatin and repetitive elements. It binds heterochromatin protein 1 (HP1) which is responsible for transcriptional repression and the actual formation and maintenance of heterochromatin. HP1 also recruits DNA methyltransferase 3b (DNMT3b), demonstrating the interplay between histone methylation and DNA methylation.	[73]
H3K27ac	Mark of active regulatory elements; may distinguish active enhancers and promoters from their inactive counterparts. Like H3K9ac, associated with transcriptional initiation and open chromatin structure.	[74]
H3K27me3	Inactive chromatin. A repressive mark established by polycomb complex activity associated with repressive domains and silent development genes. Critical for the repression of developmental genes. An important mark of the inactive X chromosome (Xi).	[75, 76]
H3K36me3	Elongation mark associated with transcribed portion of genes, with preference for 3′ regions after intron 1. It serves as a mark for HDACs to bind and deacetylate the histones. Involved in defining exons.	[77]
H3K79me2	Transcription-associated mark, with preference for 5′ end of genes. Its methylation is cell cycle dependent.	[78]
H4K20me1	Preference for 5′ end of genes. Associated with transcriptional activation. Also important for cell cycle regulation.	[79]

4.8 Histone Modifications and Fibrosis

The wide variety of reversible histone modifications regulates the structure of chromatin and gene transcription occurring in a context-dependent manner and plays a critical role in determining the gene-protein-phenotype axis. The emerging specific

inhibitors or agonists targeting each individual PTM of histones in biomedical research, typically in the cancer field, shed light on our understanding of how the epigenetic regulation leads to phenotypic changes in vivo due to early intervention of gene transcription activity.

Histone Acetylation

Chromatin state within the nucleus is regulated by the balance of acetylation and deacetylation of histones thereby regulating the access of transcription factors to DNA. Both histone acetylation and deacetylation are linked to the development of fibrosis. It is worth noting that H3 hyperacetylation through decreased expression of histone deacetylase is consistently associated with pulmonary fibrosis [43, 44]. Alteration of HDAC expression in patients with IPF results in TGF-β-induced myofibroblast differentiation, and excess collagen and matrix metalloproteinase-1 production [44]. However, acetylation is variable despite the fact that an increased acetylation of H3 was seen along with decreased acetylation of H3 on lysine K9 and K18 and increased acetylation on lysine K14 and K56 (unpublished data). Acetylation levels of H3 where shown to regulate expression of genes that are key to fibrosis including cyclooxygenase-2, IFN-gamma-inducible protein 10 (CXCL10), and Thy-1 cell surface antigen [36, 80]. In hepatic stellate cells histone acetylation state regulates expression of profibrotic genes including α-SMA, collagen I, tissue inhibitor of metalloproteinases 1 and TGF-β1 via Histone3 lysine4 methyltransferase I [81]. In systemic sclerosis, increased p300 acetyl transferase activity induces acetylation of Fli-1 proto-oncogene thereby relieving the transcriptional repression of collagens IαI and Iα2, the major collagen species in fibrosis [56].

Distinct patterns of histone H3 and H4 acetylation are present in Crohn's disease [82, 83]. Mokry et al. recently provided evidence that many of the GWAS risk loci overlap with DNA regulatory elements in the intestine including Histone H3 lysine 27 (H3K27ac) and p300 which is responsible for H3K27 acetylation and H3K4 monomethylation (H3K4me1) [84]. Sadler et al. have demonstrated that collagen Iα2 expression induced by the cytokines interleukin-1β, TNF-α and TGF-β is regulated by hyperacetylation of histone H4 [85]. We have recently shown a global increase of H4 acetylation on lysine K5, K8, K12, and K16 in myofibroblasts of affected ileum compared to normal ileum in the same patient with stricturing Crohn's disease. In the strictured ileal myofibroblasts an increased expression of both p300 and HDAC1 with the increase in HDAC 1> p300 was noted. This suggests that the balance between HAT and HDAC activities needs to be considered jointly to understand the regulation of histone acetylation in the epigenome. HDAC1 inhibition represents a potential therapeutic approach in IBD patients as seen in cancer treatment. Evidence on the use of HDAC inhibitors with other fibrotic diseases includes lung fibrosis, kidney fibrosis but not yet intestinal fibrosis. The fibrotic signaling pathways common to all fibrotic diseases with characteristic features suggest this may be effective.

Histone Methylation

We have also found a global increase of H3 methylation on lysine K4, K9, K27, K36, and K79 in affected ileum compared to normal ileum in the same patient with

stricturing Crohn's disease (unpublished results). Interestingly, we noticed that histone methylations occur in H4 lysine K20 as mono, bi, tri methylations, H4 arginine 3 methylations on 2a and 2s, and phosphorylation of Serine 1. The underlying mechanism of methylation has not yet been identified but it appears methylation of histone lysines is more discriminative than acetylation of histones (unpublished data). Trimethylation of histone lysine 9 of histone H3 or trimethylation of K27me3 are usually associated with transcriptional silencing.

4.9 MicroRNA

RNA interference of gene expression by microRNA (miR), small ~18–24 nucleotide non-coding single-stranded RNA molecules, is implicated in the epigenetic regulation of fibrosis [45, 86]. In general, miRs post-transcriptionally repress gene expression by targeting mRNA for degradation. miR genes are located throughout the genome. They can be found in introns of coding regions, in introns or exons of non-coding genes or in intergenic regions. In some cases they are transcribed independently from their own specific promoters as is the case with primary microRNA-21 (pri-miR-21) despite its location within the VMP-1 gene [29, 46].

