UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE

Academic Unit of Clinical and Experimental Sciences

A Study of MicroRNA in the Mucosal Epithelium of Inflammatory Bowel Disease

by

Richard Keith Felwick

Thesis for the degree of Doctor of Philosophy

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ABSTRACT

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A STUDY OF MICRORNA IN THE MUCOSAL EPITHELIUM OF INFLAMMATORY BOWEL DISEASE

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Introduction: The intestine is lined by a layer of epithelial cells. These form a selectively permeable barrier to luminal antigens and are vital in the innate immune regulation of the gut barrier. Responses are tightly controlled but become dysregulated in inflammatory bowel disease (IBD) leading to increased intestinal permeability, inflammation and diarrhoea. MicroRNAs are small non-coding RNA molecules which repress protein translation. They show altered expression in IBD as does TNFAIP3, an important negative regulator of TNF driven NFκB responses. It was hypothesized that microRNA are implicated in the dysregulation of the colonic epithelia barrier in Crohn's disease (CD) via effects on TNFAIP3.

Methods: Colonic biopsies were collected from patients with active CD inactive CD and heathy donors. Using laser capture microdissection, epithelial areas were isolated from biopsy sections. RT-qPCR was used to quantify expression of selected microRNAs. TNFAIP3 protein expression and distribution was characterized immunohistochemically on serial sections. Binding of upregulated microRNAs to predicted TNFAIP3 mRNA targets was confirmed with luciferase reporter assays and functional studies of the barrier and innate cytokine release were investigated with cell culture and explant culture studies.

Results: Expression of microRNA 23a was significantly up regulated in the colonic epithelium of Crohn's disease patients compared to healthy controls. Bioinformatic analysis predicted that microRNA 23a could target the 3'UTR of TNFAIP3. Luciferase reporter assays confirmed that microRNA23a repressed translation of TNFAIP3 in transfected epithelial cells and increased NFkB transcription. Immunohistochemical staining of TNFAIP3 in colonic biopsies showed a sub-apical, epithelial cell junction distribution, which was reduced or lost in adjacent Crohn's disease sections, correlating inversely with microRNA 23a levels from the same tissue. Overexpression of microRNA23a increased epithelial barrier permeability in a cell culture model and increased inflammatory cytokine release in an explant biopsy culture system.

Conclusion: MicroRNA23a is overexpressed in the colonic epithelium of Crohn's disease and represses TNFAIP3 leading to enhanced cytokine release and increased intestinal permeability.

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Academic Thesis: Declaration of Authorship

I, **Richard Keith Felwick**, declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

A Study of MicroRNA in the Mucosal Epithelium of Inflammatory Bowel Disease

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. None of this work has been published before submission

Signed: .....

Date:

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Definitions and Abbreviations

ATG16L	Autophagy related protein 16-1
AP-1	Activator Protein 1
CD40	Cluster of differentiation 40
CD	Crohn's Disease
DC	Dendritic Cell
DEPC	Diethyl Pyrocarbonate
DGCR 8	DiGeorge Syndrome Chromosomal Region 8
DSIF	DRB Sensitivity Inducing Factor
DTT	Dithiothreitol
ELIE	Elongation Inhibitor Element
EMT	Epithelial Mesenchymal Transition
FITC	Fluorescein Isothiocyanate
FOXP3	Forkhead Box P3
GUK	Guanylate Kinase
IBD	Inflammatory Bowel Disease
IEL	Intra Epithelial Lymphocyte
IFN-γ	Interferon Gamma
IgA	Immunoglobulin A
IL-1β	Interleukin 1 Beta
IL-6	Interleukin 6
IL-10	Interleukin 10
IL-13	Interleukin 13
IL-17	Interleukin 17

IL-23	Interleukin 23
IL23R	Interleukin 23 Receptor
IKKα	Inhibitor Kappa B Kinase Alpha
IKKβ	Inhibitor Kappa B Kinase Beta
IKKγ	Inhibitor Kappa B Kinase Gamma
JAK	Janus Kinase
JAM	Junctional Adhesion Molecule
LCM	Laser Capture Microdissection
LPS	Lipopolysaccharide
MCP-1	Monocyte Chemoattractant Protein 1
MDCK	Madin-Darby Canine Kidney
MLC	Myosin Light Chain
MLCK	Myosin Light Chain Kinase
mRNA	Messenger Ribonucleic Acid
miRNA	MicroRNA
MSD	MesoScale Discovery
MYD88	Myeloid differentiation primary response gene 88
UC	Ulcerative Colitis
NEMO	NF kappa B essential modulator
NFκB	Nuclear Factor Kappa B
NOD2	Nucleotide -binding oligomerization domain containing protein 2
PBS	Phosphate Buffered Saline
PRR	Pattern Recognition Receptors
Rac1	Ras-related C3 botulinum toxin substrate

Rho A	Ras Homolog Gene Family Homolog A
ROCK	Rho Associated Kinase
RT-qPCR	Reverse Transcription quantitative Polymerase Chain Reaction
SH3	SRC Homolgy Domain 3
STAT	Signal Transducer and Activator of Transcription
TBS	Tris Buffered Saline
TER	Transepithelial Resistance
TGF-β	Transforming Growth Factor Beta
Th1	T helper cell type 1
Th17	T helper cell 17
TLR	Toll Like Receptors
TNBS	2,4,6 Trinitrobenzenesulfonic acid
TNFα	Tumour Necrosis Factor Alpha
TNFAIP3	Tumour Necrosis Factor Alpha Inhibitory Protein 3
TNFR	Tumour Necrosis Factor Receptor
TNFR2	Tumour Necrosis Factor Receptor 2
Treg	Regulatory T Cell
TSLP	Thymic Stromal Lymphopoietin
p120CTN	p120 Catenin
ZnF	Zinc finger
ZO	Zonula Occludens
3'UTR	3 Prime Untranslated Region
5'UTR	5 Prime Untranslated Region
5ASA	5 Amino Salicylic Acid

Chapter 1:

Introduction

Chapter 1: Inflammatory Bowel Disease

1.1 Introduction

Inflammatory bowel disease (IBD) is a generic term encompassing two inflammatory conditions of the intestinal tract; Crohn's disease (CD) and ulcerative colitis (UC). Both are characterised by chronic relapsing inflammation within the intestinal tract and can result in significant morbidity and complications. Over half of patients with Crohn's disease will require at least one episode of hospitalisation within 10 years of diagnosis (1). The chronic nature of these diseases has a significant impact on quality of life. Education, economic productivity and the opportunity for an individual to achieve their full potential can all be hampered by repeated bouts of intestinal inflammation and the associated debilitating symptoms. The costs of treating IBD in the United Kingdom alone amount to more than £131 million per annum (2). This represents 13% of the annual NHS budget.

1.2 Prevalence

The prevalence of the inflammatory bowel diseases is greatest in the developed western world. European data reports a prevalence of 505/100,000 for UC and 322/100,000 for CD. The annual incidence of UC in Europe is 24.3/100,000 and 12.7/100, 000 for CD (3). Similar figures are seen from the North American population. This contrasts with the Asian population and southern hemisphere nations, which traditionally have had a low prevalence of IBD. However, globally, the overall prevalence of both inflammatory bowel diseases is now increasing (3).

1.3 Clinical Features

1.3.1 Crohn's Disease

Crohn's disease is characterised by patchy inflammation affecting any location of the intestinal tract from the mouth to the anus, although it commonly affects the colon and terminal ileum (4). A characteristic is the 'skip lesion' where affected segments are separated by normal mucosa. The disease is classified according to the Montreal classification (Table 1.1) which assigns a code for disease location, activity and age at diagnosis (5). Ileal disease was once the commonest

phenotype, although recent trends in Europe have seen isolated colonic disease becoming more common (6). Inflammation in CD is typically transmural affecting all layers of the intestinal wall (7).

Onset of symptoms typically occurs between the ages of 15 – 40, and include abdominal pain, diarrhoea with or without blood /mucus, fever and weight loss. Aside from affecting the intestine, Crohn's disease can also cause inflammation at sites distant from the bowel. These are termed extra intestinal manifestations and occur in up to 40% of patients, with joint involvement most common. (6). Other features may include uveitis, scleritis, aphthous stomatitis erythema nodosum or pyoderma gangrenosum. These occur in up to 15% of patients (6).

As a consequence of deep seated inflammation patients can also develop a fistula. This is defined as an abnormal connection between two epithelialized surfaces. Perianal fistulas account for the majority, although they can also occur between intestinal loops (enteroenteric), bladder (colovesical), vagina (recto-vaginal) or directly onto the skin (enterocutanoeus) (8). Perianal fistulas develop in 20% of people after 20 years (9). Fistulising disease is challenging to treat and can have a profound impact on quality of life (8, 10).

The natural history of Crohn's disease is characterised by repeated episodes of intestinal inflammation followed by periods of quiescence. Although disease location tends to remain stable over time, the phenotype changes, becoming less inflammatory and more penetrating/stricturing (4, 6). One third initially present with stricturing or penetrating disease i.e. fistula or abscess, with a similar proportion developing complex disease over 10 years (4, 6). This is a consequence of intestinal damage following repeated periods of inflammation. These episodes ultimately lead to fibrous scarring of the intestine and stricture formation. Ultimately 50% of patients with CD will require surgery within 10 years of diagnosis and almost three quarters of patients will require at least one operation during their lifetime (1).

Table 1.1: Montreal classification of Crohn's Disease

Location	Behaviour	Age of Onset
Terminal Ileum (L1)	Non stenotic/Non penetrating (B1)	≤16 (A1)
Colon (L2)	Stenotic (B2)	17–40 (A2)
Ileo-Colonic (L3)	Penetrating (B3)	≥40 (A3)
Upper GI tract (L4)	Perianal Disease Modifier (p)	

1.3.2 Ulcerative Colitis

Whereas Crohn's disease affects any portion of the gastrointestinal tract, ulcerative colitis is limited to the large intestine. Bloody diarrhoea, with or without mucus, is the characteristic symptom (11). Involvement always begins at the rectum and spreads proximally to include a varying portion of the colon. At its most severe the entire large intestine is affected. In other cases involvement is confined to the rectum. The anatomical distribution forms the basis of the Montreal score for UC (5). Unlike CD, this inflammation is confluent and superficial (7).

Similar to Crohn's disease, the natural history of UC follows a relapsing remitting course. Disease extent initially occurs in equal distributions between proctitis, left sided and extensive disease. Of those with disease limited to the rectum 28% will progress over time with 10% developing extensive colitis (12, 13). Over a 10 year period 83% of patients will experience at least one relapse (13). However, overall disease activity tends to diminish as time progresses (12, 13). Despite this a third of people have ongoing inflammatory activity and experience a chronic relapsing course even 10 years following diagnosis (13). Uncontrolled inflammation or its complications can lead to colectomy. This occurs in 10% of people over 10 years, with the highest incidence of colectomy in the first 2 years of diagnosis (13).

Table 1.2: Montreal classification of ulcerative colitis

Maximal extent of Inflammation observed at colonoscopy	Grade
Proctitis	E1
Left sided – up to splenic flexure	E2
Extensive	E3

Ulcerative colitis is also associated with extra intestinal manifestations, albeit slightly less frequently than CD (14). The manifestations are similar to those found in Crohn's disease with joint involvement most common. (14). Ulcerative colitis is also associated with a small but increased risk of developing dysplasia or colorectal cancer (15, 16). This risk increases with the duration of disease and extent of inflammation (15, 17).

1.4 Diagnosis

The diagnosis of inflammatory bowel disease is suggested by the clinical history in an individual who presents with symptoms of abdominal pain and /or altered bowel habit as described above. In addition, laboratory investigations may show evidence of an inflammatory response (typically elevated serum C reactive protein, raised total white cell count, low serum albumin). Faecal calprotectin, a stool inflammation marker, is also being increasingly used as surrogate marker of intestinal inflammation (18). The diagnosis of IBD is confirmed by visualisation of the colon and terminal ileum with ileocolonoscopy. This allows an assessment of mucosal inflammation, the extent of disease to be determined and samples taken for histological confirmation. Although there is no single pathognomic lesion for colitis, a combination of features aids the histological diagnosis (19). Typical features accepted for CD include discontinuous chronic inflammation with crypt distortion. Non-caseating granulomas may also be seen. This contrasts with UC which is characterised by continuous inflammation and crypt distortion. Granulomas are absent, but crypt abscesses are seen (19). Despite this, in a

proportion of cases it is not possible to distinguish between CD and UC, leading to a diagnosis of inflammatory bowel disease unclassified (19).

1.5 Aetiology and Pathogenesis

The precise aetiology of inflammatory bowel disease remains uncertain. It can be thought of in terms of genetic susceptibility, environmental antigens and abnormal immune homeostasis. Current thinking suggests IBD is caused by an aberrant immune response to luminal antigens in genetically susceptible individuals (20).

1.5.1 Genetics

Family studies underpin the importance of genetic susceptibility in IBD. First degree relatives of an affected individual and monozygotic twins have the greatest risk of developing IBD, with the highest risk observed in Crohn's disease (21, 22). It is important to note that genetics confers only susceptibility, and not an absolute risk of developing disease. This is highlighted by studies of monozygotic twins. Tysk et al., (1988) published the first data on concordance rates in mono and dizygotic twins observing that the concordance rate for CD in monozygotic twins was 58%, with 6.3% concordance for UC (23). Twin studies in Denmark, Germany, Sweden and the UK, collectively involving 784 twin pairs, have subsequently corroborated these findings. In these cohorts concordance for CD was 38–63% for monozygotic twins and up to 6.7% for dizygotic twins (24). Less heritability was seen with UC. For monozygotic twins concordance rates were 6 – 27 % and for dizygotic pairs up to 8% (24).

Despite the lack of absolute heritability, genome wide association studies (GWAS) have identified a number of possible susceptibility loci for IBD. With the most recent publication, 200 IBD associated risk loci have now been identified accounting for 13% of the variance in disease liability for CD and 8% for UC (25). A number of genes identified within these loci are common to both UC and CD, suggesting shared common features of the disease pathogenesis. Figure 1.1

shows the current genes found in risk loci associated with susceptibility to CD, UC and IBD in general.

Traditionally, risk loci from European cohorts were thought to be disparate from non-Caucasian cohorts (26-28); however this view has recently been challenged. Two risk loci identified via GWAS in a Korean cohort also showed significance in the International Inflammatory Bowel Disease Genetics Consortium database, which includes Caucasians (29). The most recent study in which 38 new loci were identified also included both European and non-European cohorts. The majority of the new risk loci identified in this study were found to be shared across divergent populations (25). Although there are clear exceptions to this (i.e. NOD2 (28)) this new evidence suggests that perhaps the global IBD population is not as genetically divergent as first thought.

Pathway analysis of risk loci reveals genes that are involved in a number of pathways including, but not limited to, epithelial barrier integrity/restitution, innate mucosal defence, T and B cell regulation, immune tolerance, autophagy and cell migration (30). A number of these genes and pathways have also been associated with other inflammatory and autoimmune diseases, with 51 IBD loci having some degree of overlap with 23 different disease entities (31). These disease associations include Crohn's disease and ankylosing spondylitis,(32, 33), and coeliac disease (34) or type 1 diabetes with inflammatory bowel disease (35).

The depth of literature reporting the risk loci and variants in IBD is extensive and beyond the scope of this thesis. However, several prominent IBD associated genes are worthy of particular mention as they now have an established disease association.

The first gene identified as a susceptibility gene was the Nucleotide-binding oligomerization domain-containing protein 2 (NOD 2) (36). This produces the cytosolic NOD2 protein which is the intracellular receptor for the bacterial wall peptidoglycan muramyl dipeptide. Stimulation of NOD2 by its ligand leads to activation of NFκB-mediated innate immune responses and enhanced antimicrobial defences (37). Two missense single nucleotide polymorphisms and a frame shift mutation have been identified, conferring a 3 fold increased risk of CD for heterozygotes, rising to 20 times increased risk for homozygotes (36, 38) . However only 35% of CD patients carry these mutations (39). Loss of function mutations have been shown make NOD2 less responsive to bacterial antigens, thus potentially impairing bacterial clearance from epithelial surfaces, leading to an enhanced adaptive immune response (40). NOD2 mutations are associated

with ileal or ileocolonic CD. There is no association with UC (38). They have also been associated with younger age at disease onset, increased need for ileocaecal resection and an increased post-operative recurrence and surgical intervention rate (39).

Closely associated with NOD2 function is the Autophagy-related protein 16-1, encoded by the ATG16L1 gene. Autophagy is the process where cytoplasmic constituents or foreign antigens are packaged into vesicles and enzymatically degraded. This can be coordinated by the NOD2 protein, with mutant proteins resulting from CD associated frame shift mutations leading to a failure of ATG16L1 recruitment and impaired autophagy (41). Alterations in autophagy have also been associated directly with mutations within the ATG16L1 gene (42). Numerous studies have now reported polymorphism in this gene which are all implicated in CD (43). At a functional level, it has been shown that impaired autophagy of antigens leads to inflammation (44). Similar to NOD, ATG16L1 variants seem to be associated with increased risk for ileal disease (45, 46).

Variants in the Interleukin 23 receptor (IL23R) show association with Crohn's disease and ulcerative colitis (47, 48). Interleukin 23 has been shown to be essential for driving T cell mediated mucosal inflammation which importantly provides a functional mechanism for the gene variant seen in disease pathogenesis. (49). In addition to disease associated alleles, several variants in this gene also appear to be protective. In particular G149, V3621 and R381 variants all confer some protection from the development of colitis due to reduced protein stability or cytoplasmic retention (50, 51).

A number of other mutations have since been identified, the majority of which are associated with immune cell development or function. For example, a number of loci linked to regulatory T cell and TH1/TH17 differentiation have been identified (52). Mutations in the IL10 receptor, again linked to regulatory T cell function have been associated with early onset Crohn's disease (53) . A summary of these is shown in Figure 1.1.

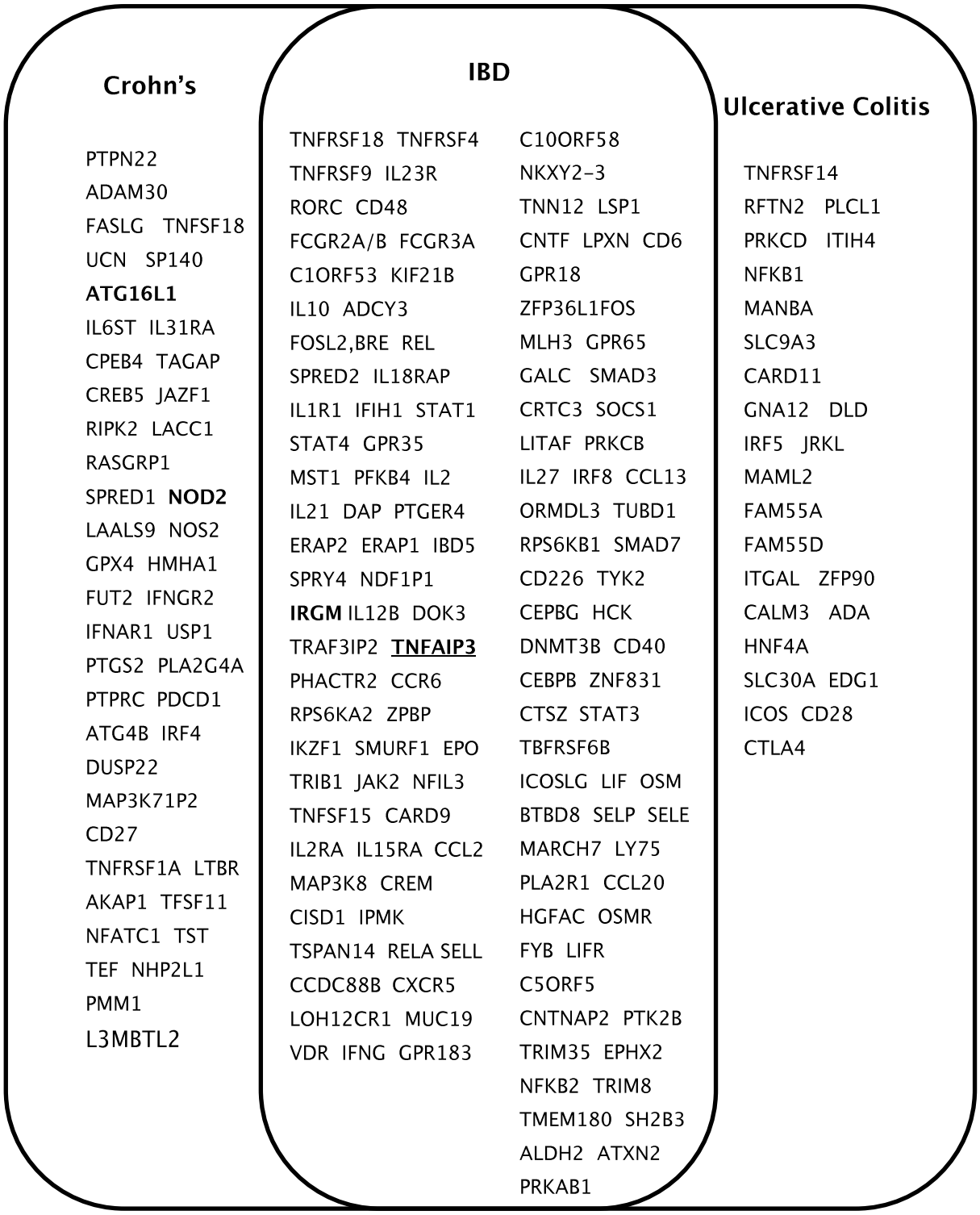


Figure 1.1 Genes identified within risk loci for CD , UC and IBD

Adapted from Jostins et al. 2012(54) and Liu et al. 2015(25)

1.5.2 Environmental Antigens

Although recent advances in genome wide studies have been informative, genetics does not fully explain the development of IBD. If a disease was truly genetically determined, the concordance in monozygotic twins would be expected to be close to 100%. Twin studies confirm this is not the case (22). It is therefore likely that there are other triggers which can result in disease if encountered in an individual with a susceptible genetic background. A number of potential environmental antigens have been considered. These include smoking, appendicectomy, hygiene, diet, antibiotic use, vitamin D levels, medication use and stress (55). Only smoking and appendicectomy have shown an association with IBD, with smoking and appendectomy being protective against UC but detrimental in Crohn's disease (55).

