Mbd2 enables tumourigenesis within the intestine while preventing tumour-promoting inflammation

Citation for published version:

Digital Object Identifier (DOI):
10.1002/path.5074

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published in:
Journal of Pathology

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.
Mbd2 enables tumourigenesis within the intestine while preventing tumour-promoting inflammation

Stephanie May1, Heather Owen2, Toby J Phesse1, Kirsty R Greenow1, Gareth-Rhys Jones3, Adam Blackwood1, Peter C Cook3, Christopher Towers1, Awen M Gallimore1, Geraint T Williams5, Michael Stürzl6, Nathalie Britzen-Laurent6, Owen J Sansom1, Andrew S MacDonald1, Adrian P Bird1, Alan R Clarke1 and Lee Parry1

1 European Cancer Stem Cell Research Institute, Cardiff University, School of Biosciences, Cardiff, UK
2 Wellcome Trust Centre for Cell Biology, University of Edinburgh, Michael Swann Building, Edinburgh, UK
3 Manchester Collaborative Centre for Inflammation Research, Manchester, UK
4 Cardiff Institute of Infection and Immunity, Henry Wellcome Building, Cardiff, UK
5 Institute of Cancer and Genetics, Cardiff University School of Medicine, Cardiff, UK
6 Division of Molecular and Experimental Surgery, Department of Surgery, Friedrich-Alexander-Universität (FAU) Erlangen-Nürnberg and Universitätsklinikum Erlangen, Erlangen, Germany
7 Cancer Research UK Beatson Institute, Glasgow, UK

*Correspondence to: Lee Parry, European Cancer Stem Cell Research Institute, Cardiff University, School of Biosciences, Maindy Road, Cardiff, CF2 4 4HQ, UK. E-mail: parryl3@cardiff.ac.uk

Keywords: colon cancer; DSS colitis; inflammation; epigenetics

Abstract

Epigenetic regulation plays a key role in the link between inflammation and cancer. Here we examine Mbd2, which mediates epigenetic transcriptional silencing by binding to methylated DNA. In separate studies the Mbd2−/− mouse has been shown (1) to be resistant to intestinal tumourigenesis and (2) to have an enhanced inflammatory/immune response, observations that are inconsistent with the links between inflammation and cancer. To clarify its role in tumourigenesis and inflammation, we used constitutive and conditional models of Mbd2 deletion to explore its epithelial and non-epithelial roles in the intestine. Using a conditional model, we found that suppression of intestinal tumourigenesis is due primarily to the absence of Mbd2 within the epithelia. Next, we demonstrated, using the DSS colitis model, that non-epithelial roles of Mbd2 are key in preventing the transition from acute to tumour-promoting chronic inflammation. Combining models revealed that prior to inflammation the altered Mbd2−/− immune response plays a role in intestinal tumour suppression. However, following inflammation the intestine converts from tumour suppressive to tumour promoting. To summarise, in the intestine the normal function of Mbd2 is exploited by cancer cells to enable tumourigenesis, while in the immune system it plays a key role in preventing tumour-enabling inflammation. Which role is dominant depends on the inflammation status of the intestine. As environmental interactions within the intestine can alter DNA methylation patterns, we propose that Mbd2 plays a key role in determining whether these interactions are anti- or pro-tumourigenic and this makes it a useful new epigenetic model for inflammation-associated carcinogenesis.

© 2018 The Authors. The Journal of Pathology published by John Wiley & Sons Ltd on behalf of Pathological Society of Great Britain and Ireland.

Keywords: colon cancer; DSS colitis; inflammation; epigenetics

Introduction

Epigenetic regulation of the genome plays a major role in human health and disease [1]. DNA methylation, via addition of a methyl group to the fifth carbon of cytosine in a CpG dinucleotide [2], is a fundamental epigenetic modification. As DNA methylation can be influenced by the environment, it plays a large role in the biology of diseases with a strong environmental component, such as the intestine [3,4]. DNA methylation is synonymous with transcriptional silencing as it can either inhibit binding of transcription regulators, such as c-Myc [5], or recruit co-repressor complexes that trigger the formation of repressive chromatin [6]. These co-repressor complexes are recruited by proteins which bind directly to methylated DNA to mediate a cell’s complex multi-layered transcriptional programme. These methyl-binding proteins act as ‘master controllers’ by mediating the effects of DNA methylation; thus, they can regulate many genes simultaneously, including aberrantly methylated genes that lead to disease [4]. One of these proteins, methyl binding domain protein 2 (Mbd2), has been shown to play crucial roles in various biological processes and diseases [4,7]. The importance of its role has been highlighted using mouse models, where its deficiency has been shown to influence intestinal inflammatory responses [8–13] and epithelial cell biology [14–16]. These multiple roles...
Opposing roles for Mbd2 in inflammation and tumourigenesis

potentially make it a unifying player in the myriad of biological processes that contribute to the initiation and development of intestinal cancer.