4.10 MicroRNA and Fibrosis

A number of miRs have been identified that have a similar role in the regulation of fibrosis in the lung, liver, heart, kidney or skin in addition to the intestine. While these miRs can have organ and tissue-specific regulation and effects, two are consistently associated with fibrosis and with the expression of TGF-β: miR-21 and miR-29. MiR-21 is pro-fibrotic and is implicated in the transcriptional regulation of Sprouty homolog 1 (Spry-1), phosphatase and tensin homolog (PTEN), peroxisome proliferator-activated receptor-α (PPAR-α), signal transducer and activator of transcription-3 (STAT3) and Smad7 [25, 37, 38, 62, 87]. It is worth noting that miR expression can itself be subject to epigenetic regulation. Transcription of miR-21, for example, is regulated by promoter methylation [38]. During intestinal barrier dysfunction, miR-21 is increased to impair the tight junction integrity and to increase barrier permeability through targeting the Rho GTPase, RhoB [94]. MiR-29a, b, c are anti-fibrotic and are implicated in the silencing of collagen, MMP and Spry1 expression [26, 27, 39, 40, 47, 62, 88–91]. MiR-29 expression is downregulated by the TGF-β-dependent Smad3 transcription factor [88]. The miR17–92 cluster is also an important determinant of fibrosis that is regulated by IL-6 in the fibrotic intestine. Transcribed from this cluster are several miRs that can target key proteins in fibrosis including Collagen IαI (miR-18a, b and miR19a, b), TGF-β (miR-17 and miR-19a, b) and MMPs (miR-17, miR-18a, b and miR-19a, b) [28, 33, 92] (Table 4.3). Unique miRNAs expression profiles in tissue samples and

Table 4.3 Summary of non-coding RNAs associated with intestinal fibrosis in Crohn's disease

microRNA	Human tissue	Expression level	Target	Pathway/ function	Methods	References
miR-21	Ileal CD tissue with different phenotypes	Increased	Smad7, Smad3, RhoB, PDCD4	TGF-β, Th1	qRT-PCR, DNA-ChIP	[37, 38, 93–95]
miR-29/ miR-29b	Mucosa overlying strictures, mucosal fibroblasts, human dendritic cells from CD	Reduced	Collagen I and III, Smad3, IL-12p40, IL-23	TGF-β, EMT	qRT-PCR	[26, 40, 47, 89, 90]
miR-200 family	Intestinal epithelial cells, intestinal biopsies	Decreased	Smad2, E-cadherin, ZEB1	TGF-β, EMT	qRT-PCR	[91]
miR-192	Active UC tissue, colonscopic pinch biopsies from the sigmoid colon	Downregulated	MIP-2α, SIP1, NOD2, Smad3	Cytokine production	qRT-PCR, in situ hybridization	[96–98]
miR-143/145	Intestinal mesenchymal cells	Decreased	IGFBP5, PI3K/Akt	IGF	qRT-PCR	[99]
miR-155	Intestinal myofibroblasts, colonic mucosa pinch biopsy, intestinal epithelial HT29 cells	Increased	FOXO3a, IL-8, IκBα	Th17	qRT-PCR	[100]
miR-19b	Intestinal pinch biopsy, intestinal epithelial cells	Decreased	SOCS3	JAK/STAT	qRT-PCR	[11, 12, 30, 33]
CDKN2B-AS1	Colonic samples, colonocyte cell line	Decreased	TGF-β	TGF-β	qRT-PCR	[101]

peripheral blood were reported between patients with UC and CD to differentiate the diagnosis of IBD [93, 94, 102]. The study of the role of miRNAs in IBD has yielded significant insights with a deeper understanding yet to be gleaned. Even though there is much progression in anti-inflammation treatment of IBD in clinical trials and practice, the frequency of stricture complication post-surgery and after immunotherapy is still high and no cure for targeted fibrosis is currently available [7–10].

4.11 Long Non-Coding RNA and Fibrosis

The understanding of the regulatory role of long non-coding RNA (lncRNA) on gene expression as it relates to fibrosis is emerging. Examination of the transcriptome of lncRNAs in IBD has demonstrated expression profiles that distinguish inflamed and non-inflamed Crohn's disease and ulcerative colitis [103, 104]. The lncRNA CDKN2B-AS1 is associated with both Crohn's disease and ulcerative colitis and is downregulated by TGF-β [101]. From a functional perspective the lncRNA H19 is protective against renal fibrosis whereas Wisp2-super-enhancer associated RNA, Wisper, controls cardiac fibrosis [105, 106].

4.12 Summary

GWAS analysis of Crohn's disease has identified numerous risk loci that account for up to 25% of the genetic risk. Recent investigations of the epigenome indicate differential changes in DNA methylation patterns, histone modifications and differential expression of miRs can further contribute to the "heritable" risk of developing fibrostenotic Crohn's disease. Integration of genetic susceptibility with changes in the epigenome associated with the development of intestinal fibrosis has been demonstrated for several genes key to the development of fibrosis in Crohn's disease including STAT3, Smad3, Smad7 and SOCS3. Preclinical studies from different laboratories have supported the potential of epigenetic therapeutics including DNMTs inhibitors, HDAC inhibitors including butyrate, a natural HDAC inhibitor, and the histone methylation inhibitor EZH2. It is also important to note that the commensal microbiota have a direct impact on the host epigenome but the correlation between the two and Crohn's disease phenotype is as yet unknown.

For progress to be made in Crohn's disease efforts to understand the epigenome and it changes that relates to the identified risk loci and their associated pathways and thus the missing heritability of fibrosis will be needed. This understanding will only improve from our exploration of strictly phenotyped and genotyped patients with fibrosis using well-defined disease-relevant cell populations.

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