Over recent years there has been a growing interest in the role of the gut microbiome in the pathogenesis of IBD. The intestine is one of the largest reservoirs of microbes in the human body (56). The microbial phyla shows wide variability between individuals, giving them their own 'microbial signature' that tends to remain stable over time (57). Couple this with the mucosal immune system and its need to maintain immune tolerance in the face of large antigen presentations, and this is an intriguing relationship. Several changes in the microbiota have been observed in IBD cohorts, although the causality of these microbial changes remains unclear (56). In general, IBD patients have reduced microbial diversity compared to healthy controls (58, 59) . This is also the case in monozygotic twins who are discordant for CD with the affected twin having reduced diversity compared to its non-affected sibling (60).

Specific microbial changes which have been linked to IBD include, reductions in *Firmicutes* and *Bacteroides* species, with an increase in *Enterobacteriaceae* species, particularly invasive *E. coli* (56). The significance of these changes remains unknown.

1.5.3 Abnormal Immune Homeostasis

The gastrointestinal tract forms a large surface area continuously exposed to the luminal microbiota. The mucosal immune system is therefore faced with the task

of recognising and responding to invading pathogens, whilst promoting immune tolerance to the commensal bacteria. In IBD this delicate balance is disrupted. The importance of the immune system in IBD is highlighted by a report of Crohn's colitis developing in an individual following the receipt of a non-myeloablative allogeneic stem cell transplantation. In this case the donor was found to have a 5 prime untranslated region (5'UTR) mutation in the NOD2/CARD15 gene. The donor did not have IBD but this became manifest in the recipient (61). This supports the notion that genetic susceptibility alone is not sufficient for the development of disease and therefore must be coupled with environmental/antigenic exposure.

The immune system is typically divided into two arms, the innate response and the adaptive response. Innate immune responses are non-specific and provide an 'immediate' response to the presence of an antigen. Typically the cells of the innate system have no 'memory' so repeated stimulation will provoke the same response with each antigen encountered. In contrast, the adaptive response is antigen specific. Once presented with an antigen, the cell of the adaptive system will enhance a repertoire of cells with immunological memory. When the same antigen is encountered in future these cells can initiate a specific response directed towards that antigen. Defective responses/regulation of both the innate and adaptive responses have been described in inflammatory bowel disease (62).

It is now recognised that the dominant immunological phenotype differs between Crohn's and Ulcerative colitis, with the former exhibiting a predominate Th1/Th17 response with the subsequent production of IFN γ TNFα, IL17, IL12 , IL21, IL22, IL 2 and IL6 (4, 63, 64). Ulcerative colitis had traditionally been associated with a predominate Th2 response, mediated by IL13, IL5, IL6, TNFα and IL4.(63, 65). This is an evolving area with more recent studies in human explant cultures suggesting that IL17A and IFN are the predominant cytokines released with IL13 release being significantly lower (66). Table 1.3 displays the inflammatory cytokine profiles associated with each type of inflammatory bowel disease.

Table 1.3: Cytokine expression profiles in inflammatory bowel disease

Crohn's Disease	Ulcerative Colitis	Crohn's Disease	Ulcerative Colitis
Innate Immune Response		Adaptive Immune Response	
IL-1β	IL-1β	IL-17	IL-5
Il-6	IL-6	IL-21	IL17A
IL-8	IL-8	IFN-γ	
IL-12	IL-18	TL1α	
IL-18	TNFα		
IL-23	Light		
IL-27			
TNFα			
Light			
TL1α			

Adapted from Shi et al. Biancheri et al, and Neurath et al. (62, 63, 66)

Polarisation of naïve T cells towards a Th1 response in CD is induced by the transcription factors signal transducer and activator of transcription 1 (STAT1) and T bet, resulting in increased expression of IL12. This induces Th1 polarisation (67, 68). In turn IL 18 is upregulated on Th1 cells further amplifying the response (69). Subsequent induction of NFκB results in cytokine release including TNFα which is increased in the serum and mucosa of IBD patients (70, 71). Antibodies directed against TNFα are used in treatment and inhibit Th1 cell responses (72).

A second subset of T cells has also gained prominence in recent years. Known as Th17 cells, they secrete IL-17 and are polarised from naive T cells via the actions TGF-β and IL-6 (73). Polarised Th17cells are then stabilised and undergo lineage expansion which is driven by IL-23(73). The release of Il-17 in turn stimulates recruitment of neutrophils and production of further proinflammatory mediators (74). This Th17 pathway is separate from the Th1 pathway (75). The important role of IL23 in driving inflammation has been confirmed in murine knock out studies with the IL 23 $^{p19-/-}$ animal failing to develop colitis (76). Genome studies have also implicated the IL23 receptor in disease as previously discussed.

In order to maintain homeostasis, immunoregulatory T cells are also produced. Under the influence of forkhead transcription factor Foxp3, naïve T cells differentiate into protective regulatory T cells (77, 78). These secrete IL10 and TGFβ and play a vital role in limiting T helper cell activity (78). Regulatory T cells (Tregs)are produced from the same lineage that produces effector Th17 cells (77). Tight regulation of T cell differentiation is therefore needed.

The differentiation of T cells is orchestrated by a multitude of cytokines. Cells of the innate immune system are the source of a number of these. These can be regulated by the intestinal epithelium (79-81). In health, the intestinal epithelium and mucosal dendritic cells act as antigen presenting cells expressing a number of pattern recognition receptors (81). These have evolved to recognise a repertoire of conserved microbial motifs (78). Stimulation of these receptors promotes either immune tolerance or if the biding motif is recognised as a danger signal' induces the differentiation of naïve T cells into T helper cells which then orchestrate the adaptive immune response (78). Closely related to this is the induction of NFκB-mediated responses from the epithelium resulting in the release of inflammatory cytokines, chemokines and other antimicrobial peptides (82). The role of NFκB and the epithelium will be discussed in subsequent sections.

The loss of immune tolerance or the ability for auto regulation underpin the immunological defects in IBD. Dendritic cell function depends on pathogen pattern recognition receptors (PPR). Mutations in these, in particular NOD2, are associated with Crohn's disease (36). A subset of dendritic cells with a more pro-inflammatory phenotype have also been found in Crohn's disease (83). Loss of immune tolerance is also seen with reduced regulatory T cells in the mucosa and peripheral blood of IBD patients (84). The importance of Tregs is further demonstrated in germ free immune deficient mice which develop colitis if

injected with TH1 cells. This can be ameliorated if the donor TH1 cells are primed in the presence of regulatory T cells (85).

The immunology of inflammatory bowel disease is thus multifaceted and interwoven in complex networks. Due to its importance, modification of the immune response has been the central focus for current IBD treatment.

1.6 Current Treatment

Therapeutic options to modulate the immune response in IBD can be grouped into one of four categories; these are used singly or in combination.

1.6.1 5 Amino Salicylic Acid Compounds

5 aminosalicylic acid (5ASA) compounds are often used as a first line treatment in an acute flare of UC. They are also used as maintenance therapy (10). The active moiety, 5ASA is poorly absorbed and therefore acts topically within the intestine, with mucosal concentrations correlating to clinical effectiveness (86). It is thought these drugs work by modulating epithelial cytokine expression (87), and by the induction of anti-inflammatory peroxisome proliferator-activated receptor-γ within the epithelium (88). Although effective in UC, there is little evidence to support their use in CD (10, 89). Their relative lack of efficacy in CD may be due to their topical mechanism of action resulting in a lack of effect on the deeper mucosal inflammation which occurs in CD.

1.6.2 Corticosteroids

Corticosteroids have formed the mainstay of treatment for a wide range of inflammatory and allergic conditions for a number of years. Their anti-inflammatory effect is mediated by binding to a cytoplasmic glucocorticoid receptor. Upon translocation to the nucleus this receptor binds to DNA directly inducing anti-inflammatory gene transcription, or directly bind to the pro inflammatory transcription factors, activator protein 1 (AP-1) and NFκB, repressing inflammatory cytokine transcription (90). Corticosteroids are recommended as an initial treatment in moderate to severe relapses for both UC and CD (10).

1.6.3 Thiopurines

Thiopurines include azathioprine and mercaptopurine. Both are extensively metabolised to the active molecules, thioguanine nucleotides. Due to their similarity to the nucleotide base guanine, these are incorporated into leucocyte DNA, resulting in DNA strand breakage and dysfunctional lymphocytes. Additionally apoptosis is enhanced via the inhibition of the leucocyte apoptotic inhibitor Rac1 (91). Some reports suggest de novo purine nucleotide synthesis is also inhibited (92). All of these mechanisms lead to immunosuppression. Thiopurines are currently recommended for use in the maintenance of remission in both UC and CD (10), however their mechanisms of action mean that there is a delay of 6- 8 weeks before they become effective.

1.6.4 Tumour Necrosis Factor Alpha Antagonists

Until the mid-1990's IBD treatment was limited to a 5ASA, steroids, or a thiopurine. This limited repertoire was revolutionised in 1999 with the introduction of the TNFα antagonist, Infliximab. This is a full length, chimeric IgG1 monoclonal antibody directed against TNFα. Already used in the treatment of rheumatoid arthritis, a case report of the successful treatment of a severe Crohn's patient in 1993 (93) ignited interest in the possibility of using this drug for IBD. The landmark ACCENT1 study followed, validating its benefit in Crohn's disease patients. This showed that Infliximab was more effective than placebo at inducing and maintaining clinical remission over a year in those who initially responded (94). Further studies followed reporting benefit for a second TNF antagonist, Adalimumab (95, 96). Later, studies investigated these drugs in UC, again reporting benefit for both Infliximab and Adalimumab (4, 97, 98). These drugs are currently recommended for the induction of remission in CD and UC, and the maintenance of remission in CD (10).

1.7 New treatments in development

Until recently the anti-TNF agents represented the only major advance in treatment strategy for IBD patients. However there are now a number of new treatments in development, with clinical trials currently reporting interim or final results.

1.7.1.1 Vedolizumab

The first of these new drugs to be approved for use in patients with UC and CD is Vedolizumab (99, 100). Circulating leucocyte migration into intestinal tissue is mediated via the interaction between a cell surface glycoprotein ($\alpha_4\beta_7$ integrin) on lymphocytes (101), and a mucosal receptor, known as mucosal addressin cell adhesion molecule 1 (102). Vedolizumab is a humanised monoclonal antibody directed against the $\alpha_4\beta_7$ integrin therefore inhibiting intestine specific leucocyte migration. In placebo controlled trials Vedolizumab induced a clinical response at week 6 in 31.4% of CD patients and 47.1% of UC patients compared to 25% with placebo (103, 104). At 1 year, clinical remission was achieved in approximately 40% of patients (103, 104). Longer term data is now emerging in CD. Depending on the dosing interval, 39- 46% of patients maintained clinical remission over 3 years (105).

1.7.1.2 Ustekinumab

A second study which has recently started reporting its final results is the UNITY programme. This has been investigating the use of Ustekinumab, a human monoclonal antibody directed against the p40 subunit of cytokines interleukin 23 and interleukin 12(106). IL 23 has been shown to play an important role in chronic intestinal inflammation in mouse models of inflammation (49, 107). In humans multiple genome studies have shown an association between polymorphisms in the IL23 gene and CD (47, 108). IL12 and 23 have also been shown to be important mediators of the Th1 or Th17 mediated inflammatory response (109).

This study has shown Ustekinumab to be more effective than placebo at meeting the predefined study endpoint. At week 6, 33.7% of patients receiving Ustekinumab demonstrated a clinical response compared to 21.5% receiving placebo (106). Clinical remission was achieved in 20% of patients receiving active drug at week 8 compared to 7.3% receiving placebo (106). After 44 weeks of treatment around half of patients treated with drug were still in remission (48.8% 3 monthly dosing, 53.1% 2 monthly dosing) compared to a third of placebo treated patients (110).

1.7.1.3 Risankizumab

Risankizumab is a human monoclonal antibody to the p19 subunit of interleukin 23. This is still being trialled in patient cohorts. In a recent phase 2 dose ranging study with moderate to severe Crohn's disease 41 % achieved a clinical response with the highest dose after 3 months, with 36% in remission compared to placebo (111). Somewhat unusually for a clinical study, this trial also included the robust endpoint of deep remission. Deep remission was defined as the resolution of clinical symptoms and healing of the intestinal mucosa with the absence of inflammation on histology (111). Perhaps it not surprising that no-one achieved this in the placebo group and was only achieved in 12.2% of the highest dosing treatment arm (111).

1.7.1.4 Janus Kinase Inhibitors – Tofacitinib, Filgotinib

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) is an evolutionary conserved intracellular mechanism by which cytokines and other extracellular signals are transduced to the nucleus, thus influencing intracellular processes. A number of JAK/STAT receptors have been identified, some of which are dysregulated in IBD (112). These pathways are now emerging as potential therapeutic targets.

Tofactinib is an oral Janus kinase 1 and 3 inhibitor. In small phase 2 dose ranging CD studies clinical remission at week 8 was induced in 43% compared to placebo (36.7%), with a clinical response achieved in 70% (113) However it should be noted that in this cohort, 54-62% of patients in the placebo arm also achieved a clinical response at week 8. At week 26, clinical response was achieved in half of patients receiving the higher dose, with 41% also in remission (113). A similar proportion sustained their remission throughout the relatively short follow-on study period (114). It should again be noted that 35% in the placebo arm also demonstrated a response at week 26, with 21.5% also having sustained remission (114). In studies with ulcerative colitis, clinical response was attained 59.9% of patients with 18.5% achieving remission at week 8. Mucosal healing was included as an endpoint in the UC study and at week 8 was achieved by 31.1% in the active treatment arm compared to 15.6% receiving placebo (115).

Similar results are reported with Filgotinib, a selective oral JAK 1 inhibitor. After 6 months half of patients were in clinical remission compared to 23% with placebo (116).

1.7.1.5 Anti-IL-6

The ANDANTE study group have recently reported the results of their phase 2 induction study for CD patients using a humanised monoclonal antibody directed against interleukin 6 (117). Statistically significant results are reported for remission at week 12 with 27.4% in the 50 mg treatment arm achieving this compared to 10.9% in the placebo group. A significant difference in clinical response at week 8 and 12 compared to placebo was also reported (49.3% week 8 vs 30.6% placebo, 47.7% week 12 vs 28.6 % placebo). However, when a more robust definition of clinical response is used, although there is a trend towards greater response in the drug arm, this does not attain significance (117).

1.8 Treatment Strategies

Early treatment algorithms recommended a step wise approach to therapy starting initially with a 5ASA or short course of steroid. Repeated flares would then prompt the introduction of a second line immunosuppressant in an attempt to maintain remission and prevent repeated corticosteroid exposure. However in recent years there has been a paradigm shift towards the so called 'top down' approach. In this strategy anti-TNFα antagonists and thiopurines are used earlier in the disease course, often in combination. This has been shown to be more effective than either drug alone in controlling inflammation (118). Treatment outcomes have also shifted, with clinicians now striving to achieve healing of the mucosa, as opposed to just symptom control. This is in response to the growing appreciation that resolving inflammation and promoting healing of the intestinal epithelium provides greater longer term benefit and remission periods for patients (119).

1.9 Treatment Outcomes

Despite the introduction of the TNFα antagonists and the use of combination treatment, outcomes with current therapies remain disappointing. Only one third of UC patients will achieve remission with an oral 5ASA and of those around 2/3rds will be maintained in remission. The results are better if topical therapy is applied, although this will only be effective for left-sided disease. TNFα

antagonists achieve remission in 38% of patients with a similar number remaining in remission (120).

For Crohn's disease, results are similar. Although corticosteroids are effective at inducing remission with 83% responding, they are unsuitable for maintenance therapy. Thiopurines are ineffective at inducing remission due to their long onset of action. Despite this it is reported they maintain remission in around 2/3rd of patients. Unfortunately up to 20% are unable to tolerate them due to side effects. Induction of remission with TNF antagonists is only achieved in 39-59 %, with just under half of those remaining in remission a year later (120).

Having achieved remission, our current treatments are not effective at maintaining this when they are discontinued, suggesting they only suppress and do not resolve the underlying disease process. For example in the STORI study it was shown that approximately 50% of patients with CD who are clinically well at the time of stopping infliximab will experience a relapse within 1 year of stopping the drug (121). Similarly 39% of CD and 26% of UC patients relapse within 2 years of stopping a thiopurine (122).

1.10 Mucosal Healing

In the era of treat to target and top down strategies, clinical remission is no longer viewed as an acceptable endpoint. This has been replaced by mucosal healing and the concept of deep remission, in which symptoms have improved, the mucosa is healed and there is an absence of inflammatory cell infiltrate (123). Mucosal healing is now starting to be included as primary endpoint in trial design (124).

The benefit of mucosal healing has been demonstrated in numerous studies. In the era before the introduction of the anti -TNFα treatment, patients with active disease and deep mucosal inflammation at index colonoscopy were shown to have an increased risk over time, of a more severe disease course and an increased need for colectomy compared to patients with more quiescent mucosal inflammation. The cumulative probability of colectomy after 1 year with severe mucosal disease was 20% rising to 42% at 8 years (125). A Norwegian cohort study during the early 1990's, again before biologic therapy, reported 20% of patients without mucosal healing progressing to surgery within 5 years, compared to 11% who achieved healing of the mucosa (126). Post resection

outcomes in the early 1990's showed early return of mucosal lesions predicting early clinical recurrence (127).

Mucosal healing also predicts long term steroid free remission, with 70% of patients maintaining steroid free remission for 4 years if healing is achieved early in the disease course (119). Similar results were seen in the Norwegian cohort (126). It follows that steroid free remission, and healing are associated with lower rates of hospitalisation and sustained clinical remission (128, 129). All of these outcomes are supported by a recent pooled meta-analysis, with both partial and complete mucosal healing showing benefit; the most benefit being achieved with complete mucosal healing (130).

Mucosal healing does not only keep a patient well, it allows successful withdrawal of certain treatment. We know from the landmark STORI trial that in patients who are in clinical remission on an anti-TNF agent, just under half of them will relapse within 1 year of stopping, even if they continue with an immunomodulator (121). Data from a recent meta-analysis of 27 studies support this. If the decision to discontinue an anti-TNFα agent is based solely on clinical remission at the time of discontinuation, the average clinical relapse rate is 42% at 1 year (131). However, if mucosal healing is also achieved, the 1 year relapse falls significantly to 26% (131).

Despite the known benefits, the attainment of complete mucosal healing, similar to the induction of clinical remission, remains a significant challenge with the treatment options currently available. The historic backbone of IBD treatment, corticosteroids, have no role to play in inducing healing. Although they produce symptomatic benefit, an endoscopic study after 7 weeks of steroid therapy revealed only 29% of those in clinical remission also had healing of their mucosa. (132).

The thiopurines are broadly similar in their response. Reported rates of mucosal healing range from 16-60% depending on the study design and cohort demographics (118, 133-136) .

This just leaves the anti-TNFα agents which have revolutionised treatment strategies for IBD, as discussed previously. Despite this, their ability to induce

and sustain mucosal healing remains somewhat disappointing. The EXTEND study was the first clinical trial to include mucosal healing as an endpoint. After 52 weeks treatment with Adalimumab mucosal healing was achieved in just 24% (124). Treatment with Infliximab produced mucosal healing in 45% (129). More recently the SONIC trial compared infliximab monotherapy with thiopurine monotherapy or combination. At week 26 mucosal healing occurred in 30% of those receiving infliximab. Addition of azathioprine produced only a modest improvement in this figure to 44% (118).