Using the Apc\(^{+/-}\) model [17], we have previously demonstrated that the Mbd2\(^{-/-}\) mouse is resistant to Wnt-driven tumourigenesis [15,16] – the most common type of human colorectal cancer (CRC). Further, we demonstrated that Mbd2 deficiency in a Wnt-activated intestine reduces the expression of Wnt target genes, including c-Myc [15]. Potentially, this attenuation of tumourigenesis is due to a lack of Mbd2-dependent silencing of tumour suppressor genes; however, the mechanism of Apc\(^{+/-}\)Mbd2\(^{-/-}\) tumour suppression remains unknown. During the course of these studies, we reported that Mbd2 deficiency in Wnt-activated intestinal epithelia relieved silencing of genes associated with immune responses [15]. This is in accordance with previously reported roles for Mbd2 in guiding cells down the different epigenetically regulated T-cell lineages [8–13]. This presents within the intestine of the Mbd2\(^{-/-}\) mouse as an excessive type 1 response upon immune challenge [8], characterised by an increase in the expression of the pro-inflammatory cytokine interferon gamma (Ifng). Evidence from mice and humans suggests an anti-tumourigenic role for Ifng in colorectal cancer (CRC), as a loss of type 1 cytokines accompanies the adenoma–carcinoma sequence in the colorectum [18]; intestinal tumourigenesis is promoted in mice deficient for Ifng or its receptors [19]; and Th1 cytokines lead to cancer senescence [20]. This role is due, at least in part, to Ifng-mediated c-Myc inhibition [21,22] and increased expression of the HLA-DR antigen [21] – features we previously reported in the Wnt-activated Mbd2-deficient intestine [21]. However, in its inflammatory role, Ifng is causally involved in inflammatory bowel diseases, where chronic inflammation drives cellular and molecular inflammatory mechanisms that underlie tumour initiation [23,24]. Evidence from other tissues indicates that the tussle between the anti- and pro-tumourigenic functions of Ifng seems to be dependent on the contexts of tumour specificity, microenvironmental factors, and signalling intensity [25].

Here we examine how the increased inflammatory response and tumour suppression phenotype interact in the Mbd2-deficient mouse intestine. Our findings highlight separate roles for Mbd2 in controlling intestinal inflammation and enabling epithelial tumourigenesis.

Materials and methods

Animal models

All animal procedures were conducted in accordance with institutional animal care guidelines and UK Home Office regulations. In brief, mice were maintained in a specific pathogen-free (SPF) barrier facility in conventional open top cages on Eco-Pure Chips 6 Premium bedding (Datesand, Manchester, UK) under a 12 h light cycle, with IPS 5008 diet (Labdiet-IPS Ltd, London, UK) provided for nutritional support. To enrich the environment, irradiated sunflower seeds (at weaning only), Techniplast mouse houses (Techniplast, Leicestershire, UK), and small chewsticks (Labdiet-IPS Ltd) were provided. All mice were from a mixed background and were homozygous with respect to the C57Bl/6 Ppla2g2a allele (also called Mom-1) allele. Experimental animals were between 10 and 15 weeks old, with siblings used as controls. The alleles for Ah-cre [26], Apc\(^{+/-}\), Ifng\(^{-/-}\) [27], Lgr5creERT\(^{2}\) [28], Mbd2\(^{-/-}\) [9], Mbd2\(^{flo/flo}\) [13], and vil-creERT\(^{2}\) [29] have been described previously. Induction of the Ah-cre transgene was performed by administering three intraperitoneal (i.p.) injections of β-napthoflavone (BNF; Sigma, Gillingham, Dorset, UK) at 80 mg/kg in a 24 h period. Induction of the Lgr5creERT\(^{2}\) and vil-creERT\(^{2}\) transgenes was achieved by administering a single injection of tamoxifen (TAM; 80 mg/kg i.p.; Sigma) for four consecutive days. For induction of colitis (acute inflammatory insult), mice were given dextran sodium sulphate (MW 36 000–50 000; MP Biomedicals, Fisher Scientific, Loughborough, UK) ad libitum in drinking water at the concentration (w/v) and duration stated. Colitis disease severity was measured according to published protocols [30]. For survival analysis, mice were harvested at either a specific time point or a humane endpoint when mice displayed phenotypes indicative of acute colitis (weight loss and diarrhoea) or tumour burden (pale feet, bloating, prolapse or piloerotion). Tumour burden was measured at point of death by removing the entire intestine and mounting en face in methacarn fixative (4:2:1 methanol, chloroform, and glacial acetic acid) to determine the number of macroscopic lesions and their size.

Reverse transcription–quantitative PCR (RT-qPCR) analyses

The following methods were performed according to the manufacturer’s instructions unless otherwise stated. For analysis of gene expression in the intestine, three to five mice from each control and experimental group were harvested. RNA was extracted either from a 0.5 cm portion of the whole large intestine taken ∼1 cm distally from the caecum or from crypt epithelia extracted from the whole large intestine; samples were stored at −80°C in RNAlater (Sigma, Dorset, UK) [31]. Total RNA was extracted using the RNeasy kit (Qiagen, Manchester, UK) and DNase-treated using the Turbo DNase kit (Fisher Scientific). Complimentary DNA (cDNA) was reverse transcribed from 1 μg of RNA using random hexamers (Promega, Southampton, UK) and the Superscript III kits (Fisher Scientific, Loughborough, UK). For relative quantitation, all samples were run in duplicate on the StepOnePlus PCR machine using Fast SYBR Green master mix (Applied Biosystems, Oxford, UK) or Taqman Universal Mastermix II (Fisher Scientific). The threshold cycle (Ct) values of each gene analysed were normalised to a reference
gene. For expression analysis, Ct values were normalised against the Actb gene (mouse) or RPL37A (human). Oligonucleotide sequences used for relative quantification are available upon request. Differences between groups were assessed using the 2−ΔΔCT method [32]. Two-tailed Mann–Whitney U (MW) tests were performed on the ΔCT values and differences with P values less than 0.05 were considered significant [33].

Reporter visualization, immunohistochemistry (IHC), and cellular analysis

For IHC, tissue was fixed in ice-cold 10% neutral sodium phosphate-buffered formalin (Sigma) and processed into wax blocks by conventional means. Section were cut at 5 μm thickness, dewaxed, and rehydrated into PBS. Staining was performed using the Envision+ mouse or rabbit kit (Dako, Agilent Ltd, Stockport, UK) according to the manufacturer’s instructions. To identify cells which had lost Apc, we used nuclear β-catenin as a surrogate marker, using a mouse monoclonal anti-β-catenin antibody (Cat No 610154; BD Biosciences, Wokingham, UK) at 1:200. For Paneth cell detection, we used a rabbit polyclonal anti-lysozyme antibody (Cat No RB-372; Neomarkers/Labvision, Fisher Scientific) at 1:200. CD4 cells were stained with a mouse anti-CD4 antibody (Clone 4Sm95; eBioscience, Fisher Scientific; 1/50). To stain CD8 cells, we used a mouse anti-CD8 antibody (Clone 53-67.2; eBioscience, Fisher Scientific; 1/50). To visualise mucin and goblet cells, slides were stained with Alcian Blue. The cells between the base of the crypt and the junction with the villus were designated as the proliferative zone. Cellular analysis was performed on more than 25 whole crypts from at least three mice of each genotype. Slides were scanned for analysis using the Axioscan Z1 slide scanner (Zeiss, Cambridge, UK) and images were excised from scans using the Zeiss Axioscan Zen software.

T-cell analysis

To label the Th1 and Th2 cytokines in serum, we used a Cytometric Bead Array Mouse Th1/Th2/Th17 Cytokine kit (BD Biosciences) following the manufacturer’s instructions and using a FACS Canto II (BD Biosciences). To characterize the immune cell populations, single cell suspensions of lymphocytes were prepared from large intestine lamina propria [34, 35]. Lymphocytes were stimulated for 4 h using ionomycin, phorbol myristate acetate (Sigma), and 1 μg/ml Golgistop (Sigma). Prior to flow cytometry, lymphocytes were stained with fluorescent conjugated antibodies against CD4 (RM-4-5, 1/800), Il17 (XMG1.2, 1/200), IL-13 (eBIO 13a, 1/200), TCRα/β (MR5.2, 1/200) (all Fisher Scientific), CD45 (30-F11, 1/200), CD8 (53-6.7, 1/200), IL-4 (11B11, 1/200) (all Biolegend, London, UK), IL-17 (TC11-18H10.1, 1/100) and TNF (MP6-XT22, 1/200) (both BD Biosciences), and a live/dead marker. Data were analysed using FCAP Array v3.0 (BD Biosciences) and FlowJo (FlowJo LLC, Ashland, OR, USA) software. Statistical analysis was performed using three to five animals per genotype and three independent experiments. Significance was calculated using a three-way full factorial fit model and a joint F-test to assess the effects of genotype, treatment, and experiment day on the cytokine response [36].

Statistical analysis

All preclinical data were evaluated with GraphPad Prism software, version 7.02 (GraphPad, La Jolla, CA, USA). When two variables were compared, a two-tailed Mann–Whitney test was performed. Survival data were analysed using the Kaplan–Meier test. The relationship between genotype and phenotype was assessed using Fisher’s exact test. If not indicated otherwise, the statistical mean is presented and error bars represent SEM. On graphs, P values are indicated as follows: *p < 0.05; **p < 0.01; ***p < 0.001.