1.11 IBD Conclusion

Inflammatory bowel disease is a lifelong chronic condition for which there is no cure. Although progress is being made, the precise aetiology remains elusive. The most recent publication takes the number of identified IBD associated genetic risk loci to 200 (25) but it is known that simply having 'susceptibility genetics' does not equate to developing IBD. There are additional factors involved in the development of IBD than solely genetic differences. There is now an increased understanding of the complex roles of the various T cell subsets in disease pathogenesis. Additionally we know that the intestinal microbiome is altered in IBD. Whether this change is causative or simply an effect of the proinflammatory environment within the mucosa remains an issue of debate. Despite our increase in knowledge many questions remain. We cannot definitively identify the cause of IBD, nor can we 'cure' it. The introduction of the anti-TNF agents in the late 1990's heralded a frameshift in treatment approach and their introduction has undoubtedly brought considerable benefit for patients. Unfortunately a significant proportion do not respond, with remission only attained in around half of patients (120). Even in those who do respond, symptoms often return when the drug is discontinued (121), suggesting we are only suppressing disease activity. It is disappointing that despite advances in the understanding of disease pathophysiology, medical treatment still offers only 40-60% response rates. Based on the emerging trial data, the newer agents in development are not fairing much better (103, 111, 114, 117).

All of the current treatments, including the novel agents, exert their effect by modifying the aberrant immune response. As our knowledge of the complex inflammatory signalling increases, so too does the variety of cytokines and receptors which can be targeted for drug development. A central component of

the innate mucosal immune system which is often overlooked is the epithelial barrier.

There is a growing appreciation of the importance of mucosal healing if lasting remission is to be achieved (119). Clinical trials are now starting to include deep remission (absence of symptoms and mucosal healing) as a primary endpoint (111), recognising its importance. To fully understand mucosal healing and the role the epithelium plays in IBD development, there needs to be an appreciation of not only how the epithelium maintains a physical barrier within the intestine, but also how its innate immune response is regulated. Understanding how mucosal disruption occurs and how it could be prevented in IBD may open up avenues for the development of the new treatments. These could be used in combination with existing approaches to bring greater effectiveness and the lasting clinical benefit that IBD patients so desperately need.

1.12 Intestinal Epithelium

The epithelium refers to a single layer of cells, lining hollow cavities and glands in the intestine, comprising mainly non-ciliated columnar epithelial cells (137). Critical for the absorption of water and nutrients, this rapidly renewing cellular monolayer is continuously exposed to the microbial milieu of the intestinal lumen. This presents numerous challenges. Nutrients must be selectively absorbed, whilst the passage of potentially damaging antigens restricted. In addition the epithelium undertakes vital innate immune functions acting as a first point of contact for luminal antigens and orchestrating the subsequent innate immune response (138). An intact barrier is therefore essential for normal physiological function and immune homeostasis of the intestine. Central to the barrier function is the coordinated expression and interaction of numerous junctional proteins including circumferential tight and adherens junctions which regulate the passage of potentially damaging macromolecules into the submucosa. Dysfunction of the intestinal epithelium with disruption of barrier integrity is implicated in a number of diseases, including inflammatory bowel disease (139). Before this can be appreciated it is first necessary to understand

the individual constituents of the barrier and how they support the structure and function of the barrier.

The basic structure of the cell junctions is shown in Figure 1.2.

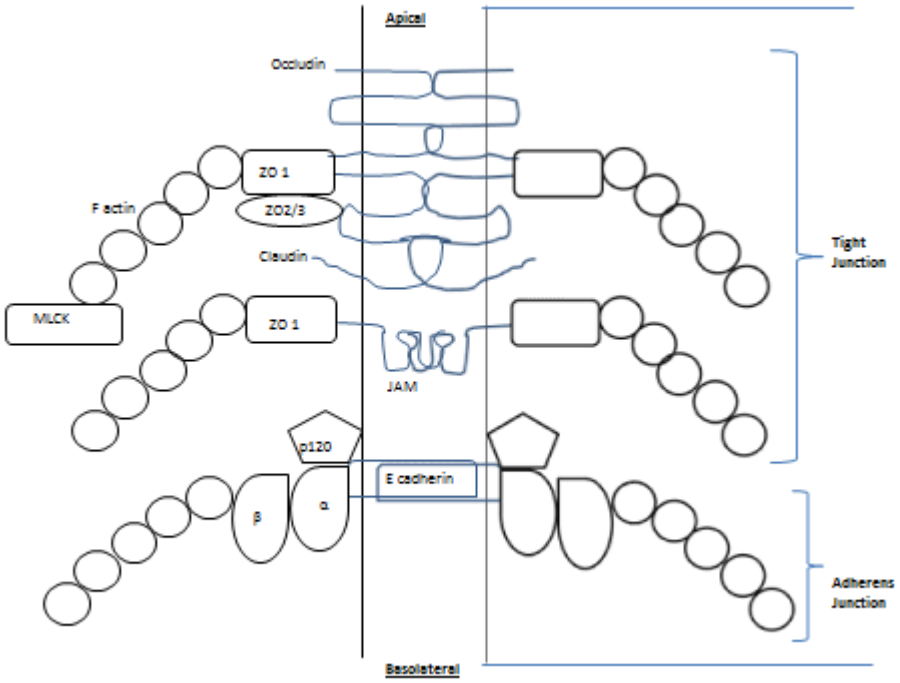


Figure 1.2: Simplified diagram of the tight and adherens cell junctions

ZO1/2/3 = Zonula Occludens, p120 = p120 catenin, MCLK =myosin light chain kinase, JAM = junctional adhesion molecule, α =α catenin, β= β catenin

1.13 Tight Junctions

Critical to any barrier is the effective sealing of gaps between cells. However, aside from forming a physical barrier, the epithelium must be able to absorb water and solutes. This is achieved via one of 2 pathways. The transcellular pathway utilises apical co transporter proteins to move solutes into the cell often against a concentration gradient, to maintain homeostasis in cells and tissues. The absorption of glucose via the sodium glucose co-transporter 1 is an example of this (140). The second pathway is the paracellular route which allows selective

diffusion between cells, particularly when rapid co-ordinated large solute fluxes are required, including from the ileal lumen during digestion and from the proximal kidney tubule during solute reabsorption. For a luminal barrier to be maintained and the transcellular absorption route to function, the paracellular space must therefore possess a regulated seal. Dysregulation of the barrier has the potential to disrupt the concentration gradients which the transcellular route relies on for its function (141) and to lead to leakage of plasma proteins and inappropriate antigen exposure in the mucosa. Functional control of the barrier is provided by a circumferential network of protein strands collectively known as the apical junction complex. This complex comprises of the tight junction and adherens junctions (141-147).

Tight junctions represent the most apical contacts in epithelial sheets and were first identified in the 1960's as a network of protein strands encircling each cell (148). There are thought to be at least 40 individual proteins intricately linked into the formation and function of these junctions (142) giving structural integrity to tissues in addition to creating a polarised selectively permeable barrier (146). Fundamental to the formation of the barrier and its permeability function are the transmembrane proteins occludin and claudin. These play important roles in regulating the paracellular pathway.

The tight junction proteins limit solute and ion flux along the paracellular pathway. This is more permeable than the transcellular pathway and is thus a major determinant of epithelial permeability (141, 146). Permeability across the junction is via leak or pore channels and is determined by size and charge selectivity of solutes. The leak pathway allows the passage of macromolecules from 6 to 60 Angstroms (149) including some proteins, and can be modified by inflammatory cytokines (150-156). It is thought that occludin plays a role in this with studies using occludin mutants showing this pathway can be regulated independently of ion permeability (157, 158). In contrast the pore pathway, which is facilitated by the claudins, permits the passage of ions up to 4 Angstroms in size (159). Ionic permeability varies from tissue to tissue depending on the expression of the claudins. It too can be modified by inflammatory cytokines(160). The barrier is permselective, with ion and solute permeability varying widely amongst different tissue types (142, 145).

Other integral membrane proteins include the tricellulins and junctional adhesion molecules (145, 161, 162). These are linked to cytoplasmic scaffolding proteins

including the PDZ domain containing zonula occludens proteins ZO-1, ZO-2, ZO-3, the MAGI proteins 1, 2 and 3 and associated cell signalling and cell cycle regulators. (145, 161, 163). Aside from their barrier function, the tight junction has a diverse range of functions including cell proliferation, signalling and transcription regulation (142, 143, 145).

The function and structure of the junction is tightly regulated and intricately linked to the phosphorylation or dephosphorylation of its constituent proteins (164). Modulation of the perijunctional actin ring by myosin light chain kinase has also been shown to have a key role in junction stability (165, 166)

1.13.1 Occludin

First identified in 1993 by Furuse et al, Occludin is 65kD tetraspan protein consisting of 504 amino acid residues (167). The protein has 4 transmembrane domains, each 21-24 amino acids long with 2 extracellular loops. The N and C terminals reside in the cytoplasm. The C terminal has numerous binding domains allowing interaction with scaffolding and signalling proteins (168).

Initial studies in occludin null fibroblasts suggested occludin was essential for cell adhesion, and was directly involved in the formation of intercellular contacts (169). It was suggested that the first extracellular loop was critical for this function (169). In support of this freeze fracture studies found occludin flanking tight junction strands on the protoplasmic and exoplasmic fracture faces, as would be expected of an integral junction protein (170). Further studies in Sf9 insect cells supported this. When these cells were transfected with chick occludin the paracellular spaces were observed to collapse and be crossed by intramembranous stands. These strands localised antibodies to occludin suggesting it is an integral membrane protein (171).

The essential role of occludin in tight junction assembly has since been disputed. Initial work in occludin deficient embryonic stem cells has shown they are able to form cellular contacts which are morphologically identical to the tight junctions found in occludin containing cells (172). In a rat TNBS/ethanol colitis model, inflamed areas had less occludin but normal tight junction morphology on freeze fracture replicas. Importantly there was no reduction in tight junction strand number suggesting that loss of occludin does not affect the structure of the junction (173). Finally an occludin knockout mouse was generated. In keeping with the findings from earlier studies these animals had morphologically normal tight junctions. Strand number was not reduced and barrier function, as

measured by transepithelial resistance was not perturbed. However there were a number of phenotypic abnormalities. Most striking was these animals were growth retarded. Histological examination revealed gastritis and mucosal inflammation first occurring at 10 weeks of age and increasing in severity with advancing age (174). It therefore seems that although occludin is dispensable as far as the structural formation of the junction is concerned it is clearly important in regulating cellular homeostasis and innate immune regulation.

The importance of occludin lies in its role in supporting barrier function and via its links to adaptor and scaffolding protein, facilitating changes at the barrier to initiate intracellular signalling cascades (175). By introducing full length and truncated occludin into Xenopus embryos it was shown that the truncated occludin resulted in a less intact barrier with increased paracellular permeability, despite being appropriately localised to the tight junction (176). Similar results were seen when COOH truncated occludin was introduced into Madine Darby Canine Kidney (MDCK) cells (158). Truncating the N terminal of the protein is also detrimental. Although localised appropriately to the tight junction, paracellular permeability was adversely affected (177). Further studies have reinforced the regulation of the paracellular 'leak' pathway by occludin with depletion or knockdown causing an increase in macromolecule flux across the junction (157, 158, 177-179). The effect on the ionic 'pore' pathway is more contentious, with some studies showing an increase in permeability (177, 179) and others finding no effect (157, 158, 178). This appears to depend on the cell line used.

Overexpression of occludin results in a tightening of the barrier (180). This was shown when chick occludin was overexpressed in MDCK cells, resulting in a reduction in mannitol flux and an increase in transepithelial resistance (180). The second extracellular loop of occludin is responsible for these properties (181).

Aside from contributing to barrier properties of the junction, occludin also functions as a linker protein with the numerous scaffolding proteins around the junction (175). This is achieved via a cytoplasmic COOH domain which contains an amino acid sequence compatible with the linker proteins (182). The first of these to be shown to associate was Zonula Occludens 1 (168). ZO-1 directs occludin to the junction. Loss of ZO-1 results in mis-localisation of occludin from the junction, suggesting it is a necessary binding partner for stability of occludin at the junction (169, 180). The N-terminal of ZO-1 associates with occludin

whilst the C-terminal associates with F actin, thus providing a link between the tight junction and the active cytoskeleton (183). This link is important as the actin cytoskeleton can be modified in disease with subsequent effects on the barrier (184). Other proteins associating with occludin include ZO-2 , ZO-3 (185) and the junctional adhesion molecule (JAM) (175). Similar to the zonula occludin protein JAM also assists in localising occludin to the junction (186). Although not directly associated as a linker protein loss of occludin also alters the expression of claudins, another constituent of the tight junction. Reductions in the expression of claudins 1 and 8 with over expression of 2, 4 and 15 have been reported in mouse and cell models (178, 179).

Under normal conditions, occludin is mobile at the tight junction with 70% available for trafficking from the cytoplasm to the junction. Only 30% of total occludin is within the junction (187). This implies that tight junction characteristics can be modulated. Phosphorylation or ubiquitination of amino acid residues within occludin are ways in which this is achieved (188).

Phosphorylated occludin appears to be preferentially expressed within the junction. This phosphorylation is mediated by a wide variety of kinases including Src, Rho, mitogen activated protein and protein kinase C. Numerous cell models have demonstrated that phosphorylation of the tyrosine residue destabilises occludin from the junction, whilst threonine phosphorylation promotes occludin inclusion and stabilisation (164).

Ubiquitination flags proteins for degradation via the proteasome (189). Occludin is ubiquitinated in vivo by the E3 ligase ITCH and removed from the junction (190). More recently it has also been shown to be deubiquitinated by another ubiquitin editing protein - Tumour necrosis factor alpha-induced protein 3(TNFAIP3) (191). The protein was shown to inhibit LPS induced occludin loss from mouse intestinal tight junctions therefore adding to junction stability.

There is now evidence to suggest that phosphorylation and ubiquitination are closely related. Using a primary bovine retinal endothelial cell model treated with vascular endothelial growth factor, it was found that occludin phosphorylation occurred at serine residue 490 prior to subsequent ubiquitination and loss from the tight junction. Reduced interaction of occludin with other junction proteins following serine phosphorylation are postulated as a potential mechanism, allowing the subsequent ubiquitination (192).

1.13.2 Claudins

The first Claudin was identified in 1998.(193) Integral to the regulation of paracellular ion permeability, the 18-25 kilodalton (kDa) claudin family comprise at least 27 proteins of variable co expression patterns and properties (194, 195). They have 4 hydrophilic transmembrane domains and 2 extracellular loops, which appear to be involved in the homophilic/herterophilic interactions needed for tight junction formation. The WWCC motif in the first extracellular loop is conserved amongst members of the claudin family. The cytoplasmic tail however, although relatively constant in length, varies in sequence amongst the claudins. This tail contains several phosphorylation sites and a PDZ binding domain which enables interaction with intracellular scaffolding proteins. Claudin expression is tissue specific with combinations of claudin pairs accounting for the ion and water permeability characteristics of epithelial cells in different tissue types (142, 145, 194-196). They are also affected in disease states. Of relevance TNFα has been shown to increase expression of the pore forming claudin 2 in ulcerative colitis (197-199), along with claudin 1(200). Other claudins (3, 4, 5, 7 and 8) have been shown to be down regulated in inflammation with a concomitant increase in epithelial permeability (139, 198, 201).

1.13.3 Zonula Occludins

There are three Zonula Occludin proteins (ZO-1-3) which form the tight junction membrane-associated guanylate kinase-like homologue family. Each of these contains three PDZ domains, one SH3 domain and one GUK domain (202). They function as scaffolding proteins and as recently mentioned interact with occludin and the claudins to stabilise the tight junction complex (143). They are essential for localising the claudins to the lateral membranes and are also important in the localisation of occludin (169, 180). ZO-1 appears to be the most important of these. Knockout studies have shown a complete lack of tight junction formation in ZO1 deficient cell lines.(202, 203) During physiological cell shedding, ZO1 redistributes towards the apical and basolateral cell membrane, thus allowing the tight junction to dissociate (144).

1.14 Adherens Junction

The presence of adherens junctions is considered a defining feature of epithelial sheets. The junction is built upon the calcium dependent homophilic interactions between adjacent E-cadherin molecules (204-206). E-cadherin belongs to the classical cadherin family and is characterised by an extracellular domain with 5-6 calcium binding cadherin repeats and a cytoplasmic domain that is required for adhesive functions. Whilst the extracellular domain allows calcium-mediated binding to other E-cadherin molecules, the cytoplasmic domain is highly conserved and mediates binding to cytoplasmic proteins involved in junction stability and cell signalling (207).

1.14.1 p120 Catenin

p120ctn is an armadillo repeat protein that closely associates with junctional E-cadherin and is essential for intracellular signalling and cadherin stability. It functions by binding to the juxtamembrane domain of the cytoplasmic cadherin tail, thus stabilising E cadherin at the cell surface (208-215). The strength of the junction is in part dependent on the amount of E cadherin at the cell surface. In E-cadherin deficient cell lines p120ctn is poorly localised. This contrasts with normal epithelial cells in which E-cadherin and p120ctn co localise in a stoichiometric complex resulting in stronger cellular adhesion(215).The amount of E-cadherin available at the cell membrane is influenced by Numb promoted clathrin pit endocytosis. This enables E-cadherin to be internalised and the junction recycled. p120 plays a key role in regulating this process. Through its association with the membrane proximal region of E-cadherin it masks the dileucine motifs which bind Numb, thereby inhibiting this mechanism (216). Studies in p120ctn knockout mice show that the absence of p120ctn results in intestinal inflammation and disruption of the adherens junction with reduced E-catenin. All animals developed bloody diarrhoea similar to that seen in human inflammatory bowel disease.(214) Longer term studies show the development of a barrier defect and neutrophil influx with incomplete p120 knockout mice and eventually tumour development (217). Additionally p120ctn may play an important role in the regulation of inflammatory responses by suppressing NFκB(218).

1.14.2 Beta catenin

B-catenin is also an armadillo repeat protein and forms a complex with E cadherin at the cell membrane. The integrity of the cadherin-catenin complex is negatively regulated by the phosphorylation of β-catenin by receptor tyrosine kinases and cytoplasmic tyrosine kinases (Fer, Fyn, Yes, Src) (219) REF? Smad7 prevents its dephosphorylation and stabilises the E-cadherin-β catenin complex at the cell membrane(220). Once released from E-cadherin, it translocates to the nucleus and functions in the canonical wnt pathway(221)

1.14.3 Alpha Catenin

α-catenin binds indirectly to E-cadherin via β-catenin and links to the actin cytoskeleton. α-E-catenin is the most prevalent in mammalian epithelial sheets(208) . Loss of function mutations in α- catenin have been found in a variety of human tumours(208), whilst cells deficient in α- catenin but replete in E cadherin failed to adhere into epithelial sheets(222, 223).

1.15 Intestinal permeability

As discussed, intestinal permeability is regulated by the proteins of the tight junction, principally occludin and the claudins. (160). Defects in the epithelial barrier and their junctions, leading to alterations in intestinal permeability have been associated with a variety of human diseases including bacterial infections, coeliac disease and inflammatory bowel disease (141, 224-227).

In Crohn's disease, increased intestinal permeability is a well-documented observation. Using a variety of molecular probes, intestinal permeability has repeatedly been shown to be increased in both active small bowel and colonic disease (228, 229). This leads to the question of whether the barrier defects are intrinsic to the pathogenesis of the disease or a consequence of the disease process itself. Although the precise aetiology remains uncertain, it is plausible that barrier dysfunction represents an early event in the disease process in susceptible individuals and is not solely associated with inflammatory activity. This was first observed in a small cohort of sixteen patients by Cosellas et al in

1986. Compared to healthy controls, all patients with active Crohn's disease had increased intestinal permeability. This persisted even when the inflammation then resolved (230). These findings have been repeated more recently in larger cohorts with increased permeability still evident in the absence of any clinical symptoms (231, 232). A persisting barrier defect also predicts the risk of clinical relapse with 44-70% of patients who are clinically well at the time of measurement subsequently developing active disease within 1 year (231, 232). In a single case report, a patient was reported to have increased intestinal permeability 8 years before the onset of Crohn's disease (233) suggesting barrier defects can be early events in the disease process. All of these studies have evaluated permeability using the measured absorption of low molecular saccharides or Ethylenediaminetetraacetic acid (EDTA.) Due to advances in techniques it is now possible to directly visualise these barrier defects in patients in real time. Using fluorescein dye Kiesslich et al observed increased fluorescein leakage between epithelial cells in patients with inactive Crohn's disease compared to healthy controls. In keeping with earlier studies, this increased permeability was predictive of clinical relapse within 12 months (234).

In an attempt to further understand the significance of the barrier defects, permeability studies have been extended to include first degree relatives and non-related partners of Crohn's disease patients. Between 10-54% of first degree relatives have increased permeability in the absence of clinical features of Crohn's disease (235-239), suggesting an inherited component.

Animal studies have supported the notion that increased permeability can, and does precede the onset of clinical symptoms. Transgenic mice over expressing myosin light chain kinase (discussed in next section) were found to have structurally normal tight junctions, but increased intestinal permeability. Although these mice developed normally and were free from any symptoms suggestive of disease, they were found to have subclinical mucosal inflammation. This was characterised by a 40% increase in $CD4^+$ lymphocytes in the lamina propria and a mild increase in neutrophils. (240). By 6 weeks of age mucosal elevated transcripts for cytokines TNFα, IFNγ, TGFβ and IL-10, indicative of a Th1 responses, were observed (240). Further work using the same model has shown that that the onset of colitis in animals with pre-existing barrier defects is more rapid and the disease more severe. In the group studied only 20% of animals were alive at day 84, compared to 50% in the control group without pre-existing barrier dysfunction (240).

A summary of studies is shown in table 1.4.

Table 1.4: Summary of intestinal permeability studies in Crohn's disease patients, first degree relatives and healthy controls.

		Numbers			Permeability compared to control	
Author /year	**Crohns Disease**	**Relative**	**Controls**	**Probe**	**Crohn's Disease**	**1st Degree Relative**
Hollander etal 1986	11	32	17	PEG400	Increased	Increased
Katz etal 1989	25	29	41	L/M	Increased	normal
				Rhamnose	normal	normal
				Mannitol	normal	normal
Ainsworth etal 1989	15	20	28	51-Cr EDTA	Increased	Normal
Teahon etal 1992	28	32	25	PEG-400	normal	normal
				51-Cr EDTA	Increased	normal
Ruttenberg etal 1992	45	30	31	PEG-400	Normal	Normal
May etal 1993	36	38	31	L/M	Increased	normal
Wyatt etal 1993	72	0	30	L/M	Increased	/
Peeters etal 1994	19	0	10	51-Cr-EDTA	Increased	
				PEG-400	Normal	/
Yacyshyn etal 1995	15	13	10	L/M	Increased	Increased
Peeters etal 1997	25	67	36	L/M	Increased	Increased
Soderholm etal 1999	39	34	29	L/M	Increased	Normal
Buhner etal 2006	128	129	96	L/M	Increased	Increased
Gerova etal 2011	32	0	25	Iohexol	Increased	/

l/m = lacose/mannitol

PEG400 = polyetheylene gylcol

References : (232, 235-239, 241-247)

1.16 Tight junction regulation health and disease

Tight junctions are dynamic structures, modulated by numerous stimuli (145, 248). This modulation can occur as part of a normal physiological response or as the result of inflammatory cytokine release in disease. It is known that intestinal permeability is a feature of IBD. There is now convincing evidence that the tight junction complex is also disrupted. Freeze fracture images of epithelial cell junctions in IBD patients show discontinuous, disrupted tight junction strands (139). The number of strands forming a junction is reduced compared to healthy individuals with redistribution of proteins. (249) The proteins occludin and claudins 4, 5 and 8 are reduced and localised away from the junction (139, 197, 198). Claudin 2, a pore forming claudin, has also been consistently shown to be over expressed in IBD (139, 198). These structural changes alter the physical properties of the barrier, with decreases in electrical impedance evident (139, 198), indicating an increase in permeability. Interestingly, this does not happen during physiological cell shedding, during which junctional proteins are reorganised to the lateral cell membrane without any detriment to the barrier (250).

The mechanisms of tight junction modulation are complex (184). An established mechanism is contraction of the actin cytoskeleton (205).This was first identified with the discovery that the tight junction structure is linked to the actin cytoskeleton (251). The identification of connections between these 2 structures suggested that the alterations in their morphology may also be linked (251). This was then proved to be correct with the discovery that phosphorylation of myosin II regulatory light chain (MLC) by myosin light chain kinase (MLCK) leads to contraction of the perijunctional actin ring causing tight junction disruption and increased permeability (252, 253). This mechanism has physiological relevance. Absorption of glucose by the apical sodium glucose cotransporter, SGLT1, causes MLCK activation, MLC phosphorylation and increased epithelial permeability (253). In this way glucose is absorbed via both transcellular and paracellular pathways leading to increased uptake.

The MLCK pathway is relevant in disease also, particularly IBD. In murine overexpression models animals with constitutively active MLCK had increased paracellular permeability indicative of tight junction disruption (240). Increased expression of MLCK is observed in the mucosa of active Crohn's disease and ulcerative colitis, suggestive of a role the disease pathogenesis (254).

Linked to this are the proinflammatory cytokines. Cytokine mediated alterations to paracellular permeability and junctional protein re-localisation have been observed in a number of ex vivo culture models of IBD.(153). The pro inflammatory cytokine TNFα is of particular importance. Elevated in the serum and intestinal mucosa of IBD patients, particularly Crohn's, but also UC this has been shown to disrupt the epithelial barrier, in part by activation of MLCK and MLC phosphorylation, although additional mechanisms are postulated. MLCK is inducible by TNFα (255). In the Caco 2 intestinal epithelia cell line TNFα induces transcription of NFκB which in turn upregulates the MCLK promotor. This causes actin myosin contraction, disruption of the junction and removal of ZO-1(155, 256, 257). TNFα may also increase permeability by reducing tight junction complexity. Freeze fracture images of epithelial monolayers after TNF treatment had shown reduced tight junction strand number compared to control. It should be noted that this study found no reduction in ZO-1,a although this may reflect the use of different cell line (HT29) (258). TNFα can also act directly on tight junction proteins. Loss of occludin from the junction in response to this cytokine is mediated by caveolar dependent endocytosis, causing removal and internalisation (259). Over expression of occludin diminishes the detrimental effect of TNFα on barrier permeability, adding support to this (259). Reduction of occludin is also possible by direct repression of occludin transcription via the inhibition of the occludin promoter (260). Tight junction complexity can also be simplified. Actin depolymerisation and gap formation have been observed, although this was in a bovine endothelial cell line and may therefore not be directly applicable to the actions of TNFα in the intestine (261).

One of the classic and often embarrassing symptoms for patients with IBD is diarrhoea. The pathophysiology of this is related in part to a leak flux mechanism whereby a leaky barrier allows an ingress of antigens causing inflammation, and an efflux of water, causing diarrhoea (262). Using a mouse T cell activation model Clayburgh et al provides a link between TNFα, barrier dysfunction and diarrhoea (263). Using an antiCD3 antibody T cell activation was induced. The resulting TNFα release increased transcription of MLCK, MLC phosphorylation and caused disruption of the junction. Occludin was localised away from the junction and all animals developed an increase in paracellular permeability (263). Studies in the epithelial Caco 2 cell line and a mouse model also show that TNF results in accelerated degradation of occludin with a concomitant increase in permeability (264). In human IBD the use of TNF neutralising antibodies can ameliorate

diarrhoea in some patients therefore providing further evidence of the detrimental effects of TNF on the barrier (120).

Interferon gamma (IFNγ), increased in the mucosa of IBD patients also causes disruption of the tight junction. Its mechanism of action appears separate from that of TNFα (153). T84 monolayers exposed to this cytokine show alterations in cell morphology, with disrupted cellular organisation and reduced cell height (151). Subtle disruptions to F actin strands are also seen with reductions in actin at the apical junction (265, 266). Internalisation of tight junction proteins is also observed, Further studies in T84 cells have revealed there is reduced transcription of ZO-1, coupled with increase protein turnover (266). Reductions in this important scaffolding protein then contribute to the destabilisation of the junction. Redistribution of occludin and claudins 1 and 4 are also seen with these proteins being internalised and trafficked away from the junction (152, 267). Unlike TNF which induced caveolin mediated endocytosis, IFNγ resulted in internalisation by micropinocytosis into F actin containing vacuoles. These vacuoles were not associated with the cytoplasmic lysosymes meaning the redistributed proteins are not necessarily degraded and may be recycled back to the junction(152, 267, 268). These processes are dependent on the phosphorylation of myosin light chain. Unlike TNF which induces MCLK, the phosphorylation was induced by the small GTPase RhoA, which is transcriptionally activated by IFNγ (268). RhoA in turn increases expression of a Rho associated kinase (ROCK) which then phosphorylates and actives MLC (268). Rho A in itself is also capable of remodelling actin fibres (269). This helps to explain how IFNγ can cause actin remodelling and contraction of the cytoskeleton. Precisely how the micropinocytosis is achieved remains uncertain(153).

Capable of individually causing barrier dysfunction, the combined effects of IFNγ and TNFα are synergistic (151, 255). Interestingly IFNγ primes epithelial cell cultures for TNFα responses by inducing the expression of the TNFR2 cell surface receptor (270). Although each cytokine acts individually via different mechanisms, they converge at the phosphorylation of MLC and disruption of the actin cytoskeleton.

Numerous other cytokines have been shown to have detrimental effects on barrier integrity. The pro inflammatory cytokine IL1β is increased within the inflamed mucosa of IBD patients(271) and plays an important role in mediating chronic inflammation(272) Cell culture studies have shown that it is also able to increase barrier permeability via NFκB mediated activation of myosin light chain

kinase (273, 274). IL13 results in increased permeability associated with increased expression of claudin 2(198). IL6 has also been associated with increases in barrier permeability, although the results are variable and dependent on the cell line used(156).

1.17 Immune functions of the epithelium

The human intestine contains vast quantities of bacteria with individuals harbouring up to 160 different species (275). These commensal bacteria are essential for health, facilitating the digestion of food, metabolism of vitamins and contributing to the development of the mucosal immune system (276). Enteropathogenic bacteria compete with the commensal flora for space and nutrients, therefore a robust commensal population can help prevent enteric infection (277). To sustain this, the mucosal immune system is faced with the complex task of promoting tolerance to commensal organisms, whilst being able to mount an appropriate rapid response to invasive pathogens. The intestinal epithelium plays a fundamental role in this. In contact with both the lamina propria and the luminal microbiota, the epithelium is much more than just a physical barrier between these domains. As the first point of contact for luminal bacteria, the epithelium is a critical component of the innate immune response (276).

This is elegantly demonstrated by the B cell deficient mouse, which does not produce Immunoglobulin A (IgA), the major immunoglobulin at mucosal surfaces (278). Exposure of the intestinal epithelium to the luminal microbiota results in a change of epithelial gene expression, with upregulation of immune response pathways and downregulation of those concerned with metabolism. These responses are not seen in the absence of bacteria, with or without the presence of IgA B cells suggesting the intestinal epithelium is able to respond to the local environment and is capable of recognising and responding to luminal antigens itself (279).

To facilitate this epithelial cells express innate immune receptors, termed pattern recognition receptors (PRR's) although the repertoire is less diverse than those

expressed by professional immune cells (276). These receptors are contained on the cell membrane or within the cytosol and can be categorised as follows: Toll Like Receptors (TLR) are an evolutionary conserved diverse group of receptors which can be further sub categorised into those found predominantly at the cell surface(TLR4, TLR2, TLR5) and those contained within endosomes (TLR3, TLR7, TLR9) (81). They respond to highly conserved ligands known as pathogen associated microbial patterns found on bacteria, viruses and parasites (81, 276). Cytosolic receptors include NOD-like receptors and retinoic acid inducible gene I like receptors (RIG) (81). NOD like receptors respond to bacterial peptidoglycan ligands, whilst RIG receptors recognise viral RNA (81).

As previously discussed the presence of a tight junction complex permits the formation of a polarised epithelial layer with distinct apical and basolateral domains. In turn this allows the compartmentalisation of PRR's thus allowing specific transcriptional responses depending upon the location of the stimulated receptor. TLR5 is found only on the basolateral surface and therefore responsive to invasive pathogens (280). In contrast TLR9 is expressed on both apical and basal surfaces, with apical stimulation promoting a tolerogenic response to further stimulation. However stimulation of the basolateral receptor triggers a proinflammatory transcriptional response (281). Depending upon the stimulus, epithelial cells are able to directly secrete proinflammatory cytokines and chemokines including, but not limited to interleukin 8, TNFα, IL-6, TGF-β, IL-15 and MCP-1 (277, 282-284).

In addition to its own innate immune responses, the epithelium is also capable of conditioning the response of dendritic cells and intraepithelial lymphocytes contained within the lamina propria (137, 138, 283). Immature dendritic cells have no capacity for differentiating between commensal or pathogenic ligands (285) However, this is important if immune homeostasis is to be maintained. Another cell essential for immune homeostasis is the intraepithelial lymphocyte (IEL). These are numerous with one IEL for every 10-15 epithelial cells (286). Both of these cells are intimately linked to the epithelium which is dependent on the tight junction. IEL's have been observed to perform a sentry-like function, migrating along the subepithelium up to the tight junction, which is not broached (286). Similarly dendritic cells are able to open the tight junction allowing them to directly sample luminal antigens (287, 288). Both cell types have been found to interact directly with tight junction occludin, thus allowing them to function without disrupting the barrier (286, 287). Disruption of tight junction occludin is sufficient to impair the migration of IEL's (286).

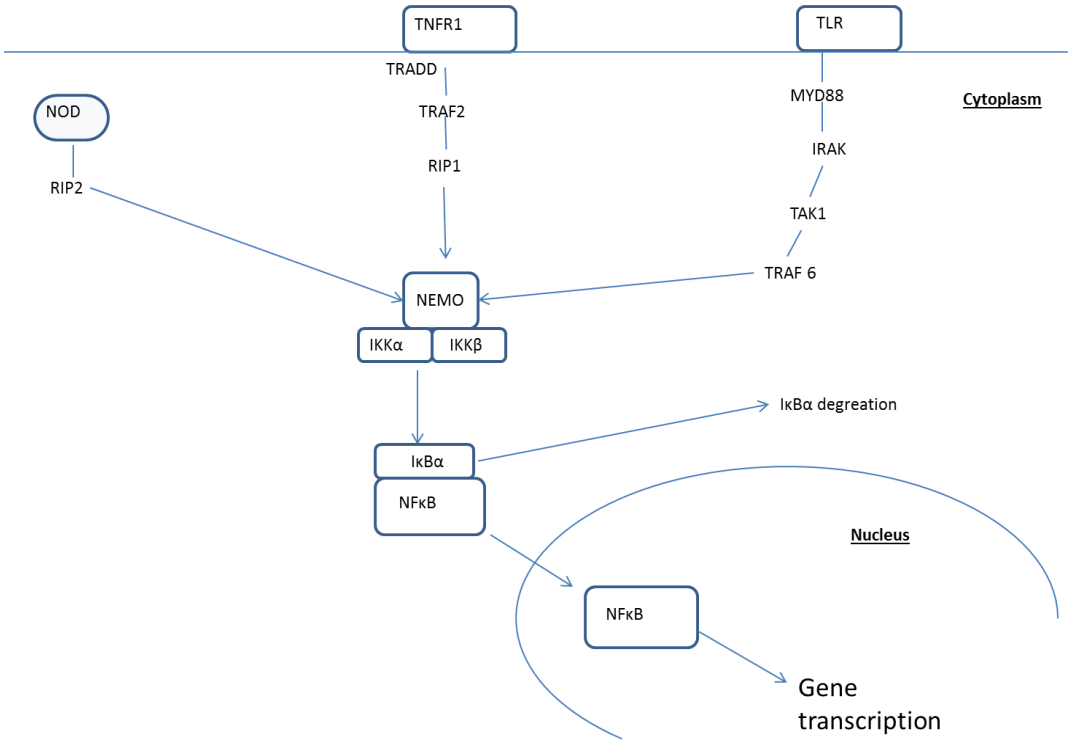
Due to the vast numbers of antigens that are encountered in the intestine, regulation of dendritic cell responses are vital for immune homeostasis, As well as facilitating luminal sampling by dendritic cells, the epithelium is also able to condition their response towards a more tolerogenic phenotype. Dendritic cells cultured with epithelial supernatants produce less TNFα (289) , more IL-10(290) and prime T cells towards Th2/Treg phenotypes (289, 290). This is mediated by the release of thymic stromal lymphopoietin (TSLP), retinoic acid and transforming growth factor beta (TGFβ) from epithelial cells (79, 80, 283, 289, 290). This is particularly evident in the colon which has a greater proportion of DC's able to promote Treg cell differentiation, despite the large bacterial antigenic loads encountered (291). In the absence of epithelial conditioning, dendritic cells become pro-inflammatory promoting Th1 T cell maturation (290).

Interestingly, a recent murine model of colitis has shown that inflammation does not develop if the intestinal epithelium is the only cell exposed to endogenous TNFα. This same model was also used to show that with an intact mucosal immune system, TNF production by the epithelium was sufficient to induce mesenchymal cell activation and mucosal inflammation (292). Together this data supports the notion that the epithelium and its responses to inflammatory cytokines or stimuli are critical in preventing or permitting inflammation to occur. One of the transcription factors integral to this, induced by TNFα and critical for the production of inflammatory response is NFκB.(82, 293, 294)

1.18 NFκB

Integral to epithelial immune responses is the transcription factor nuclear factor kappa beta (NFκB). Consisting of 5 proteins families (p65/RelA, c-Rel, Rel-B, p50 and p52) these form homodimers which bind to DNA sequences in the regions of genes which regulate transcription. In the unstimulated cell these proteins are inactive, forming a complex with members of the IκB family; IkBα and IkBβ in the cytoplasm (294). Upon stimulation of the appropriate cell receptor, adaptor molecules are recruited which lead to the phosphorylation and ubiquitination of the IKK complex. This contains 3 proteins IKKα, IKKβ and a regulatory IKKγ, otherwise known as NEMO (295). When stimulated the IKKα and β subunits facilitate the phosphorylation and subsequent degradation of the NFκB inhibitory

IkBα complex (294, 295). This allows the active NFκB subunits to translocate to the nucleus where they regulate gene transcription. An overview of the constituents of the canonical NFκB pathway is shown in Figure 1.3. For clarity only key pathways are shown. Individual phosphorylation and ubiquitination stages have been omitted intentionally. Full nomenclature of proteins is provided in the glossary.



Adapted from (296, 297)

Figure 1.3: Overview of canonical NFκB signalling pathway. Stimulation of the appropriate cell membrane receptor by its ligand leads to the phosphorylation and ubiquitination of the IKK complex. The IKKα and IKKβ subunits of this complex then facilitate the phosphorylation and degradation of IκBα from NFκB, allowing the NFκB subunits to locate to the nucleus where affect gene transcription

To maintain homeostasis within the epithelium, NFκB must be tightly regulated, with over or under activity resulting in inflammation (294). Increased NFκB is seen in the mucosa of IBD patients(293) suggesting it is involved in disease pathogenesis. It has been shown in murine colitis models, that blocking NFκB

pathways leads to resolution of colitis(298). Using a transgenic mouse with an epithelial specific over expression of a mutant super repressor version of IkBα (299), it was shown that inhibiting NFκB activity in response to T cell mediated immune activation limits intestinal permeability increases and occludin loss from the tight junction compared to wild type animals (300) However although this is an attractive idea, amelioration of NFκB also has potentially detrimental effects.

Mice lacking NEMO, a critical component of the NFKB pathway, are unable to mount an appropriate response to luminal antigens and develop severe colitis with reduced expression of antimicrobial peptides and increased sensitivity to TNFα induced apoptosis(301). If the adaptor molecule MYD88 is also absent, therefore inhibiting the TLR pathway, inflammation is also absent, suggesting the NFκB pathway is important in producing responses to bacterial antigens (301). Similar results are seen if IKKβ is absent. In this situation NFκB cannot be released from its inhibitor, and therefore shows reduced activity. This results in reduced TSLP expression from the epithelium, reduced conditioning of dendritic cells and initiation of a pro inflammatory Th1 response (302). NFκB activity via the TLR2 pathway also functions to stabilise the tight junction barrier and thus limit intestinal inflammation (303). Therefore it is essential that appropriate regulation of NFκB activation is achieved.

A failure to regulate the complex functions of the epithelium can lead to dysregulation of both its structure and immune functions leading to an increase in permeability and adaptive immune system activation. These features are seen in inflammatory bowel disease. The barrier and immune functions of the epithelium are closely interwoven, with disruptions in one potentially leading to disruption of the other. It is therefore essential that mechanisms ensuring homeostatic regulation of NFκB activity and barrier stability are maintained and regulated. (294). There are several inducible proteins which fulfil this function. One, which is of particular interest within the context of IBD is tumour necrosis factor alpha induced protein 3 (TNFAIP3).

1.19 TNFAIP3

The first line of defence against invading pathogens is the innate immune system. This response is initiated by the recognition of pathogen associated molecular patterns (PAMPs) located on invading organisms. These are detected via a variety

of surface and intracellular receptors including NOD like receptors, Toll like receptors (TLR) and RIG-I receptors. Upon stimulation these receptors trigger an intracellular signalling cascade which ultimately leads to the up regulation of NFκB and transcription of pro inflammatory genes. This allows the release of numerous cytokines and chemokines, propagating the immune response and facilitating the recruitment of T cells to the site of damage. In turn, T cell activation leads to stimulation of the adaptive immune system and a more sustained response. In order to prevent prolonged activation of the adaptive immune response and excessive tissue damage the innate immune system therefore needs to be tightly regulated. Tumour Necrosis Factor Alpha Inhibitory Protein 3 (TNFAIP3) is an important homeostatic regulator of the acute innate immune response, mediating its effect by control of NFκB responses. It plays a pivotal role in maintaining the homeostatic balance between appropriate immune activation and excessive tissue damage.