Results

Intestinal epithelial loss of Mbd2 is sufficient to suppress tumourigenesis

Potentially, the intestinal tumour resistance of the Mbd2−/− mouse is due to an enhanced anti-tumourigenic Th1/Il17 response, a loss of Mbd2 epigenetic regulation within the intestinal epithelia or a combination of both. To clarify this situation, we utilised a conditional Cre-Lox mouse to delete Mbd2 solely within the intestinal epithelia, allowing us to establish the epithelial contribution of Mbd2 to tumourigenesis. Mice carrying the Ah-cre and Mbd2ex1 transgenes were crossed to generate Ah-creMbd2ex1 mice, which following BNF induction deleted exon 1 of Mbd2 specifically within the crypts of the intestinal epithelia. Cohorts of four to six mice were examined 4 days after deletion. In comparison to control cohorts, there was no alteration to the expression of Wnt target genes (c-Myc and Axin2), the size of the crypt proliferative zone, Paneth cell localisation (Figure 1A–C) or genes characteristic of the differentiated cell types (supplementary material, Figure S1). To investigate the epithelial role of Mbd2 in Wnt signalling, Ah-creMbd2ex1 mice were crossed to mice carrying an ApcΔfs allele, to generate Ah-creApcΔfsMbd2ex1 mice and control cohorts. Upon Ah-cre-driven Apc loss, we observed an upregulation of Wnt target genes, an increase in the size of the crypt proliferative zone, and mislocalisation of Paneth cells (Figure 1A–C), as we have previously reported [37]. In comparison, the increase in expression of Wnt target genes Axin2 (p = 0.0159) and c-Myc (p = 0.0159), due to Apc deletion, was significantly attenuated by additional loss of Mbd2 (p = 0.0159) (Figure 1A). This corresponded with a decrease in the size of the proliferative zone within the crypts of Leiberkühn (Figure 1B) and a partial rescue of the mislocalisation of Paneth cells (a characteristic of Ah-cre-driven Apc loss) (Figure 1C). These findings are consistent with our previously reported data using the Ah-creApcΔfs Mbd2−/−
model [15], in which Mbd2 is absent systemically. To investigate whether these changes would influence the initiation of intestinal tumourigenesis, cohorts of Ah-creApc+/flx (N = 20) and Ah-creApc+/flxMbd2ex1/ex1 (N = 23) mice were induced at 10–12 weeks of age and harvested at 180 days post-induction (dpi). These mice are equivalent to the Apcphem model, as they require the spontaneous loss of the remaining wild-type allele for tumour initiation. The Ah-creApc+/flxMbd2ex1/ex1 cohort showed a significant increase in survival compared with the Ah-creApc+/flx control cohort (Figure 2A). Expression analysis on the whole intestine from six to eight animals within each cohort confirmed the continued absence of Mbd2 within the epithelia (Figure 2B). At 180 dpi, the Ah-creApc+/flxMbd2ex1/ex1 cohort had significantly fewer tumours and reduced burden compared with the control cohort (Figure 2C). In summary, the absence of Mbd2 within the intestinal epithelia is well tolerated and sufficient to suppress Wnt signalling and tumourigenesis, replicating the phenotype observed in the ApcphemMbd2−/− mice and demonstrating a cell intrinsic mechanism for Mbd2. We next sought to investigate the role that Mbd2 plays in intestinal inflammation.

Mbd2 deficiency exacerbates DSS-induced colitis

As previous reports demonstrated that the Mbd2−/− mice have an enhanced CD4+ T-helper type 1 response, characterised by an increase in Ifng levels, we first sought to verify this in our mice [8,9]. Lymphocytes were isolated from the large intestine lamina propria of wild type (WT) and Mbd2−/− mice and characterised using flow cytometry. The Mbd2−/− mice displayed a significant increase in the numbers of CD4+Ifng+ (Figure 3A) and CD8+ cells expressing IL-4, IL-17, Ifng, and TNF, with CD8+ cells also displaying increased expression of IL-13 (Figure 3A, B), similar to previously published data [8]. The significantly elevated Ifng levels were further confirmed by an increase in mRNA expression and Ifng presence in the serum (supplementary material, Figure S2A, B). To investigate the impact of these changes on the large intestine, cohorts of ≥ 6 mice were administered 2% DSS (w/v) in drinking water ad libitum for 6 days to induce acute inflammation; experiments were repeated three times to assess reproducibility. The Mbd2−/− mouse demonstrated a significant increase in the disease activity index (DAI), histology score, weight loss, and large intestine atrophy compared with control mice (Figure 3C and supplementary material, Figure S2C–E). Analysis of lymphocytes from the lamina propria indicated an altered cytokine profile with a significant increase in CD4+ cells positive for IL-17, Ifng, TNF, and CD8+Ifng+ cells (Figure 3B, D). As a key role for CD4+ cells and Ifng in DSS-induced colitis has been previously shown, we repeated this experiment using Ifng−/− mice and neutralising CD4+ antibodies to determine their contribution to the phenotype. The absence of Ifng suppressed colitis, as previously shown [38], and significantly decreased severity in the Mbd2−/−/Ifng−/− double-knockout mice (Figure 3C). To address the importance of the CD4+ lymphocyte population, Mbd2−/− and control mice were administered a CD4 neutralising antibody −3, −1, and 0 days prior to induction of acute inflammation. The blockade of CD4+ cells reduced the DAI and weight loss scores to the levels previously observed by deleting Ifng (Figure 3C). To investigate whether the epithelial loss of Mbd2 played a role in the altered immune response, we generated cohorts of vil-creERT2Mbd2ex1/ex1 to drive Mbd2 deletion in the large intestinal epithelia. Four days after deletion of Mbd2, mice were exposed to 2% DSS in drinking water ad libitum. Six days following DSS exposure, the vil-creERT2Mbd2ex1/ex1 mice, in comparison to control mice, displayed a significant four-fold decrease in Mbd2 expression within the large intestine crypt epithelia but no difference in overall disease severity (supplementary material, Figure S3A, B). Further expression analysis within the large intestine indicated no alteration to genes characteristic of Th1 (Ifng), Th17 (Tbx21), and Tregs (Foxp3), which we previously demonstrated to be altered in the Mbd2−/− setting [8,10,12] (supplementary material, Figure S3C). In summary, the Mbd2−/− mouse is highly susceptible to DSS-induced colitis, at least in part, due to the loss of Mbd2 function specifically within the CD4+ cells and loss of appropriate regulation of the pro-inflammatory cytokine Ifng. However, additional roles for Mbd2 within the non-epithelial cells of the stromal compartment cannot be discounted due to the epithelial specific nature of the vil-creERT2 model. We next sought to establish the role of this inflammation susceptibility in a cancer setting.