Tumour necrosis factor alpha inhibitory protein 3 (TNFAIP3) was first identified as a rapid TNFα responsive gene in human umbilical vein cells (304). Subsequently it has been shown to be expressed in numerous different cell types and in response to several other stimuli including lipopolysaccharide, CD40 and viral infection (305-307). In humans the gene is located on chromosome 6q23, producing an mRNA transcript 4KB in length with an open reading frame of 2370 nucleotides. This produces a 90kDa protein containing 790 amino acids (308). The gene is rapidly induced by TNFα with maximal transcription occurring one hour post induction (304). Under basal conditions transcription is initiated by the binding of RNA polymerase II and the general transcription factor TFIID to core promoter elements. These are recruited under the control of the transcription factor specificity protein 1 (309). However this complex is unable to initiate repeated rounds of transcription, resulting in low basal levels. In addition, elongation of the transcript is inhibited by a reduction in RNA polymerase II activity by DRB sensitivity inducing factor (DSIF) (310). This is under the control of another promoter complex known as the elongation inhibitory element (ELIE) which is composed of an E box and USF1 protein. Under conditions of TNF stimulation NFκB binds to κB response elements within the promoter and re-initiates multiple rounds of transcription rapidly increasing TNFAIP3 levels (310). In addition NFκB displaces USF1 from the promoter and gains control of DSIF from ELIE (311) thus also controlling transcript elongation. Under the control of NF-kB this permits RNA polymerase II to continue mRNA elongation, resulting in increased synthesis.

1.20 Structure and Function

TNFAIP3 modifies the ubiquitin status of key proteins in the NFκB cascade to have a regulatory effect. In particular TLR, NOD2, TNFR and CD40 mediated NFκB signalling pathways are regulated in this way (305, 312-314)

Polyubiquitination of substrates is an integral part of the NFκB signalling cascade (315, 316). Ubiquitin is a small protein found in all eukaryotic cells which has a role in the posttranslational modification of proteins. Via a multistep enzyme linked process of activation, conjugation and ligation, individual ubiquitin molecules are linked to substrate proteins to form polyubiquitin chains (315). The linkage between ubiquitin molecules takes place between the glycine residue of ubiquitin and one of the seven lysine residues from a substrate bound ubiquitin. The effect of ubiquitination depends on the lysine links within the chain. Lysine 48 (Lys 48) linked ubiquitin chains target a protein for degradation with in the proteasome. Lysine 63 linked chains allow the coordination of other signalling processes (317).

The dual effect of TNFAIP3 on ubiquitin stems from 2 distinct domains within the protein. The amino terminal of the protein contains an ovarian tumour domain (OTU) with a catalytic site composed of Cys103, His256, Asp70 amino acid residues (318). The cysteine residue preferentially cleaves Lys 48 and or Lys 63 polyubiquitin chains (312, 319) and may also inhibit E3 ligase interaction, thus limiting synthesis and addition of new ubiquitin chains (320)

The C terminal contains zinc finger (ZnF) domains which facilitate E3 ligase activity thus catalysing the addition of Lys 48 ubiquitin chains to substrates (319). This targets them for degradation. Although there are 7 zinc fingers in total, there is a degree of redundancy with a minimum of 3 domains being sufficient for catalytic activity (321). Of these ZnF 4 has been shown to be crucial (319, 322). This residue has 3 ubiquitin binding sites (322).

The results of early studies suggested that each domain of TNFAIP3 acted in isolation to either ubiquitinate or deubiquitinate its substrate. However more recent studies have suggested there may be dimerization of proteins and synergistic action between each functional domain (323).

As mentioned previously, TNFAIP3 regulates NFκB signalling driven by TNF, NOD TLR and CD40 receptors. The ubiquitin status of key substrates in each of these pathways is modified by TNFAIP3, there limiting further NFκB transcription. This is summarised in Figure 1.4. Important targets for TNFAIP3 as shown are RIP1 , TRAF 6 and RIP 2 in the TNF, TLR and NOD2 pathways respectively (312, 313, 319).

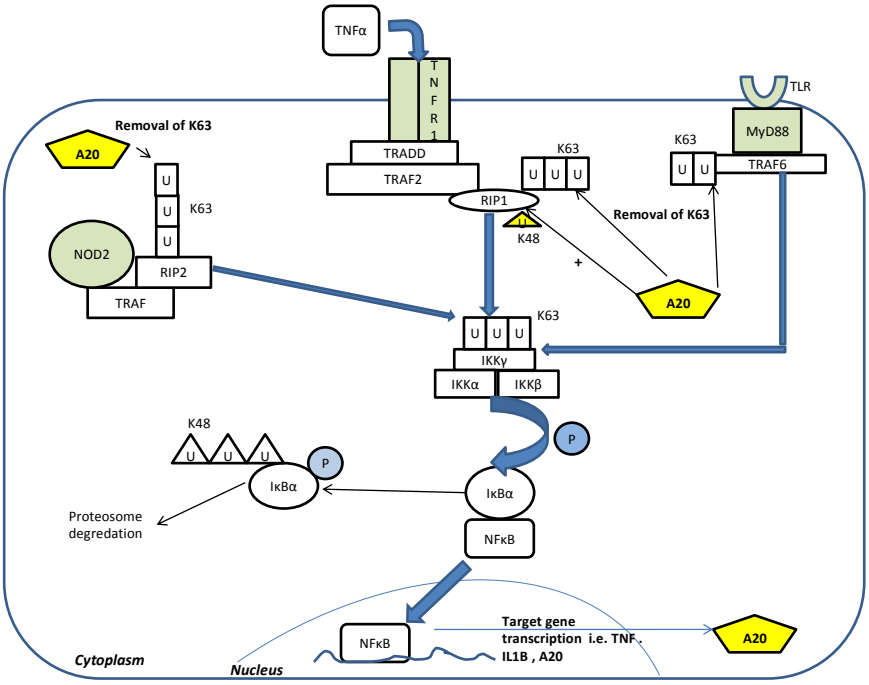


Figure 1.4: TNFAIP3 regulatory pathway.

Termination of NF-κB signalling by TNFA1P3 (A20). Removal of K63 ubiquitin chains from RIP2 (NOD complex), RIP1 (TNFα pathway) and TRAF6 (TLR pathway) prevents the K63 ubiquitination of the IKK complex and the subsequent K48 mediated degradation of IκBα. This prevents NF-κB translocation to the nucleus and gene transcription. Additionally TNFAIP3 mediates the degradation of RIP1 and Ubc15 via the addition of K48 ubiquitin chains.

In addition to its function regulating NFκB, TNFAIP3 also prevents TNF mediated cytotoxicity (324) and has recently been shown in a mouse model to stabilise occludin within the tight junction by deubiquitinating it (191).

1.21 TNFAIP3 and IBD

The biological importance of TNFAIP3 is highlighted by studies of TNFAIP3$^{-/-}$ mice. Although such mice develop and are born successfully, suggesting TNFAIP3 is not needed for prenatal development, they rapidly develop multi organ inflammation and premature death. This has been shown to driven by unregulated toll like receptor responses to commensal bacteria (325) .These mice are hypersensitive to even small doses of lipopolysaccharide (LPS) and TNF, displaying exaggerated NFκB responses (314). In such mice, the intestinal epithelium is also disrupted with the loss of occludin and increased intestinal permeability (191). In contrast TNFAIP3$^{+/+}$ mice mount appropriate NFκB responses and survive (314). Thus TNFAIP3 is vital for limiting inflammatory responses, particularly in response to commensal organisms. Further evidence is provided by the study of TNFAIP3$^{OTU/OTU}$ and TNAFIP3$^{zf4/zf4}$ mice which have a point mutation in the ovarian tumour or zinc finger domains respectively (323). In contrast to the TNFAIP3$^{-/-}$mouse, these animals survive and are grossly normal until at least 4 months of age. However when intestinal inflammation is induced with dextran sodium sulphate, these animals exhibit more inflammation with greater amounts of inflammatory cytokines (323).

The intestinal microbiome poses a significant challenge for the intestinal epithelium. The barrier formed by the epithelium must allow the absorption of water and nutrients whilst at the same time preventing translocation of bacterial antigens in the lamina propria, where they will encounter the cells of the innate and adaptive immune system. There is now a growing body of evidence to suggest that TNFAIP3 is of critical importance in maintaining this intestinal epithelial barrier function and that disruption of this may contribute to inflammatory bowel disease. Deletion of TNFAIP3, specifically within the intestinal epithelium (TNFAIP3^{IEC-KO}), results in mice which are normal at birth, but when challenged with dextran sulphate sodium (DSS) develop severe colitis. This does not resolve when the insult is removed (191, 324). Additionally these mice are hypersensitive to even sub-therapeutic doses of TNFα displaying profuse diarrhoea and inflammatory cytokine release with mortality in all animals (191). This is associated with higher circulating levels of the inflammatory cytokines MCP-1 and IL-6 following TNF stimulation with a greater number of apoptotic

cells compared to wild type mice. Concomitant deletion of the TNFR1 receptor negates these effects demonstrating that TNFAIP3 is important in restricting harmful TNF mediated signalling (324).

Permeability studies on explanted intestinal loops from TNFAIP $^{IEC-KO}$ mice reveals an increase in FITC dextran flux suggesting impairment of epithelial barrier function. There is also an associated loss of occludin from the apical cell surface which is not observed in mice overexpressing TNFAIP3(191). This suggests TNFAIP3 may act directly on tight junction proteins to support barrier function. Confirmation of this is provided from cell culture work showing that TNAFIP3 is able to deubiquitinate occludin, thus preventing its removal from the tight junction (191).

A picture therefore emerges of TNFAIP3 being critical for the stability of the epithelial barrier with protective effects mediated through the regulation of inflammatory signalling and tight junction stability. It follows that the loss of barrier function may allow ingress of bacterial antigens to come into contact with the lamina propria immune cells, further adding to the inflammatory response. Treating TNFAIP3^{IEC-KO} mice with broad spectrum antibiotics still results in extensive barrier damage and cellular apoptosis, but protects against the TNF lethality seen in mice not given antibiotics (324). This suggests that an intact epithelial barrier is needed to prevent a more sustained systemic inflammatory response. TNFAIP3's role in supporting the barrier and regulating the innate epithelial cytokine response is therefore critically important.

Removal of TNFAIP3 from the epithelium is detrimental. Conversely epithelial cell over expression protects against DSS-induced colitis, reduces the inflammatory response to LPS stimulation and reduces NF-kB activation (191, 326). Villin transgene TNFAIP3 mice recover more rapidly from a DSS insult, with less weight loss and preservation of barrier function (326)

In humans single nucleotide polymorphisms (SNPs) have been identified that define TNFAIP3 as a susceptibility gene for Crohn's Disease both in Caucasians (327-329) and African American populations (330). The mutation in the African population has been mapped to exon 3 where it causes reduced activity of the deubiquitinating domain, thus affecting the function of the TNFAIP3 protein (330). The functional significance of the other SNPs remains unknown.

In a separate cohort, intestinal biopsies from Crohn's disease patients show reduced TNFAIP3 mRNA expression from inflamed and non-inflamed mucosa

compared to healthy controls (331, 332).This lack of difference between inflamed and non-inflamed tissue suggests the reduced expression may be inherent in the disease pathogenesis and not simply indicative of the inflammatory phenotype. In this cohort the lowest expression levels of TNFAIP3 were found to be associated with more severe disease and a poor response to anti TNF therapy (331).

1.22 TNFAIP3 Summary

TNFAIP is an intriguing protein. Able to modify the ubiquitin status of other proteins it has an important function as a negative regulator of NFκB. This is critically important in locations such as the intestinal epithelium, which at the interface with luminal microbiota, must be able to tightly control its immune activity. Closely related to this is the importance of maintaining an intact epithelial barrier. Recent evidence is now suggesting that TNFAIP3 may be important here also by having a direct effect on the stability of the tight junction.

Perhaps unsurprisingly TNFAIP3 is now being associated with a number of human immune mediated diseases (333). IBD is no exception. However, although genome wide studies have shown an association (328) and reduced TNFAIP3 mRNA is seen within colonic tissue (331), the reasons why this is reduced in IBD remain unknown. In this context I became interested in a class of molecules called microRNA. These small inhibitory RNAs showed altered expression in disease (see below) and may provide some insight into alternative mechanisms impacting on TNFAIP3 expression and the control of innate immunity.

1.23 MicroRNA

The coding exons of the human genome account for only 1.2% of the entire sequence (334). In recent years there has been a growing interest in the non-coding genome, and the role this may play in epigenetic modification. These modifications may alter expression of the protein coding genes thus influencing normal physiology and disease pathogenesis. Two modifications in particular are providing key insights - DNA methylation and histone acetylation (335-338). However, non-coding RNA sequences are also emerging as key regulators of gene expression with both long and short variant's being identified (339). In particular,

short RNA sequences called MicroRNAs (miRNA) are emerging as a key regulators and are being implicated in a number of disease processes (340-343).

1.24 Biogenesis of MicroRNA

MicroRNAs are small 18-24 nucleotide, non-coding RNAs which regulate gene expression via the translational inhibition or degradation of their target messenger RNA (mRNA) (344). First identified in 1993 by Lee et al in Caenorhabditis Elegans (C.Elegans) (345) there are now 28645 identified miRNA in over 100 species [mirbase v21 accessed 15 Dec 2014, and checked August 2017](346). MicroRNA genes are located throughout the genome and have been found in the introns of coding genes, introns and exons of non-coding genes and in intergenic regions (347). They often share the same promoter as their host gene but in some cases can be transcribed independently (348).

MiRNAs are transcribed from the genome by RNA polymerase II to produce double stranded pri-miRNA transcripts with a stem loop structure (349). These are then cleaved in the nucleus by the RNase III enzyme Drosher (350) in conjunction with the microprocessor complex, DiGeorge syndrome chromosomal region 8 (DGCR8) (351). DGCR8 interacts with the pri-miRNA transcript allowing Drosha to cleave in the correct position producing a ~ 70 nucleotide stem loop pre- miRNA (351, 352). The pre-miRNA is then exported from the nucleus to the cytoplasm via exportin 5 (353) where the RNAse III enzyme, Dicer, undertakes a further cleavage to produce a mature ~22nt miRNA duplex. (354). This miRNA is loaded into an effector complex known as the RNA – induced silencing complex (RISC), which is formed from Dicer, Argonaute protein 2 and an RNA binding protein; human immunodeficiency virus I transactivating response RNA binding protein (TRBP) (355, 356). Within the complex it was initially thought Argonaute 2 cleaved the miRNA duplex into the mature single stranded microRNA which is retained within the complex and the second 'passenger' strand was discarded (357). However this thinking has now been revised with the recognition that both the upside 5' to 3' sequence and the downside 3' to 5' side of the stem loop can be used as miRNA (358, 359) with Argonaute3 involved in strand selection(360). Either can also be discarded. The opposite strands are different 5' to 3' sequences (even though complementary) and will target different genes. Therefore the mRNA repressed by a microRNA may differ depending on which strand of the stem loop is processed to the active strand.

This is summarised in figure 1.5:

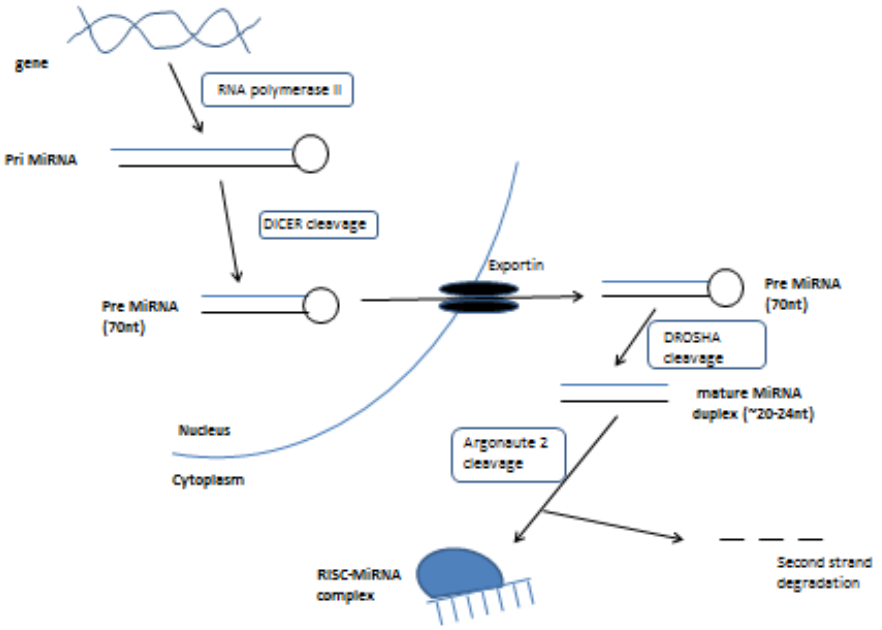


Figure 1.5: Summary of microRNA biogenesis.

As described in the text, a double stranded stem loop (pri-Mir) is cleaved to a ~70nucleotide stem loop (pre mir)and then exported from the nucleus. Further cleavage then occurs to produce a mature ~22 nucleotide duplex miRNA. This is loaded into the RISC complex where the active strand is selected.

The mature microRNA facilitates binding of the RISC complex to a mRNA via partial Watson Crick base pairing to complementary sequences in the 3' untranslated region (3'UTR) of the mRNA(345, 361, 362). This partial complementarity is centred on a short 6-8 nucleotide sequence in the 5' untranslated region (UTR) of the microRNA known as the seed sequence. To date 5 seed types have been identified (363).

Seed Types

8mer	Exact seed match to positions 2-8 of the mature miRNA followed by adenosine
7mer-8m	Exact match to positions 2-7 of the mature miRNA (seed + position 8)
7mer1A	Exact match to positions 2-7 of the mature miRNA followed by adenosine
6mer	Exact match to positions 2-7 of the mature miRNA
6mer offset	Exact match to positions 3-8 of the mature miRNA

This incomplete base pairing means that each microRNA seed will show partial complementarity to a number of 3'UTR mRNA sequences (361, 364). As result initial estimates suggested that microRNA -mRNA interaction may affect approximately 30% of all human genes (365). A recently revised estimation now suggests that >60% of all human protein coding genes contain sequences compatible with microRNA pairing (366).

1.25 MicroRNA Function

Although it is clear protein levels are affected by microRNA, there is no consensus on precisely how this is achieved (367). Initial work in C. Elegans found that although protein levels were repressed, mRNA expression was unaltered (368, 369) suggesting translational repression as opposed to degradation of the mRNA. This has also been observed in cell culture models (370, 371). Further work has suggested that repression can occur after protein translation has started with miRNA acting directly on actively translating polyribosomes (372). In other cases premature drop off of ribosomes from the elongating protein strand has been observed (373), or inhibition of translation initiation (374).

In contrast studies using C. Elegans and cell models have observed reductions in mRNA levels associated with microRNA function (375, 376) suggesting miRNA may also exert their effect via the degradation of mRNA. De-adenlylation of the mRNA strand by miRNA has been shown to lead to its rapid degradation in cell culture models (377). Argonaute proteins, which bind miRNA have been shown to localise to cytoplasmic structures called Processing bodies (P bodies) (378).

miRNA targeted mRNA has been found in these structures suggesting they may function to sequester mRNA or assist with their degradation/translational repression (379). Thus miRNA functions via a number of possible mechanisms (380).

1.26 Effects of microRNA

MicroRNAs have a wide repertoire of effects, determined principally by the mRNA that is being repressed. The effect of microRNA appears to be dependent upon the cellular context in which it is expressed with organ specific knockout studies resulting in distinct phenotypes (381). They are crucial in normal embryonic development with the failure of embryos from Dicer knock-out mice to develop beyond day 7 of gestation attesting to this (382). Additionally they appear to control a range of developmental and cellular metabolic functions (345, 383-385, 386). More recently they been gaining emerging importance as key regulators in immune cell development and differentiation (341, 387, 388). Binding of a ligand to its immune cell receptor induces the transcription of specific microRNAs which then regulate the cellular immune responses (340, 341).

Up to 37 % of the known microRNAs exist as clusters within the genome (389) with 51 clusters currently identified (390). These clusters act in a coordinated manner to regulate a network of proteins (391) with the most highly expressed microRNA being found at the start of the cluster (392). Generally the effects of a microRNA on protein expression are subtle (361). In this way microRNAs can be thought of as homeostatic regulators, 'fine tuning' protein expression and cellular responses.

It follows that disruption to this homeostatic control may lead to aberrant physiological responses and disease phenotypes. Given their roles in immune cell function, they have been implicated in a number of autoimmune / inflammation mediated diseases in a diverse range of tissues, including rheumatoid arthritis, systemic erythematosus, diabetes and inflammatory bowel disease (340, 341, 393, 394). Dysregulation of microRNA networks have also been found in a variety of haematological and solid organ malignancies (390, 395). Of particular interest is their role in epithelial -mesenchymal transition and the development of metastatic malignancy.

Epithelial –mesenchymal transition (EMT) is a reversible embryonic programme which allows a partial or complete transition between two cellular phenotypes; the stationary epithelial phenotype, and the more mobile mesenchymal. EMT is crucial in normal development but is also associated with the development of malignancy and metastatic disease. A number of microRNAs have now been implicated in this (396) Some of these including miR200, miR 9 and miR23a repress the essential junctional protein E- cadherin in a variety of different tumours. (397-402). This ability to manipulate cell adhesion in malignancy suggests that microRNAs may also function as regulators of epithelial barriers in other diseases. Although vital in all tissues, given its diverse range of functions, maintenance of the epithelial barrier in the intestine is of particular importance.

1.27 Intestinal Epithelial Barrier

MicroRNAs are critical to the normal development and polarisation of the intestinal epithelium. One way of determining their importance is to remove them from the tissue of interest. This has been done by producing a Dicer 1 $^{loxp/loxp}$;Villin Cre mouse which lacks microRNA within the intestinal epithelium . These mice have a structurally disorganised epithelium with the loss of key junctional proteins. The epithelium is dysfunctional with a significant increase in intestinal permeability thus highlighting the importance of microRNA for the normal development and function of the intestine (403).

Determining the role of individual microRNA is more challenging although data is now beginning to emerge. MicroRNAs have been shown to affect the integrity of the intestinal epithelial barrier in several disease phenotypes. In irritable bowel syndrome microRNA 29a is overexpressed in both blood microvesicles and colonic mucosa, compared to healthy controls. This has been shown to repress the translation of glutamate synthase, resulting in an increase in intestinal permeability (404). A second study by the same group in a larger patient cohort also found an increase in intestinal permeability associated with an over expression of miR 29a and 29b (405). In further work these microRNAs were also shown to repress the tight junction protein claudin 1 and the NFκB regulator nuclear-kB-repressing factor (NKRF). In a murine model, deletion of these microRNA improved junction permeability with preserved claudin 1 and NKRF expression (405).

MicroRNAs have also been linked to intestinal barrier dysfunction in pancreatitis, alcohol induced liver disease and Human Immunodeficiency Virus infection(406-408) suggesting microRNA dysregulation can in occur tissue separate from the primary pathology.

A summary of microRNA identified to repress key tight junction proteins is shown in **Table 1.5.** Table adapted from (409).

Table 1.5: Currently known tight junction proteins affected by microRNA

Tight Junction Protein	MicroRNA	Reference
Claudin 1	miR-155-5p miR-874	(410, 411)
Claudin 6	miR-7a miR-218	(412)
Claudin 10	miR-204	(413)
Claudin 16	miR- 595 miR-874 miR-204	(413, 414)
Claudin 19	miR-204	(413)
Occludin	miR122a miR-212a miR-874 miR- 429	(264, 409, 411, 415)
ZO-1	miR-105 miR-212a	(406, 416)
ZO-2	miR-203	(414)
Rho -A	miR-155-5p miR 185-3p	(407, 417)
Rho-B	miR-21a	(418)

1.28 MicroRNA and Epithelial Barrier in IBD

Specifically in IBD several microRNAs have been investigated. MiRNA 21 has been found to be overexpressed in colonic mucosal biopsies from several CD and UC cohorts (392, 419-421). MiRNA $21^{-/-}$ knock out mice have less severe colitis when exposed to DSS with reduced weight loss and a later onset of diarrhoea compared to wild type animals. Intestinal permeability is reduced compared to wild type, with Rho-related GTP-binding protein (RhoB) expression preserved with the epithelium (422). Studies in Caco 2 cells have replicated these findings with overexpression of miR21 increasing monolayer permeability. Reduced RhoB protein (Ras Homolog Family Member B) levels were also observed (421). However the authors reported association between mir 21 and RhoB is based purely on the observed expression levels of each. Direct evidence of association between miRNA 21 and the 3'UTR of RhoB mRNA is not provided. Therefore although these experiments suggest miRNA21 plays a role in stabilising the barrier the precise mechanism by which it does this is not certain.

More direct evidence of microRNA regulation of the barrier is provided by Ye and colleagues. They have shown in a Caco 2 cell line and subsequently a mouse model, that TNF α promotes the expression of miRNA 122 in intestinal epithelia. Via its interaction with complementary sequences in the 3'UTR of the tight junction protein occludin mRNA, miRNA122 reduces translation of the tight occludin, resulting in an increase in intestinal permeability. These changes could be reversed via the inhibition of miRNA122 with an antisense oligonucleotide (264).

MicroRNA 223 is elevated in the intestinal mucosa of IBD patients and induced by IL-23 (423). The tight junction protein claudin 8 has complementary base pairing in its 3'UTR for this miRNA. In a Caco 2 epithelial model, over expression of miR 223 disrupted the tight junction with reductions in claudin8 and a decrease in transepithelial resistance (423).

MiRNA 150 has also been shown to have effects on the barrier, potential via apoptosis. Initial studies in a DSS mouse model of colitis identified this microRNA as one of several that were induced during inflammation. DSS treatment was found to induce miRNA155 and reduce levels of its predicted protein target c-Myb, involved in the apoptosis pathway (424). These findings were confirmed in a colonic epithelial cell line where overexpression of miRNA150 resulted in reduced c-Myb protein, and increased cell apoptosis (424). The relevance to human

disease was demonstrated with mucosal samples from active UC also having increased miR150 and reduced c-Myb protein levels (424).

1.29 Inflammatory Bowel Disease and MicroRNA

In addition to potential effects on the epithelial barrier over recent years there has been a growing interest in the potential roles microRNAs may play in inflammatory bowel disease in general. As previously discussed expression of some microRNAs have been shown to be altered in IBD with effects on the intestinal barrier. Other studies have shown potential pathways regulating adaptive immune responses via T cell differentiation (425).

Innate immunity responses are also regulated. Relevant to IBD, the autophagy gene ATG16L1 is regulated in 2 microRNA cell culture systems. Overexpression of miR 106b and 142-3p reduced ATG16L1 protein and consequently autophagy responses (426, 427) . NOD2 can also be reregulated by miRNA. Expression of this important protein can be repressed by miRNA122 reducing NOD and leading to inhibited LPS induced apoptosis (428). Although undertaken in a cell line this study provides some insight and suggests how microRNA may affect NOD2 function. MiRNA192 also reduces NOD2 (429). This may be relevant to IBD as the rs313550 single nucleotide polymorphism in the 3'UTR of NOD2 reduces the ability of miRNA192 to inhibit via its binding site within this 3'UTR.(429). In dendritic cells microRNA are induced by NOD2. Induction of miRNA29 functions to limit IL23 and IL17 cytokine release, both key proinflammatory mediators (430). Polymorphism in the NOD2 gene alters this balance. Dendritic cells homozygous for the 10078sinC mutation or compound heterozygous for any of the known NOD mutations fail to induce miR29 and thus have a pro-inflammatory phenotype (430). Further miRNA-SNP interactions have been implicated in CD, with a variant of the autophagy gene, immunity-related GTPase family, M (IRGM), having an altered miRNA binding site for miR 196 leading to altered function and strengthening the concept of misregulation of miRNA in CD (431).

In addition several groups have now also published microRNA profiling data from the mucosal tissue and serum of UC and CD cohorts. Using microarray techniques to interrogate mucosal biopsies and peripheral blood samples from

these cohorts, differential expression of microRNAs has been reported between IBD patients and healthy controls, as well as between active and inactive disease groups (419, 420, 432–434). Table 1.6 summarises the findings from these studies.

Table 1.6: Summary of microRNA studies in IBD

Sample	Phenotype	Control Group	Increased miR	Decreased miR	Reference
Colon pinch biopsy	Active UC	Healthy	16, 21, 23a, 23b, 24, 26a, 29a, 126, 195, 199a, 203, Let-7f	19b, 192, 320, 375, 422b, 629	(420)
Colon pinch biopsy	Inactive UC	Healthy	21, 23a, 23b, 26a, 29a, 126, 195, 199a, Let 7f	19b, 422b, 629	(420)
Colon pinch biopsy	Active UC	Inactive UC	200b	16, 21, 24, 126, 203	(420)
Colon pinch biopsy	Active CD	Healthy	23b, 106a, 191	19b, 629	(434)
Ileum pinch biopsy	Active CD	Healthy	16, 21, 223 594	-	(434)
Colon pinch biopsy	Active UC	Healthy	7, 31, 135b 29a, 29b, 126, 127-3p, 324-3p	188-5p, 215, 320a, 346	(419)
Colon pinch biopsy	Inactive UC	Healthy	196a, 29a, 29b. 126, 127-3p 324-3p	188-5p, 215, 320a, 346	(419)
Sample	**Phenotype**	**Control Group**	**Increased miR**	**Decreased miR**	**Reference**
Colon pinch Biopsy	Active CD	Healthy	9, 126, 130a, 181c, 375, 21,22, 29c, 31, 106a, 146a, 146b-5p, 150, 26a, 29b, 30b, 34c-5p, 126, 127-3p, 133b, 155, 196a, 324-3p	-	(419)

Colon pinch biopsy	Inactive CD	Healthy	9, 30a, 30c, 223, 26a, 29b, 30b, 34c-5p, 126, 127-3p, 133b, 155, 196a, 324-3p,21, 22, 29c, 31, 106a, 146a, 146b-5p, 150	-	(419)
Colon Pinch biopsy	Active UC	Healthy	31, 27a, 665, 363, 193b, 503, 29b, 1273d, 146b, 99b	196b, 192, 141, 378a, 1973, 200a, 429, 196b, 192,10b, 215, 200b, 200a, 196a, 194, 335, 10b	(435)
Colon Pinch Biopsy	Active UC	Inactive UC	424, 138	338	(435)
Colon Pinch Biopsy	Pooled active and inactive	Pooled healthy and inactive	650, 223, 708, 155, 150, 146b, 29b-2, 212, 584, 551b, 21, 146a,let-7i	422, 147b, 3201, 378a, 194, 378c, 375, 200b, 4284, 200c	(435)
Peripheral blood	Active CD	Healthy	16, 23a, 28-5p, 29a, 106a, 107, 126, 191, 199a-5p, 200c, 362-3p 532-3p	19b, 21, 31, 143, 151-5p, 155, 223, 422b	(432)
Sample	**Phenotype**	**Control Group**	**Increased miR**	**Decreased miR**	**Reference**
Peripheral Blood	Active UC	Healthy	16, 21, 28-5p, 143, 151-5p, 155, 199a-5p,	19b, 23a, 29a, 31, 106a, 107, 126, 191, 200c, 223, 362-3p, 422b 532-3p	(432)
Peripheral blood	Active CD	Healthy	199p-5a, 362-3p, 340, 532-3p	149	(433)
Peripheral blood	Active UC	Healthy	28-5p, 151-5p, 199a-5p,	505	(433)

			340, 103-2 362-3p, 532-3p		
Peripheral blood	Active UC	Active CD	28-5p, 103-2, 149, 340 151-5p, 532-5p	505	(433)

Although interesting, these studies provide little insight in the mechanistic role microRNAs play in the disease pathogenesis. There are also a number of flaws in the methodology used which makes direct comparison between studies difficult. Principally there is little homogeneity between groups. In particular control groups are not well defined, with biopsies taken from un-characterised patients, those with polyps or functional abdominal pain. Although histologically normal, in those with abdominal pain this does not exclude irritable bowel syndrome (IBS) as a diagnosis. MicroRNA have been shown to dysregulated in IBS with adverse effects on the intestinal epithelium (404, 405). This will therefore confound the results of these studies. There is also a wide variation in the medications being taken by the included patients in a cohort, ranging from none to potent anti-TNF agents. This is again a confounding factor. Medication usage has been shown to alter microRNA expression (436). Although not necessarily an issue, these results have not been stratified according to medication usage.

In studies utilising intestinal biopsies, the location of the biopsy is often not well defined. A further difficulty arises due to the heterogeneous cell population. A colonic pinch biopsy will contain multiple cell types in varying abundance (enterocytes, lymphocytes, neutrophils goblet cells etc.). It is therefore difficult to know with any degree of certainty if the expressed microRNAs are from all cell types within the sample or cell specific. MicroRNA's are important in immune cell differentiation and function (340, 425). An inflamed biopsy will contain an abundance of these further adding to the confusion. Fundamental questions remain unanswered: Where are the mucosal microRNAs originating from and what is their function? A similar issue arises from the study of peripheral blood samples with the additional difficultly of determining if the circulating microRNA is contributing to the disease process directly or simply reflects microRNA activity at a distant site.

To resolve these issues microRNA study needs to be undertaken in carefully controlled, well characterised populations with groups appropriately controlled

for medication usage and biopsy location. Clearly defined healthy controls are also essential if the results are to be meaningfully interpreted.

1.30 Conclusion

It is clear that the epithelial barrier is dysfunctional in inflammatory bowel disease. This is intricately linked with an aberrant immune response, driven by the transcription factor NFκB. Regulation of TNF driven NFκB responses is essential if the integrity of epithelial barrier is to be maintained and immune responses appropriately terminated. TNFAIP3 being one such regulator of NFκB shows altered expression in Crohn's disease in addition to being located within a GWAS IBD susceptibility locus. However the reason for reduced expression remains unknown.

MicroRNA have also been shown to be differentially expressed in IBD. Some insights into their function have been provided however their cell specific expression and specific effects on epithelial barrier function remain largely unknown. Focussed cell specific analysis is needed to provide insights into their function, particularly within the intestinal epithelium.

By determining how epithelial cells regulate their tight junctions and NFκB responses it may be possible to identify a new therapeutic target. An epithelial specific approach is therefore needed.

1.31 Hypothesis and Aims

This thesis will test the following hypothesis:

Specific MicroRNA are altered in the colonic epithelium in Crohn's disease and modify the expression of the TNFAIP3 gene.

The principle aims of this work are:

- Identify microRNA predicted to repress TNFAIP3 and investigate if they show differential expression within the colonic epithelium in Crohn's disease.
- Characterise the expression of TNFAIP3 protein in colonic epithelium in Crohn's disease
- Test the hypothesis that microRNA mediated altered expression of TNFAIP3 adversely effects barrier properties of the epithelium and immune regulation

Chapter 2:

Materials and Methods

Chapter 2: Methods

2.1 Ethics

The use of patient samples in this study was approved by the Southampton University Hospitals Regional Ethics Committee (Rec No: 10/H0502/69). Written informed consent was obtained from all participants prior to sample or data collection.

2.2 Colonic Biopsy Collection

Sigmoid colon pinch biopsies were collected from patients with inflammatory bowel disease undergoing colonoscopy on a dedicated IBD list as part of their routine care. 1- 2 pinch biopsies were collected per patient and immediately placed into 10% neutral buffered formalin before being embedded in paraffin blocks and archived for later use as described in section 2.3. The diagnosis of active Crohn's disease was defined as the presence of mucosal inflammation and confirmed by histopathology on a separate biopsy taken from an immediately adjacent area as part of routine clinical care. Inactive Crohn's disease was defined as the absence of mucosal inflammation and was also confirmed by histopathology on an immediately adjacent biopsy. Healthy controls for comparison were provided by patients undergoing colonoscopy for the investigation of anaemia, polyp surveillance or rectal bleeding and had macroscopic and histologically normal colonic mucosa.

2.3 Biopsy Fixation, Processing and Embedding

All biopsy processing into wax was undertaken by staff of the Immunohistochemistry Research Unit. After fixation in 10% neutral buffered formalin at room temperature for 24 hours, biopsies were passed through sequential graded alcohols and clearene at 37°C according the sequence below. Each biopsy was then embedded in Surgipath Histology Paraffin wax for 20 minutes at 60°C, before being stored at room temperature for later use.

Table 2.1: Processing for colonic biopsies

Step	Reagent	Time (minutes)
1	70% Alcohol	20
2	80% Alcohol	20
3	90% Alcohol	20
4	Absolute Alcohol 1	20
5	Absolute Alcohol 2	20
6	Absolute Alcohol 3	20
7	Absolute Alcohol 4	20
8	Clearene 1	20
9	Clearene 2	20
10	Clearene 3	20

2.4 Laser Capture Dissection of Colonic Epithelium

Epithelial cells were isolated from colonic biopsies using Laser Capture Microdissection (LCM). Microscope slides for cutting were prepared by the Immunohistochemistry Unit. 10μm sections were cut from paraffin blocks and floated on 0.1% Diethylpyrocarbonate (DEPC) treated water (Aldrich Chemistry) before being mounted onto Arcturus PEN Membrane Glass Slides (Applied Biosystems). Slides were dried for 24 hours at 37°C prior to use. 30 tissue sections per biopsy were prepared.

Prior to use, the mounted tissue sections were de-waxed and dehydrated by successive washes; Clearene 2x 10 mins, absolute alcohol 5 mins followed by 3 mins, 70 % alcohol 3 mins. Tissue sections were then stained with 0.1% w/v cresyl violet (Sigma), washed in 100% ethanol and allowed to dry overnight prior to laser capture microdissection.

Tissue was cut using a Lecia laser dissection microscope with a pulsed nitrogen laser (wavelength 337.1nm). Each pulse was <4nanoseconds in duration delivering 75Kw of energy at a frequency 30Hz. Each tissue sample was cut in a single session with epithelium being isolated from the lamina propria.

2.5 RNA extraction

Total RNA was extracted from epithelium using the Ambion RecoverAll™ Total Nucleic Acid Isolation Kit (Life Technologies). All reagents were supplied pre-prepared with the exception of the Wash 1 and Wash 2/3 working solutions, to which 42ml and 48ml respectively, of 100% ethanol were add prior to use. To extract total RNA 50ul of digestion buffer and 2ul of protease was added to each sample. These were then heated at 50°C for 15 minutes and 80°C for 15 minutes. To each sample 60ul of isolation additive and 138ul of 100% ethanol was added and the solution mixed by pipetting. The material was placed on micro filters from the Ambion RNAqueous® Micro-Kit and centrifuged at 10,000g. The supernatant was discarded. 500ul of Wash 1 working solution was added to each sample and then centrifuged for 30 seconds at 10,000g. The supernatant was discarded and 350ul of Wash 2/3 working solution was added. Samples were centrifuged at 10,000g for 30 seconds before the supernatant was discarded and the wash repeated. To remove any excess ethanol following these washes the samples were then centrifuged for 1 minute at 10,000 g. The filter was moved to an elution tube and 10 ul of elution buffer at 50°C was added. This was allowed to rest on the filter for 1 minute before being centrifuged for 1 minute at 16,000g. The process was repeated once.

The concentration of eluted RNA was determined using the Nanodrop ND-1000 Spectrophotometer with 1.5ul of the eluted 20ul sample. Samples were frozen at -80°C until required.

2.6 miRNA RT-qPCR

cDNA was prepared from the RNA samples using the TaqMan® MicroRNA Reverse Transcription kit (Life Technologies) and TaqMan®MicroRNA Assay RT primers (Life Technologies) RNA was diluted to a final concentration of10ng/ul in RNAse free water. The composition of each reaction was as follows:

Table 2.2 miRNA Reverse Transcription Template

Reagent	Volume per reaction (µl)
Buffer	0.75
Primer	1.5
dNTP	0.08
RNase Inhibitor	0.1
Reverse Transcriptase	0.5
Water	3.575
Sample RNA	1.00
Total Volume	**7.505**

The samples were then incubated in the Tetrad2 thermal cycler with the following programme:

16^{o}C for 30minutes

42^{o}C for 30minutes

85^{o}C for 5minutes

20^{o}C until reaction stopped

The cDNA was stored at -20^{o}C if not being used immediately.

qPCR was performed using the cDNA using TaqMan Fast Universal PCR Master mix (Life Technologies) and TaqMan® MicroRNA Assay TM primers (Life Technologies). Samples were loaded in triplicate on to MicroAmp optical 384 well

reaction plates (Applied Biosciences) Each reaction well had the following composition:

Table 2.3: miRNA PCR Reaction Template

Reagent	Volume per reaction (µl)
Buffer	5
Primer	0.5
Water	3.8
cDNA	0.7
Total	**10**

Prior to reading the plate was centrifuged at 1200g for 2 minutes. The plate was read using a 7900HT Fast Real Time PCR System (Applied Biosystems).

Analysis of critical threshold cT values was undertaken using SDS v2.3 software (Applied Biosystems). Once a cT value had been obtained the fold induction of each microRNA of interest was calculated. RNU44 was used as stably expressed internal control in all experiments. The fold induction was calculated using the delta delta cT method.

ΔcT = cT (sample) – cT (relevant RNU44 sample)

ΔΔcT = ΔcT (disease sample) – ΔcT (healthy control)

Fold induction =2^ (-(ΔΔcT))

2.7 mRNA RT-qPCR

cDNA was prepared from RNA samples using the TaqMan® MicroRNA Reverse Transcription kit (Life Technologies) and random hexamers (Life Technologies). RNA samples were diluted in RNAse free water to a final concentration of 30.3ng/µl prior to use. The composition of each reaction was as follows:

Table 2.4: mRNA Reverse Transcription Reaction Template

Reagent	Volume per reaction µl
Buffer	1.0
dNTP	0.4
Reverse Transcriptase	0.5
Random Hexamers	1.0
RNase Inhibitor	0.5
RNA	6.60
Total	**10**

The samples were then incubated in the Tetrad2 thermal cycler with the following programme:

25^{o}C for 10 minutes

37^{o}C for 60 minutes

37^{o}C for 59 minutes

85^{o}C for 5 minutes

20^{o}C until reaction stopped

The cDNA was stored at -20^{o}C if not being used immediately

qPCR was performed using the cDNA using TaqMan Fast Universal PCR Master mix (Life Technologies) and TaqMan® MicroRNA Assay TM primers (Life Technologies). Samples were loaded in triplicate on to MicroAmp optical 384 well reaction plates (Applied Biosciences). cDNA was diluted 1:10 with RNAse free water prior to use. Each reaction well had the following composition:

Table 2.5: mRNA PCR Reaction Template

Reagent	Volume per reaction (µl)
Buffer	5
Primer	0.5
RNAse free water	2
cDNA	2.5
Total	**10**

Prior to reading the plate was centrifuged at 1200g for 2 minutes. The plate was read using a 7900HT Fast Real Time PCR System (Applied Biosystems) and analysed using the ΔΔcT method.