DSS exposure leads to chronic mucosal colitis and tumourigenesis in the Mbd2−/− mouse

To determine whether this increased susceptibility to colitis had a long-term effect on intestinal health, cohorts of control and Mbd2−/− mice were administered 2% DSS for 6 days ad libitum and aged for 30, 60, and 170 days. The control Mbd2+/− littersmates made a complete recovery following withdrawal of DSS and at 30 (N = 9) and 170 days (N = 11) post-inflammation showed no sign of intestinal disease (Figure 4A). In contrast the Mbd2−/− mice failed to resolve the inflammation and developed a chronic mucosal colitis. At 30 days, 83% (5/6, p = 0.002) of Mbd2−/− mice showed continuing signs of colitis which included severe mucosal inflammation with severe diarrhoea, widespread crypt loss, superficial ulceration, focal active cryptitis with scattered crypt abscesses, and patches of epithelial regeneration (Figure 4B and supplementary material, Figure S4A). At 60 days, the severe diarrhoea and bleeding had subsided in all mice (N = 6); however, in the intestines of 66% (4/6) of these mice, an active chronic mucosal colitis remained, with a mononuclear cell infiltrate in the lamina propria, distortion of crypt architecture, regenerative epithelial
hyperplasia, and crypt fission (supplementary material, Figure S4B). At 170 dpi, 60% (6/10, p = 0.0039) of the mice had flat lesions which were classified as mucinous adenocarcinoma (Figure 4C and supplementary material, Figure S4C). These lesions displayed nuclear β-catenin staining indicating that they were driven via deregulation of the Wnt pathway (Figure 4D and supplementary material, Figure S4C). We have previously shown that absence of Mbd2 in the epithelia alone is not sufficient to alter the acute immune response (supplementary material, Figure S3B). To confirm there was not a longer-term phenotype, we generated cohorts of vil-creERT2Mbd2ex1/ex1 mice for analysis. At 180 days following exposure to 2% DSS in drinking water given ad libitum, these mice, in contrast to the systemic Mbd2−/− setting, showed no signs of intestinal disease (data not shown). These data suggest that the switch to a tumour-promoting environment is dependent on loss of Mbd2 in cells outside of the intestinal epithelia, with the cells of the immune system being the most likely candidates. We next sought to establish the effect of the Mbd2−/− inflammatory response on the tumour suppression observed in the Apcc/+min Mbd2−/− intestine.

Figure 1. Epithelial loss of Mbd2 in the intestine attenuates the phenotype associated with Ah-creApc deletion. (A) RT-qPCR gene expression data indicating that Mbd2 loss suppresses the expression of Wnt target genes following Apc deletion (N = 4 – 6). (B) Quantification of crypt size (left panel) indicating reduction in the size of the proliferative zone and representative images of the proliferative zone and Paneth cells (brown; right panel). (C) Cumulative frequency curve of Paneth cell localisation within the intestinal crypt, indicating partial rescue of positioning in the Ah-creApcflx/flxMbd2ex1/ex1 intestine compared with Ah-creApcflx/flx.
Opposing roles for Mbd2 in inflammation and tumourigenesis

Chronic inflammation overcomes \( Apc^{+/\text{min}}Mbd2^{-/-} \) intestinal tumour suppression

As the \( Apc^{+/\text{min}}Mbd2^{-/-} \) is resistant to intestinal tumourigenesis, we addressed whether this is still the case following an acute inflammatory insult. \( Apc^{+/\text{min}}Mbd2^{-/-} \) mice at 10–12 weeks old were exposed to 2% DSS (w/v) for 6 days and allowed to age for 30 and 180 days. At 30 days after DSS withdrawal, all \( Apc^{+/\text{min}}Mbd2^{-/-} \) mice (4/4) still presented with chronic colitis (Figure 5A). At 180 days post-DSS, 72% (8/11) of \( Apc^{+/\text{min}}Mbd2^{-/-} \) mice displayed mucinous adenocarcinoma with nuclear \( \beta \)-catenin (Figure 5B), in contrast to control \( Apc^{+/\text{min}}Mbd2^{-/-} \) mice, which remained disease-free at the same age, as reported previously [16]. These lesions presented as flat tumours in contrast to the standard polyloid-type lesions with extensive nuclear \( \beta \)-catenin that developed in the \( Ape^{+/\text{min}} \) model (Figure 5C). Thus, following the onset of a chronic inflammatory response, tumour suppression is lost in the \( Apc^{+/\text{min}}Mbd2^{-/-} \) large intestine; indicating that the protection afforded by the absence of Mbd2 in the epithelia is overcome following the onset of an Mbd2-deficient inflammatory response in the intestine.