2.8 Immunohistochemistry for TNFAIP3

4µm thick sections form the same tissue blocks used for RNA extraction were mounted on plain glass slides and incubated for 10 minutes with 0.5% hydrogen peroxide in methanol to inhibit endogenous peroxidase activity and then washed in Tris-Buffered Saline (TBS). Antigen retrieval was performed by microwaving the slides at full power for 25 minutes in 0.01M pH 6 citrate buffer. Slides were blocked with Avidin and then Biotin (Vectastain UK) for 20 minutes each and washed with TBS between each step. Tissue culture medium was applied for 20 minutes before each slide was incubated overnight at 4°C with anti-Rabbit TNFAIP3 polyclonal antibody (ProSci, USA) at a 1:2000 (500ng/µl) dilution. . Following overnight incubation slides were warmed at room temperature for 30 minutes before further TBS washes. Biotinylated swine anti-rabbit secondary antibodies (Dako, UK) at 1/400 dilution were then applied and slides incubated for 30 minutes at room temperature. After further washes with TBS, Avidin-Biotin peroxidase complexes (Vectastain ABC kit) were applied at a 1:1:75 dilution for 30 minutes before another TBS wash. 3,3'-Diaminobenzidine (DAB) substrate

(Biogenax) was then applied to each slide for 5minutes. This results in identification of the TNFAIP3 antibody by a brown colouration. Before counterstaining with Meyers haematoxylin, slides were washed in running tap water for 5 minutes. Prior to coverslips being applied each slide was then dehydrated by passage through graded alcohol (70%, 90%, 100%, 100%) and Clearene.

All sections were examined using a light microscope at 400x magnification.

Photographic images were taken using a 4 megapixel Nikon Coolpix 4500 digital camera. Images were taken in JPEG format. Scale bars were added in Image J software. Minor adjustments were made in Adobe Photoshop elements v8.0. The only adjustments made were minor cropping and adjustment of levels to ensure uniformity across all images. For all image level adjustments the black point was set at 29, grey point at 1 and white point at 197.

2.9 Immunohistochemistry Quantification

All slides were randomly coded and assessed by an observer blinded to the code. Slides were examined at 400x magnification and 5 fields were examined per slide. In each field 5 random crypts were chosen for analysis, each crypt was divided into quadrants with each quadrant given a numerical score for intensity and quantity of staining as follows:

Stain quantity

% of each quadrant +ve staining	Numerical score
Up to 100 %	4
Up to 75 %	3
Up to 50 %	2
Up to 25 %	1
0	0

Stain intensity

Stain Intensity	Numerical Score
Easily visible.	3
Staining present but not immediately obvious	2
Very faint but present	1
No Staining	0

The scores for each quadrant were added to give a final crypt score out of 28. The scores for all crypts were added to give a total score per slide of 580. This method has previously been described (198).

2.10 Cell culture

2.10.1 T84 cells

T84 cells were obtained from liquid nitrogen stores and cultured in 25mls of advanced DMEM:F12 media (Life Technologies) supplemented with 1% L glutamine 1% (Life Technologies) , Penicillin –Streptomycin 5000 IU/ml (1%) (Life Technologies) and Fetal bovine serum (FBS) 5% (Life Technologies). They were incubated at 37°C in 5% CO_2 and passaged 1:4 when 80-90% confluent. Subsequent passages were either refrozen or used for experiments and continued culture.

For cell passage culture media was removed and the cells washed 3 times with 10mls sterile calcium and magnesium free Phosphate Buffered Saline (Lonza). Cells were detached from the flask by incubation at 37°C with 500µl of Trypsin-EDTA (Sigma) for 20 -30 minutes. The trypsin was deactivated by the addition of 10ml of warmed culture media supplemented with 10% FBS and the suspension transferred to a universal container. 5µl of sterile 10mg/ml DNase1 (Sigma) was added to facilitate digestion of any cell aggregates. The cells were pelleted out of suspension by centrifugation at 1100g for 10 minutes at 4°C. The supernatant

was discarded and the cell pellet re-suspended in 10mls of warmed fully supplemented culture media before being split as needed for culture or experiment.

2.10.2 Hela Cells

Low passage Hela cells were obtained from liquid nitrogen stores and cultured in 25mls of Minimum Essential Media with Earls salts and stable glutamine (Life Technologies), supplemented with Penicillin-Streptomycin 5000 IU/ml (1%) (Life Technologies) and Fetal Calf Serum (5%) (Life Technologies). Cells were incubated at 37°C 5% CO_2 and were passaged 1:3 when 80-90% confluent. Subsequently cells were passaged between 1:2 -1:10 as needed for experiments as outlined in section 2.10.1

2.11 TNFAIP3 and miRNA23a constructs

To investigate the relationship between miRNA23a and TNFAIP3, expression vectors were used containing miRNA23a and the 3'UTR of TNFAIP3.

2.11.1 Generation of miRNA23a constructs

Pre-existing constructs in laboratory were used. These had been prepared by a previous member of the laboratory group and stored at -20°C (Geraint Dingley, unpublished). Briefly, the genomic premiR of miRNA23a was cloned into a pcDNA3.1(-) expression vector with predesigned restriction sites (forward Xba1, reverse Kpn-1).

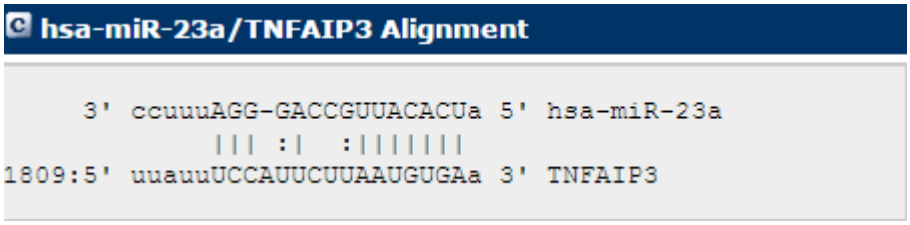
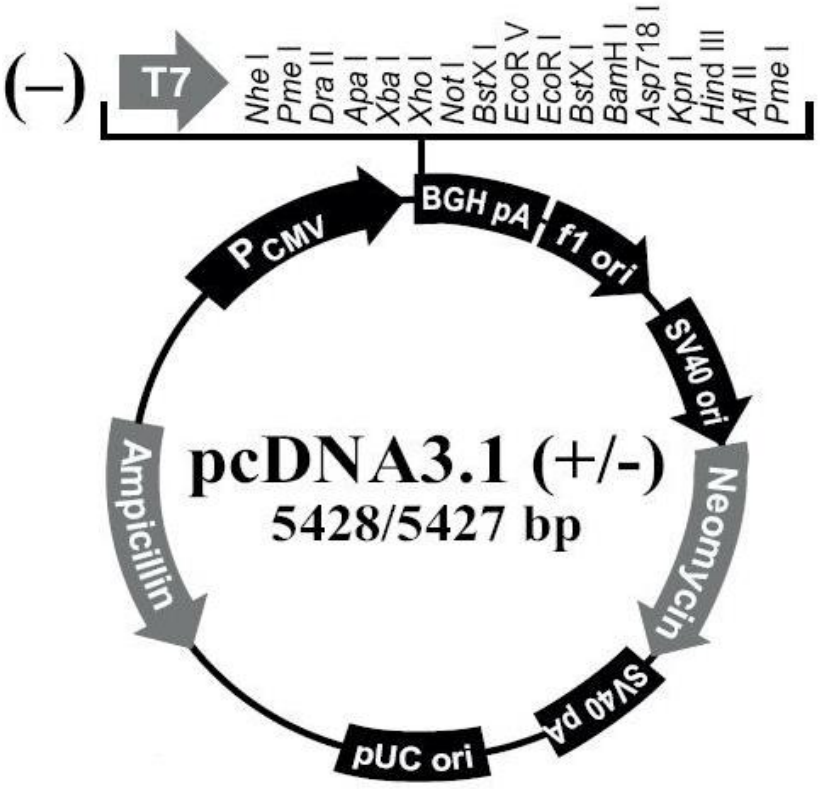


Figure 2.1: TNFAIP3 3'UTR showing predicted miRNA23a complementary sites

5'Cagg**tgccagcctctggccc**cgcccggtgcccccctcacccctgtgccac**GGCCGGCTGGGGTT CCTGGGGATGGGATTTGCTTCCTGTCACAAATCACATTGCCAGGGATTTCCAACCGACC**ctgagct ctgccaccgaggatgctgcccggggacggggtggcagagaggcCCCGAAGC**CTGTGCCTGGCCTGA GG**AGCA 3'

Figure 2.2: Product for expressing miRNA23a

The seed sequence complementary to TNFAIP3 is highlighted in yellow. Bold capital letters indicate the preMir sequence. The flanking regions are also included with the primer location sites underlined.



EXMir23aFOR

tAAGCTTTCTAGAtgccagcctctggccc

HindIII-Xba1 = AAGCTTTCTAGA

EXMir23aREV

tGGTACCACGCGTctcctcaggccaggcacag

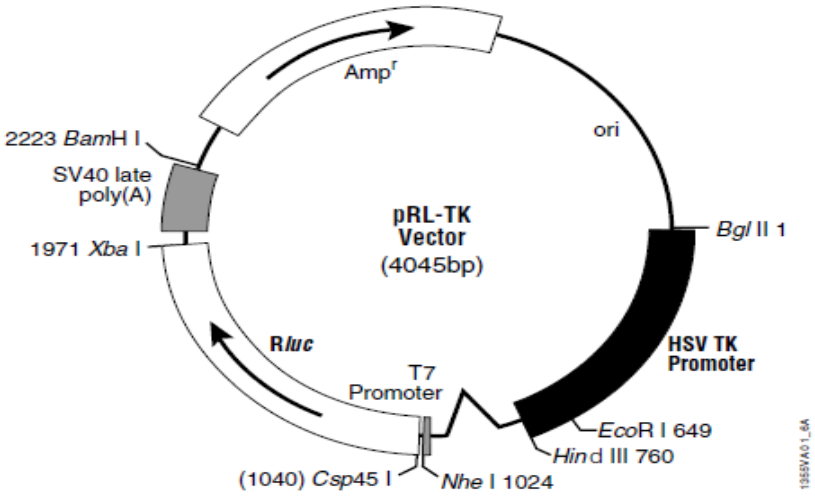
GGTACCACGCGT – Kpn1-Mlu1

Figure 2.3:Vector Map pcDNA3.1

Cloning of miR23a sequence with preMir used Xba1-Kpn1sites in the polylinker of pcDNA3.1(-) vector (personal communication, Geraint Dingley)

2.11.2 Generation of pRLTK TNFAIP3 construct

Pre-existing constructs in the laboratory were used. These were also prepared by Geraint Dingley and stored at -20°C. Briefly, the 3'UTR of TNFAIP3 was cloned into a pRLTK reporter vector 3' to Renilla luciferase using the forward Xba1 site at nucleotide position 1971 and the reverse Not1 site at position 1978 (personal communication Geraint Dingley). The forward primer had an Xba1 compatible Nhe1 site incorporated into the forward primer. This was designated TNAFIP3 wild type. The seed sequence of miRNA23a was then mutated by inserting an ECoR1 restriction site. This was designated TNFAIP3 mutant. The pRLTK vector is shown in Figure 2.4 and the 3'UTR sequences for TNFAIP3 in figure 2.5.



Cloning of the 3'UTR of TNFAIP3 (A20)

A20FOR

tt**GCTAGC**agactggcaatggtcacagg

GCTAGC – Nhe1 Xba1 compatible site is Nhe1

A20REV

ttGCGGCCGCatccaacaaagaataggtggc

GCGGCCGC – Not1

Figure 2.4: Vector map of pRLTK and cloning sequences of the 3'UTR of TNFAIP3

Wild type

5′GTGTTGATCATTATTTCCATTCTTAATGTGAAAAAAAGTAATTATTTATACTTATTATAAAAA 3′

Wild type 3'UTR TNFAIP3 -miRNA23 site shown in blue

Mutated

5′GTGTTGATCATTATTTCCATTCTTAGAATTCAAAAAAGTAATTATTTATAC 3′

3'UTR TNFAIP3 with the miRNA23a seed mutated by the insertion of an ECoR1 restriction site shown in yellow

ECoR1 site= GAATTC

Figure 2.5: Sequence for the 3'UTR of wild type and mutated TNFAIP3 showing miRNA23a seed

2.11.3 Maxiprep and Restriction Enzyme digest

As the existing 3' UTR TNFAIP3 pRLTK and pcDNA3.1 premiR23a constructs were prepared several years ago, prior to use, fresh preparations of each were made. 10ng of each construct was added to a 50µl suspension of competent cells (Ecoli DH5α - Invitrogen) and incubated on ice for 30 minutes. The bacteria were heat shocked at 42°C for 30 seconds before being placed back on ice. 500µl of pre-warmed SOC media (Invitrogen) was added to the bacteria which were incubated at 37°C with gentle agitation for 1 hour, prior to spreading on to an agar plate (Sigma) containing 50mcg/ml Ampicillin (Life Technologies). Plates were incubated overnight at 37°C. The following day single colonies were selected and

the culture expanded in 15ml of Luria-Bertani medium (Sigma). The plasmid DNA was then purified into a miniprep or the culture further expanded for a maxi prep if needed. Both preparations used commercially available kits from Qiagen, according to the manufactures instructions. All reagents were supplied in the kit.

2.11.4 Restriction enzyme digest

To check the integrity of the constructs a restriction enzyme digest was performed using the TNFAIP3 wild type and TNFAIP3 mutant constructs. Each reaction had the following composition.

Table 2.6: Composition of restriction enzyme digest of TNFAIP3 construct

Reagent	Volume (µl)
Plasmid DNA	3
RNAse free water	14
10x Buffer	2
Restriction Enzyme	1

Restriction enzymes and the appropriate 10x buffer were supplied by New England Biolabs. The enzymes used and their corresponding buffer are shown below.

Restriction enzyme	10x Buffer
Not1	NEB Buffer 3.1
EcoR1	cutsmart

Each TNFAIP3 construct was incubated for 1 hour with each of the restriction enzymes. Digestion products were then separated by gel electrophoresis using a 1% agarose gel (Life Technologies) and 1x Tris-Borate-EDTA running buffer. Each

gel had 12µl of a HighRanger Plus 100bp DNA ladder (Norgen) added to allow the molecular size of digestion products to be determined. The gel was run for 1 hour at 80 volts before being examined under ultraviolet light.

2.11.5 Sequence analysis

Samples of each miniprep for TNFAIP3 wild type and TNFAIP3 mutant were posted to Source Bioscience Nottingham UK for sequence analysis. A total of 5µl of each prep was sent along with 40µl of forward and reverse primers for pRLTK (purchased from Sigma).

2.12 Luciferase Assay

The dual luciferase assay is an invitro technique used to study gene expression. This utilises the chemical reaction used by organisms including *Photinus pyralis* (firefly) and *Renilla reniformis* (sea pansy) to emit light via bioluminescence. For this to occur a substrate is needed together with a class of oxidative enzymes known as the luciferases. The dual luciferase assay exploits the fact that the firefly and sea pansy luciferases each require different substrates. In this way both reactions can be measured in a single sample.

Firefly luciferase uses beetle luciferin as its substrate, which is oxidised to oxyluciferin. This is produced in an excited state, meaning that its electrons exist in a higher energy level. As they then return to their baseline energy state the excess energy is emitted as a photon of light.

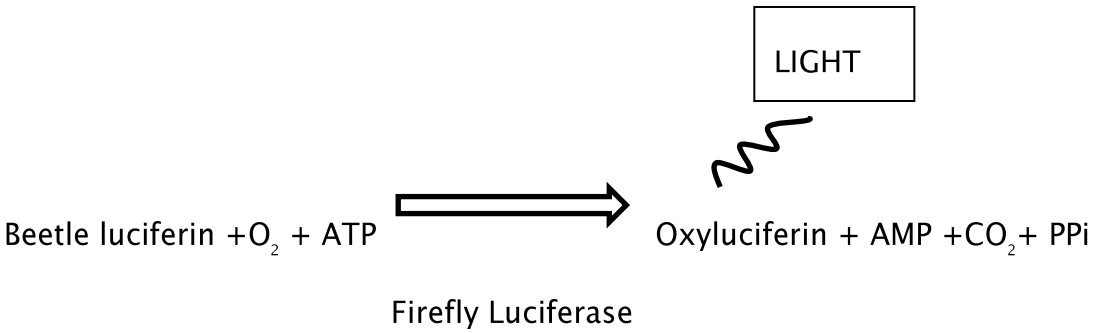


Figure 2.6: Firefly luciferase reaction

In contrast sea pansy (*Renilla)* luciferase oxidises luciferin in the form of colelenterazine. Calcium triggers the release of coelenterazine from its luciferin

binding protein. This is degraded to coelenteramide with the release of energy as light.

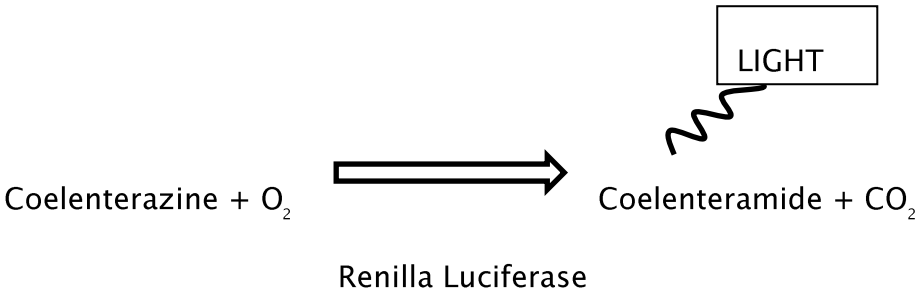


Figure 2.7: Renilla luciferase reaction

The assay works by the addition of the substrate for firefly luciferase. This signal is measured and then quenched by the addition of a second reagent which also contains the substrate for Renilla luciferase. The second signal is measured. In the Promega Dual Luciferase Assay the experimental reporter serves as an indicator of the experimental condition, whilst the co transfected 'control' reporter provides an internal control and baseline across all conditions. The experiment can be set up to use either the firefly or Renilla as control.

2.12.1 Hela Cell transfection pcDNA 3.1 miRNA23a + TNFAIP3 pRLTK

For transfection, Hela cells were grown to 80% confluence as described in 1.10.2. When at the correct confluence cells were removed from the culture vessel as described and suspended in MEM at a density of 40000cells/ml. 1ml was added to each well of a 24 well plate and the cells allowed to plate down for 24 hours at 37°C 5%CO_2. pGL3 as luciferase control vector (Promega) was added to 60ul serum free MEM to give a final concentration of 10ng / well. Appropriate volumes of expression (pcDNA3.1-miRNA23a) vector were added to give a final concentration of 900ng /duplicate wells. Reporter constructs (TNFAIP3-pRLTK) were added to a final concentration of 100ng/duplicate wells. 14µL of Superfect transfection medium (Qiagen) was added and the solution incubated at room temperature for 10 minutes. 700µl of serum free media was added and 400µl of the final solution added to each well of Hela cells. These were incubated for 2

hours at 37°C in 5%CO_2 before the media was removed and replaced with 1 ml of MEM supplemented with 10% FBS. Each transfection was performed in duplicate. Cells were then incubated for 16 hours before proceeding to luciferase assay.

2.12.2 Luciferase assay

The medium was removed and each well washed with 100µl of PBS once. Each well was then coated with 100 µl of passive lysis buffer prepared according to manufactures instructions (1 volume of 5X passive lysis buffer to 4 volumes distilled water)(Promega). Plates were rocked at room temperature for 15 minutes. The resulting lysates were transferred to 1.5ml eppendorph tubes and immediately used for luciferase assay using the Promega Dual-Luciferase® Reporter Assay system.

Aliquots of 1 ml Luciferase Assay Reagent II (LAR II) (Promega) were prepared and stored at -80°C with aliquots being defrosted in a water bath at room temperature immediately prior to use. Stop and Glow® Reagent (Promega) was prepared before use according to manufactures instructions (1 volume 50X Stop and Glow® Substrate to 50 volumes Stop and Glow® Buffer).All measurements were undertaken using an TD20/20 luminometer (Turner Biosystems, USA) set for a 2 second pre read delay and 10 second measurement. For each assay 50ul of LAR II was added to a 1.5ml eppendorph and mixed with 10ul of cell lysate by pipetting 4 times. The firefly luciferase reading was taken. When indicated by the luminometer, 50ul of Stop and Glow® Reagent was added and mixed by pipetting 4 times. The Renilla Luciferase activity was measured. Results are expressed as the ratio Renilla/Firefly for each construct compared to the empty vectors. Each transfection was repeated twice in 5 separate experiments.