Loss of Mbd2 decreases the survival of Apc-deficient stem cells

Comparison of the data from our \( Apc^{+/\text{flx}}Mbd2^{ex1/ex1} \) mice and our previously published \( Apc^{+/\text{flx}}Mbd2^{-/-} \) [15] and \( Apc^{+/\text{min}}Mbd2^{-/-} \) [16] mice indicated that the suppression of tumourigenesis, Wnt activation, and Paneth cell relocalisation phenotypes were enhanced in the \( Mbd2^{-/-} \) mouse. These data suggested that in the \( Mbd2^{-/-} \) model the epithelial and non-epithelial phenotypes synergised to suppress tumourigenesis. As we have demonstrated a significant role for \( Ifng \) in the \( Mbd2^{-/-} \) inflammatory response and it is a key player in inflammation and anti-tumour immune responses [18,20,21,23,39,40], we sought to assess the relevance of \( Ifng \) in the tumour suppression observed in \( Apc^{+/\text{min}}Mbd2^{-/-} \) mice. We generated \( Apc^{+/\text{min}}Mbd2^{-/-}Ifng^{-/-} \) mice to explore whether loss of \( Ifng \) impacted on intestinal tumour suppression. As expected, at 60 days, the \( Apc^{+/\text{min}}Mbd2^{-/-}Ifng^{-/-} \) mice displayed increased epithelial and non-epithelial phenotypes, which resulted in an increase in tumour burden and a decrease in survival compared to the \( Apc^{+/\text{min}}Mbd2^{-/-} \) and \( Apc^{+/\text{flx}}Mbd2^{ex1/ex1} \) mice. These results suggest that Mbd2 plays a critical role in the suppression of tumourigenesis in the intestine.
Figure 3. Mbd2 deficiency increases susceptibility to chronic intestinal inflammation. (A) Flow cytometry plots illustrating an increase in CD4^+Ifng^+ cells following PMA stimulation in Mbd2^{+/−} mice in comparison to WT. (B) Bar charts quantifying the cytokine expression profiles of CD4 and CD8 lamina propria lymphocytes as a percentage of the parent population in WT and Mbd2^{−/−} mice before and after DSS exposure. (C) Bar chart illustrating the DAI score 6 days after addition of 2% DSS to drinking water. (D) WT (left panel) and Mbd2^{−/−} (right panel) large intestine sections illustrating crypt loss and an increase in CD4^+ cell number (brown) following DSS exposure.

(N = 6) mice displayed significantly more nuclear β-catenin-positive lesions (average 22) in comparison to the Apc^{+/-}Mbd2^{−/−} mice (N = 6; average < 1) (Figure 6A). As we have previously demonstrated that epithelial loss of Mbd2 alone is capable of intestinal tumour suppression (Figure 1), it is of note that the Apc^{+/-}Mbd2^{−/−}Ifng^{−/−} mice (N = 6) displayed a small but significant increase in nuclear β-catenin-positive lesions (p = 0.03; average 2.6), indicating that in the absence of Ifng a small number of lesions can escape...
Opposing roles for Mbd2 in inflammation and tumourigenesis

Figure 4. Mbd2^{−/−}-driven inflammation drives tumourigenesis in the large intestine. (A) Scoring for the presence of intestinal disease, following DSS withdrawal, indicating the percentage of mice with a histologically normal large intestine at different time points following exposure. d = days. (B) Representative image of Mbd2^{−/−} intestine 30 days post-inflammation displaying a chronic mucosal colitis with superficial ulceration (↓) and mononuclear infiltrate (→). (C) Mbd2^{−/−} intestine 170 days post-inflammation displaying an adenocarcinoma stained for mucin (blue). (D) Mbd2^{−/−} mucinous adenocarcinoma displaying heterogeneous nuclear β-catenin (brown, →; inset).

The tumour resistance conferred by epithelial Mbd2 loss. In our previous work, where we investigated mice 4 days after acute Apc loss in the crypts of Mbd2^{−/−} mice, this information may have been obscured [15]. This acute Apc loss does not reflect the small number of mutated cells which initiate an individual tumour in vivo and presents over an insufficient time for an adaptive immunological anti-tumorigenic phenotype to manifest. To overcome this, we targeted gene deletion to the intestinal stem cell (ISC) using the Lgr5cre transgene to generate Lgr5creERT2-Apc^{floxx/Mbd2^{floxx}} and Lgr5creERT2-Apc^{floxx/Mbd2^{−/−}} mice. These allowed us to delete Apc specifically within a proportion of ISCs, the cell of origin for CRC [41], allowing us to observe the effects of Mbd2 deficiency on Apc-deleted ISCs over a longer (15-day) time frame. In comparison to the control Lgr5creERT2-Apc^{floxx} (N = 6) mice, we observed a significant reduction in the number of nuclear β-catenin lesions in the Lgr5creERT2-Apc^{floxx/Mbd2^{−/−/ex1}} (N = 7) cohort, which
MBD2 loss is a rare event in colorectal cancer

Our data presented here and previously [15,16] support the notion that in murine intestinal epithelium Mbd2 is required to permit tumourigenesis. To investigate the relevance of MBD2 in human intestinal cancer, we looked for evidence that it is preferentially retained in colorectal cancer, and whether its loss is associated with a positive prognosis. To achieve this, we examined MBD2 expression in a panel of human intestinal tumours (TNM stage I–IV, \(N = 7\) per stage). Expression analysis failed to detect any loss or alteration to the MBD2 profile across the different tumour stages (supplementary material, Figure S6). We also examined publicly available colorectal cancer sequencing data using cBioPortal (http://www.cbioportal.org). In silico analysis indicated that the MBD2 gene was classed as deep deleted in 6/2079 (0.28%) patients; while supportive of a role for MBD2 in enabling tumourigenesis, the small number of samples in which MBD2 was lost prevented any conclusive survival analysis.

Discussion

Based on these preclinical data, the genes transcriptionally regulated by Mbd2 within the immune system and stem cells from the normal and diseased intestinal epithelia offer a set of targets that play a key role in linking epigenetic changes to cancer. The finding that within the intestinal epithelia normal function of Mbd2 is required to permit tumourigenesis is supported by clinical data; CRC sequencing data and a small study looking specifically at MBD2 status [42] indicate that loss of MBD2 is a rare event in CRC patients [43,44]. However, assigning a crucial role to a gene that requires no alteration to elicit a tumourigenic function is extremely difficult and emphasises the value of this type of preclinical research. Which of the Mbd2 regulated genes within the intestinal epithelia are responsible for this protection remains to be established. However, it is now clear that the broad role of Mbd2 in the immune system influences Apc-deficient epithelial stem cells. Evidence is now emerging that cross talk between Th cells and MHC II-expressing ISCs regulates ISC numbers and differentiation status [45]. Recent work has demonstrated a key role for two inflammatory cytokines, Ifng and Tnf, and the JAK/STAT-1 signalling pathway in the reserve ISC regenerative response to acute intestinal inflammation [46]. Thus, the role of Mbd2 in controlling the CD4 and Ifng immune response is likely to be of great interest because ‘tumour-promoting inflammation’ is now identified as an enabling characteristic in the hallmarks of cancer [47]. Further investigation of Mbd2 functions should aid at understanding the links between a type 1 acute inflammatory response (the DSS-induced colitis model; Figure 2A) and an anti-tumour response. Potentially, in the early stages, the acute Th1–Ifng inflammatory response in the Mbd2−/− mouse is associated with tumour clearance. In humans, a Th1–Ifng response is a characteristic of cancer immune surveillance [48], associated with Th1 CD4+ and CD8+ T cells which directly regulate tumour cell cytotoxicity or induce senescence, while indirectly polarizing innate immune cells towards tumour suppression [20,49]. However, the inability of these Mbd2−/− mice to resolve the inflammation, resulting in chronically inflamed intestines, despite irritable withdrawal, suggests either an auto-immune response to a self-antigen, a neo-antigen generated by the altered
Opposing roles for Mbd2 in inflammation and tumourigenesis 279

Figure 6. Mbd2 promotes the survival of Apc-deficient stem cells. (A) Scoring of nuclear β-catenin-positive lesions indicates that at 60 days there is a reduction in Apc+/min-driven lesions due to Mbd2 deficiency which is partially dependent on Ifng (Apc+/min Mbd2−/−, N = 6; Apc+/min Mbd2−/−, N = 6; and Apc+/min Mbd2−/− Ifng−/−, N = 6). (B) Following deletion of Apc in the ISC, using the Lgr5creERT2Apcflx/flx (N = 5) model, the number of nuclear β-catenin-positive crypts in the small (left panel) and large (right panel) intestine is significantly reduced in Lgr5creERT2Apcflx/flxMbd2flx/flx mice and further reduced in the Lgr5creERT2Apcflx/flxMbd2−/− (N = 4) setting. (C) Representative image of nuclear β-catenin-positive crypts (arrows; brown) in the small intestine 15 days after Lgr5creERT2Apcflx/flx-driven Apc deletion in ISCs and (D) in combination with Mbd2ex1/ex1 deletion.

transcriptional profile [50], or a response to bacterial translocation as a result of impaired epithelia integrity [51]. This chronic pro-tumourigenic inflammatory environment is akin to the inflammation-associated cancer that develops in Crohn’s and other colitis patients. In our mouse model, the inflammation can override the Mbd2-deficient-dependent anti-tumour suppression mechanism to such an extent that Apc+/min Mbd2−/− mice develop adenocarcinomas, which are rarely, if ever, seen in this model without an inflammatory insult. This loss of inflammatory control is potentially due to the known role of Mbd2 in promoting T-reg cell function [10]. These cells suppress the immune responses of other cells and maintain self-tolerance; experimental depletion of these cells in animal experiments leads to colitis, whereas in CRC patients their accumulation is
associated with progression [52]. Thus, Mbd2 function may play a significant role in the progression from a protective acute response to a chronic tumour-promoting environment.

This work highlights the role that epigenetics plays in the cells of a tumour and its environment. These changes can affect the tumour itself and the delicate balance between acute and chronic inflammation which elicits anti- or pro-tumourigenic effects. Taking into consideration that in excess of 95% of CRC cases are sporadic, arising in individuals with no identified genetic predisposition [53], demonstrates that the aetiology of CRC is multifactorially linked to genetic mutations, diet, inflammatory processes, ageing, and, more recently, the gut microbiota. The study of the epigenetic mechanisms which underpin these gene–environment interactions is crucial to understand how to prevent and control this disease. Epigenetic regulation, via DNA methylation, is commonly used by all normal cells to ensure proper regulation of gene expression and stable gene silencing, and is invariably altered in tumourigenesis. Recent technological advances are now leading to the identification of new genes and loci associated with inflammatory bowel diseases and CRC, based on generating methylene maps [54,55]. However, concentrating solely on the DNA methylene within the intestinal epithelium neglects the fact that the majority of the CRC-associated factors impact on the epigenome within the entire body and not just the intestinal epithelium. Ultimately, interpreting the DNA methylation changes will require a more holistic approach to explain the links between the environment and CRC. We can begin to move towards this goal by exploiting existing knowledge of how DNA methylation is interpreted. The myriad of DNA methylation differences observed at an organism level are interpreted by a relatively small number of proteins [4]. Given the small number of proteins capable of interpreting the DNA methylation signal, it stands to reason that some will play a major role in the multiple changes that occur to permit tumour initiation and progression. In this study, we have demonstrated how loss of a single gene involved in the interpretation of DNA methylation has pleiotropic effects on inflammation and cancer that could be environmentally regulated. In the context of understanding the relationship between the multiple factors associated with CRC, this emphasises that non-cell intrinsic epigenetic changes beyond the target tissue should be considered in the attempt to unravel mechanisms.

Acknowledgements

LP would like to dedicate this paper to Professor Alan Clarke (1963–2015), who provided inspiration, mentorship, support, and friendship. This work was supported by a Cancer Research UK programme grant. LP is supported by a fellowship from the European Cancer Stem Cell Research Institute. SM is supported by a philanthropic donation from the Mr Lyndon and Mrs Shirley Ann Wood from the Moorhouse Group Ltd. The authors recognise the assistance of lab technicians Sarah Davies, Elaine Taylor, Matt Zverev, Mark Bishop, and Jolene Twomey. AG is supported by a Cancer Research UK Programme grant (C16731/A21200). MS was supported by grants from the German Research Foundation (DFG: KFO257 (sub-project 4) and FOR 2438 (subproject 2)) and the Interdisciplinary Center for Clinical Research (IZKF) of the Clinical Center Erlangen.

Author contributions statement

LP designed the research. LP, SM, TP, KG, AB (Cardiff), CT, NB-L, GRJ, PCC, ASM, and GW performed research. HO and AG provided reagents and critical analysis of the manuscript. LP, MS, TP, AB, ARC, and OS analysed data and provided critical analysis of the manuscript. LP drafted the manuscript.

References


**SUPPLEMENTARY MATERIAL ONLINE**

Supplementary figure legends

Figure S1. Expression analysis of intestine 4 days after epithelial Mbd2 deletion indicates that cell homeostasis is maintained

Figure S2. Deficiency of Mbd2 increases Ifng levels and enhances DSS-induced colitis

Figure S3. Intestinal DSS response is unaltered following vil-creER<sup>T2</sup>-driven epithelial loss of Mbd2

Figure S4. Following an acute inflammatory insult, the Mbd2-deficient intestine develops chronic mucosal colitis (6 days post-DSS administration)

Figure S5. Mbd2 promotes the survival of Apc-deficient stem cells

Figure S6. MBD2 expression is constant irrespective of intestinal tumour stage

---

**25 Years ago in The Journal of Pathology...**

Identification of numerical chromosome aberrations in archival tumours by in situ hybridization to routine paraffin sections: Evaluation of 23 phaeochromocytomas


Expression of keratin mRNAs and proteins in normal salivary epithelia and pleomorphic adenomas

Lan Su, Dr. Peter R. Morgan, Dolores L. Harrison, Ahmad Waseem, E. Birgitte Lane

Loss of α6 and β4 integrin subunits coincides with loss of basement membrane components in oral squamous cell carcinomas

Caroline S. Downer, Fiona M. Watt, Dr. Paul M. Speight

Proliferative activity as revealed by Ki-67 in uterine adenocarcinoma of endometrioid type: Comparison of tumours from patients with and without previous oestrogen therapy

Anette Lynge Nielsen, Henrik Christian Juul Nyholm

To view these articles, and more, please visit: [www.thejournalofpathology.com](http://www.thejournalofpathology.com)

Click 'BROWSE' and select 'All issues', to read articles going right back to Volume 1, Issue 1 published in 1892.

---

**The Journal of Pathology**

*Understanding Disease*