2.13 NFKB Reporter assay

The Qiagen Cignal reporter assay utilises the dual luciferase reaction format to determine transcriptional activity of NFκB. The reporter assay kit provides both reporter and control vectors. The NFκB reporter consists of tandem repeats of the NFκB transcriptional response elements joined to the firefly luciferase gene which is under the control a TATA box as a basal promoter element. The reporter mix also contains a construct constitutively expressing Renilla Luciferase under the control of CMV promoter. This means Renilla luciferase output acts as the internal control and NFκB transcription is represented by the firefly luciferase.

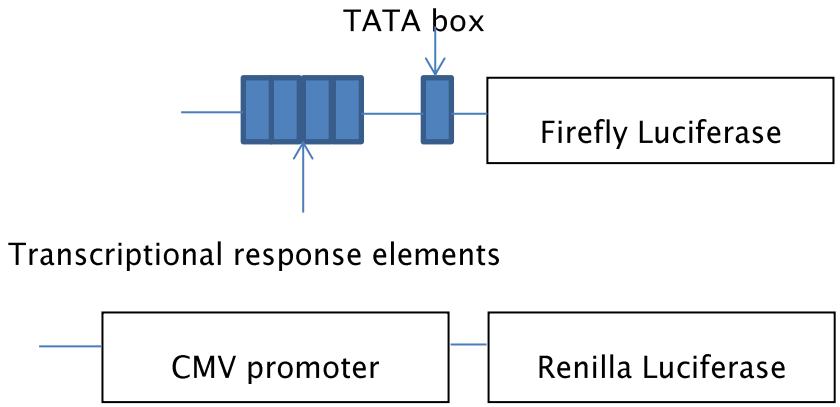


Figure 2.8: NFκB Reporter

Representation of the NFκB reporter (top) and the internal control (bottom).

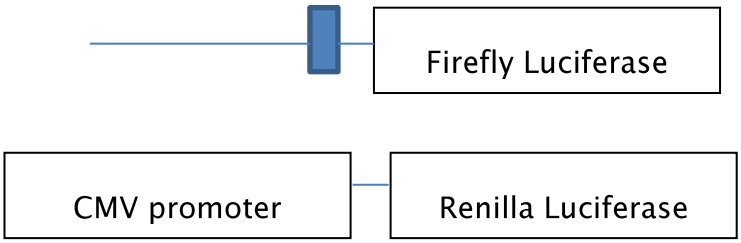


Figure 2.9: NFκB Negative Control

The negative control construct is identical except that the transcriptional response elements are not included. Therefore stimuli for NFκB should have no effect on this assay.

2.13.1 Hela transfection with Plasmid DNA pcDNA3.1-miRNA23a and NFKB reporter

Hela cells were seeded on to 24 well plates at a density of 40,000/ml well plate and allowed to plate down for 24 hours at 37°C 5%CO_2. The NFκB reporter or reporter negative control were diluted in 120ul serum free media per duplicate to give a final concentration of 20ng/well. Volumes of pcDNA3.1 -miRNA23a or empty vector (pcDNA3.1)were added to achieve 500ng per well. 14ul of Superfect (Qiagen) was added per duplicate and after gentle mixing, incubated at room temperature for10 minutes. Finally 700µl per duplicate was added. Hela cells were retrieved from the incubator and washed once with PBS before 400µl of the prepared constructs were added to the cells. Cells were incubated for 2 hours at 37°C before the construct solution was removed and replaced with 1ml of complete serum containing media. After 46 hours of incubation 1ng/ml of TNFα was added to each well. Incubation continued for a further 2 hour before proceeding to the luciferase assay.

2.13.2 Dual Luciferase Assay

The protocol is identical to that described in section 2.12.2. For this assay Renilla luciferase values act as internal control. Aliquots of 1 ml Luciferase Assay Reagent II (LAR II) (Promega) were prepared and stored at -80°C with aliquots being defrosted in a water bath at room temperature immediately prior to use. Stop and Glow® Reagent (Promega) was prepared before use according to manufactures instructions (1 volume 50X Stop and Glow® Substrate to 50 volumes Stop and Glow® Buffer).All measurements were undertaken using an TD20/20 luminometer (Turner Biosystems, USA) set for a 2 second pre read delay and 10 second measurement. For each assay 50ul of LAR II was added to a 1.5ml eppendorph and mixed with 10ul of cell lysate by pipetting 4 times. The firefly luciferase reading was taken. When indicated by the luminometer, 50ul of Stop and Glow® Reagent was added and mixed by pipetting 4 times. The Renilla Luciferase activity was measured. Results are expressed as the ratio Firefly/Renilla for each construct compared to the empty vectors. Each transfection was repeated twice in 5 separate experiments.

2.14 Functional studies of miRNA23a expression in T84 cells and ex vivo colonic biopsy culture.

2.14.1 T84 transfection with preMir 23a

To enable functional studies of microRNA expression T84 cells were transfected to overexpress miRNA23a using premade RNA preMir23a (Life Technologies). These are small double stranded RNA's which mimic endogenous precursor miRNA. To act as control a prepared premir miRNA precursor negative control was used (Life Technologies). This negative control has no mRNA site specificity and therefore is not expected to have any effect on target mRNA. For experiments to test the effect of antagonism of the microRNA cells were transfected with AntiMir23a MiRNA Inhibitor (Life Technologies). These single stranded RNA base inhibitors inhibit the activity of the endogenous microRNA. For control in these experiments an antiMir miRNA Inhibitor – Negative Control #1 (Life Technologies) was transfected. The concentration of antiMir used was the same as the preMir (100nm)

For transfection T84 cells were grown as described previously until 70% confluent. Cells were then passaged and plated in 400µl of DMEM:F12media onto 24 well plates or transwell filters coated with 3 µl Bovine collagen I (Life Technologies) at density of $5x10^5$/well . For each well or filter preMir23a or negative control was diluted to 100nM in 100µl serum and antibiotic free Advanced DMEM:F12 media. After gentle mixing, 3µl of Hiperfect transfection reagent (Qiagen) was added and mixed by briefly vortexing. The pre-miR-Hiperfect solution was incubated for 10 minutes at room temperature before adding dropwise to cells. This gave a final solution of 500ul/well or filter.

For cells cultured for western blot culture was stopped at 24 hours and the cells lysed. 1ng/ml TNF was added to selected wells 3hrs before lysis as determined by the experimental plan.

For cells plated on transwell filters media was changed daily with fresh DMEM:F12. These cells were used for measurement of barrier function.

2.15 Ex-vivo colonic biopsy culture and transfection with preMir23a

Sigmoid colon pinch biopsies were collected from patients with macroscopic and histologically normal colonic mucosa. Up to 4 biopsies were collected from each patient and immediately placed in 1ml ice cold Roswell Park Memorial Institute 1640 medium (RPMI 1640 media) containing L glutamine (Life Technologies) and supplemented with penicillin/streptomycin. Biopsies were transferred immediately to the lab where they were washed 3 times on ice in RPMI1640 media supplemented with penicillin/streptomycin. Each biopsy was placed into an individual well on a 24 well plate with 500µl RPMI 1640 media containing L glutamine, and supplemented with penicillin/streptomycin, and 10% foetal calf serum and transfected as described in section2.15.1

2.15.1 Transfection colonic biopsies ex vivo

Colonic biopsies cultured ex vivo were transfected with 100nM of either pre-MiR 23a or empty vector. An appropriate volume of PreMir23a or empty vector was diluted in 100ul of serum and antibiotic free RPMI 1640 media to give a final concentration of 100nM. 5µl of Interferin(PolyPlus) was added and the solution gently mixed before being incubated at room temperature for 15 minutes. This was then added dropwise to each biopsy, already in 500µl of media to give a final culture volume of 600ul. Transfected biopsies were incubated for 22 hours at 37°C, 5% CO_2. At this time point 1ng/ml TNFα was added to each well and culture continued for a further 2 hours. Supernatant was then collected and frozen at -20°C . Each biopsy was fixed in 10% neutral buffered formalin embedded in paraffin blocks for subsequent LCM.

2.16 Transepithelial Resistance

Electrical impedance of filter grown monolayers was investigated by measuring transepithelial resistance (TER) .12 well plate culture inserts (0.9cm^2) 0.4um pore size (Fischer Scientific) were coated with 3ul Bovine collagen I (Life Technologies) and allowed to polymerise for 30 minutes at 37°C. Cells were grown to 80% confluence in a T75 flask before being passaged at a volume of 1:1 on to the filters. The filter insert had a final media volume of 500ul of Advanced DMEM/F12. 1500ul of Advanced DMEM/F12 media was added to the culture well. Filters were incubated at 37°C in 5% CO_2 with the apical and basal media changed

every 24 hours. Transepithelial resistance (TER) was measured daily with an EVOM epithelial volt ohm meter (World Precision Instruments) and STX2 electrode. Triplicate measurements were taken for each insert. When the cells were confluent with a stable TER, Interferon γ 100ng/ml (R+D Systems) and TNFα 1ng/ml (R+D Systems) were added apically and basally, both singly and in combination. Media was changed and supplemented with cytokines every 24hours. TER measurements were made every 24 hours prior to the media change for 3 days. The TER of each filter was measured in triplicate with the average reading used.

2.17 FITC Dextran Permeability

Epithelial paracellular permeability was investigated by measuring the permeability of filter grown monolayers to 4kD Fluorescein isothiocyanate-dextran(FITC-dextran) (Sigma Life Sciences). Confluent monolayers were cultured for 72 hours with apical and basolateral media containing 1ng/ml TNFα100ng/ml IFNγ or both in combination. 20μL of FITC-dextran (50mg/ml) was added to each apical well to give a final concentration of 2mg/ml. Cells were incubated for a further 3 hours before 100μl aliquots were taken from the basolateral wells and loaded into an opaque 96 well plate. The plate was read using a fluorescent plate reader with excitation spectra of 485nm and emission spectra of 530nm. Results were compared to those obtained from a standard curve.

2.18 Western Blot

2.18.1 Protein sample preparation

T84 cells were transfected to overexpress miRNA23a as detailed in section 2.16.1. Cells were lysed using sample buffer with protease inhibitor cocktail (PIC) (Sigma Aldrich). 40ul of PIC was added to 1ml aliquots of sample buffer and heated to 95°C for 5 minutes. Culture media was aspirated from cells and the well washed with 1ml of PBS. 200ul of boiling sample buffer was added to each well and the cells mechanically disrupted. The sample buffer contained Tri buffer (Thermo Fisher) to maintain a pH of 6.8 and glycerol to increase the viscosity of

the sample and facilitate gel loading. Sample buffer also contained sodium dodecyl sulphate (SDS) (Thermo Fischer) to denature proteins and provide all of them with the same negative charge. This facilitates separation of proteins by size and not charge when run on a gel.

Lysates from duplicate wells were combined into 1.5ml eppendorph tubes. These were heated for 5 minutes at 95°C, cooled to room temperature and centrifuged at 13200rpm for 5 minutes. Aliquots were made and frozen at -20°C until use.

2.18.2 Protein concentration determination

The protein concentration of lysates was determined using a bicinchoninic acid (BCA) assay (Thermo Fisher). The assay utilises the biuret reaction. In an alkaline environment in the presence of amino acids copper is reduced from Cu^{2+} to Cu^{+}. Bicinchoninic acid reacts with the reduced cation in a ratio of 2:1 producing a purple solution. The BCA/Cu^{+} complex is water soluble with linear absorbance at a wavelength of 562nm. This increases with increasing protein concentration.

The BCA assay was performed according to the manufactures instructions. A standard curve was prepared from serial dilutions of 2mg/ml serum albumin solution to give a range 2mg/ml to 0mg/ml. Unknown protein lysates were thawed and diluted 1:5 and 1:10 in distilled water. A 96 well plate was loaded with the protein standards and the unknown samples, each in duplicate. BCA reagent was prepared 50 parts solution A to 1 part solution B before 200ul was added to each well. The plate was incubated at 37°C for 30mins before the absorbance measured at 570nm on a plate reader (Dynatech MR7000). The concentration of the samples was then determined from the absorbance of the standard curve.

2.18.3 Western Blot

For western blotting protein samples were thawed and diluted in sample buffer to give a final concentration of 16ug in 20ul. To this 2ul of 1:10 bromophenol blue/Dithiothreitol (DTT) (Sigma Aldrich) was added. This further denatured the proteins and prevented tertiary or quaternary structures form forming. Samples were briefly heated at 95°C for 3 minutes to redissolve SDS. Precast gels (BioRad MiniProtean TGX 10% pre cast gel) were loaded with 20ul of sample or 10 ul of Spectra multicolour high range protein ladder (Thermo Fisher). A voltage of 120V was run across the gel in the presence of 1x running buffer for 1hr 20 minutes. Protein samples were transferred to PVDF membranes (Trans-Blot® Turbo™

Transfer Pack Mini Format 0.2µM PVDF) using the BioRad Trans Blot Turbo Transfer system. The mixed molecular weight setting was used which applied a current of 1.3 amps and voltage of 25volts for 7 minutes. Following transfer the blot was allowed to dry for 2 hours on filter paper. Unbound sites on the transfer membrane were blocked by washing with blocking buffer (ECL blocking powder, GE Healthcare)for 1 hour at room temperature. The membrane was probed with the primary anti A20/TNFAIP3 antibody (raised in rabbit) (Cell Signalling Technology) at 1:1500 dilution in ECL blocking buffer (GE Healthcare). The blot was incubated overnight at room temperature.

Blots were washed in washing buffer (3x 15 minutes) before being incubated at room temperature with secondary antibody, ECL Anti Rabbit IgG Horseradish Peroxidase linked whole antibody from donkey, (GE Healthcare) diluted 1:2000 in ECL blocking buffer. A second wash was applied (3x 15 minutes). The blot was developed for 5 minutes using the ECL Select™ western blotting detection reagent (GE Healthcare) and viewed using the Biorad ChemiDoc Imaging system.

All blots were also developed for beta Actin as a loading control. Developed blots were stripped by incubating at 55 degrees for 20 minute in 30 mls of stripping buffer to which 210µl of β mercaptoethanol (Sigma) was added. After stripping blots were cooled for 5 minutes at room temperature before being washed (3 x15 minutes)with wash buffer. They were then incubated at room temperature for one hour with a primary HRP linked antibody to beta actin (Abcam) before 3 further washes and development with ECL.

All western blot images were viewed using BioRad Image Lab software.

2.19 MesoScale Discovery Cytokine Assay

Inflammatory cytokines were measured in cell culture supernatant using a high sensitivity MesoScale Discovery V-PLEX assay (MSD). This is a sandwich immunoassay which allows the measurement of up to 10 cytokines in a single sample. MSD provide a 96 well plate precoated with capture antibodies immobilised on an electrode surface. The sample for analysis is added. Analytes in the sample bind to the capture antibodies and are then sandwiched by the addition of SULPHO-TAG™ labelled detection antibodies. A buffer is added which

provides a suitable environment for electrochemiluminescence when a voltage is applied across the plate. The intensity of light output provides a quantitative measure of the sample analytes.

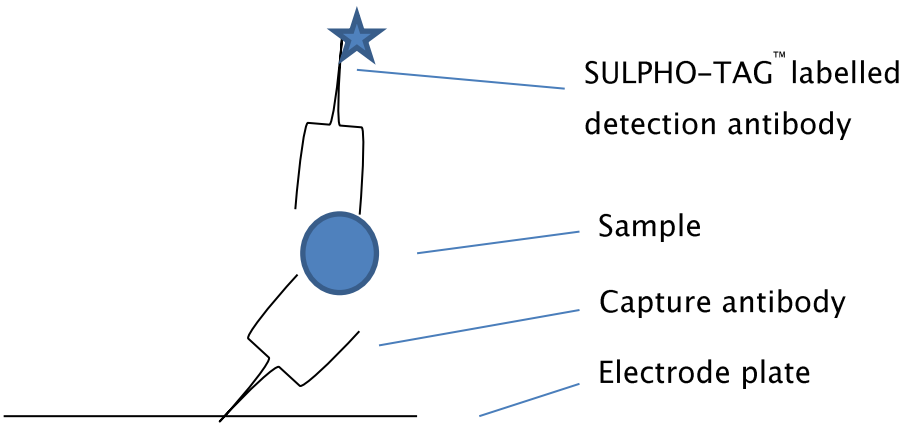


Figure 2.10: Representation of sandwich immuno MSD assay

All reagents were provided for the assay with the exception of wash buffer. As per manufacturer's instruction PBS + 0.05% Tween 20 (Sigma Life Sciences) was used for this. To perform the assay the pre supplied lyophilised multi-analyte calibrator was reconstituted in 1000µl of dilutant 2. A fourfold serial dilution was prepared to generate a 7 point calibrator series. Samples for analysis were diluted in dilutant 2. Each well of the 96 well plate was then loaded with 50µl of either the calibrator solution or sample and incubated overnight at 4 degrees.

On day 2 the plate was washed 3 times with 150µl of wash buffer. 60µl of each detection antibody was combined with dilutant 3 to give a final volume of 3000µl per plate. A volume 25µl of detection antibody solution was added to each well and the plate incubated at room temperature for 2 hours with gentle agitation. A final 3 washes with 150µl of wash buffer were performed before 150µl of 2x Read Buffer was added to each well. Plates were read immediately on the MSD Sector Imager 2400 plate reader.

2.20 Statistical Analysis

All data was analysed using GraphPad Prism v6. Data was tested for normality using the D'Agostino Pearson omnibus normality test and parametric or non-parametric tests of significance used as appropriate. A 2 tailed Students t test was used for parametric data, with results expressed as the mean and standard deviation. The Wilcoxon matched pairs or Kruskal Wallis were used for non parametric data , with results expressed as the median and range. A p value <0.05 was denoted * and taken as significant at the 95% confidence interval.

MicroRNA expression was expressed as the fold change as determined by the delta delta cT method. This is similar to other published microRNA studies which also reported microRNA expression as fold change differences.

Chapter 3:

Laser Dissection of epithelium from colonic biopsies with miRNA extraction and analysis

Chapter 3: Results

3.1 Introduction

As discussed in chapter 1, miRNA function in the homeostatic regulation of protein expression and therefore aberrant expression levels may have negative consequences in disease. Each miRNA will share partial complementary base pairing between its own seed sequence and the 3'UTR of numerous mRNA's. Conversely a single mRNA may be regulated by numerous miRNAs. Although it is known miRNA show altered expression in IBD, studies have so far been largely quantitative, based on microarray techniques from heterogeneous samples (437). Any subsequent correlations between miRNA expression and target protein expression have relied largely on cell culture or murine models (264, 420, 422, 423, 438). Focussed studies correlating cell specific miRNA expression with target protein expression in human tissue are currently lacking.

The ability to study miRNA expression in a defined cell population from human tissue samples would represent significant progress within miRNA research. One such way of isolating cell populations is with laser capture microdissection. Although this has been used to isolate epithelial cells from mouse intestine (264), this approach has not been applied to human colonic biopsies from inflamed mucosa, in relation to miRNA analysis. It is therefore unknown if this is feasible. The ability to extract and quantify miRNA from laser dissected inflamed epithelium will allow determination of the epithelium specific patterns of miRNA expression. This will rely on adequate preservation of tissue morphology during fixation of the tissue and minimal disruption to cellular morphology during the dissection process. Encouragingly miRNA have been successfully isolated from FFPE breast tissue (439), but to date microRNA quantification from isolated colonic epithelia in FFPE sections has not been reported.

Hypothesis

MicroRNA can be extracted from laser dissected colonic endoscopy biopsy epithelium, for specific and reproducible quantification of individual miRNA.

Aim

The aim of this study was therefore to determine the feasibility of isolating colonic epithelial cells from whole tissue colonic biopsies of IBD patients and healthy controls and to extract and accurately quantify specific miRNA.

Objectives

- To establish a method of extracting total RNA from colonic epithelial tissue, isolated from endoscopic biopsy sections using laser microscopic dissection.
- To determine the feasibility and reproducibility of using RT-qPCR to quantitate selected microRNA.
- Identification of a stable housekeeping gene to control for variations in RNA quality and amplification efficiency.
- To use bioinformatic analysis to identify potential microRNA whose predicted mRNA targets included essential proteins for epithelial barrier and immune regulation.

3.2 Isolation of Colonic Epithelium

Formalin Fixed Paraffin Embedded colonic biopsies were cut into 10μm sections and mounted onto Arcturus PolyEthyleneNapathalate Membrane Glass. After staining with Cresyl Violet, the epithelium was isolated from the lamina propria with laser capture microdissection using a pulsed nitrogen laser with a wavelength of 337.1nm. Using this method it was possible to isolate both single crypts and multiple adjoining crypts. Surface epithelium was also isolated. A total of 36 sections were cut from each biopsy. During dissection, damage to surrounding crypts by lateral heat dissipation was observed to be minimal. To further reduce the risk of damage to surrounding cells, where possible crypts were isolated in groups.

Inspection of dissected epithelium showed no obvious distortion to cell morphology. Figures 3.1–3.4 illustrate the LCM workflow.

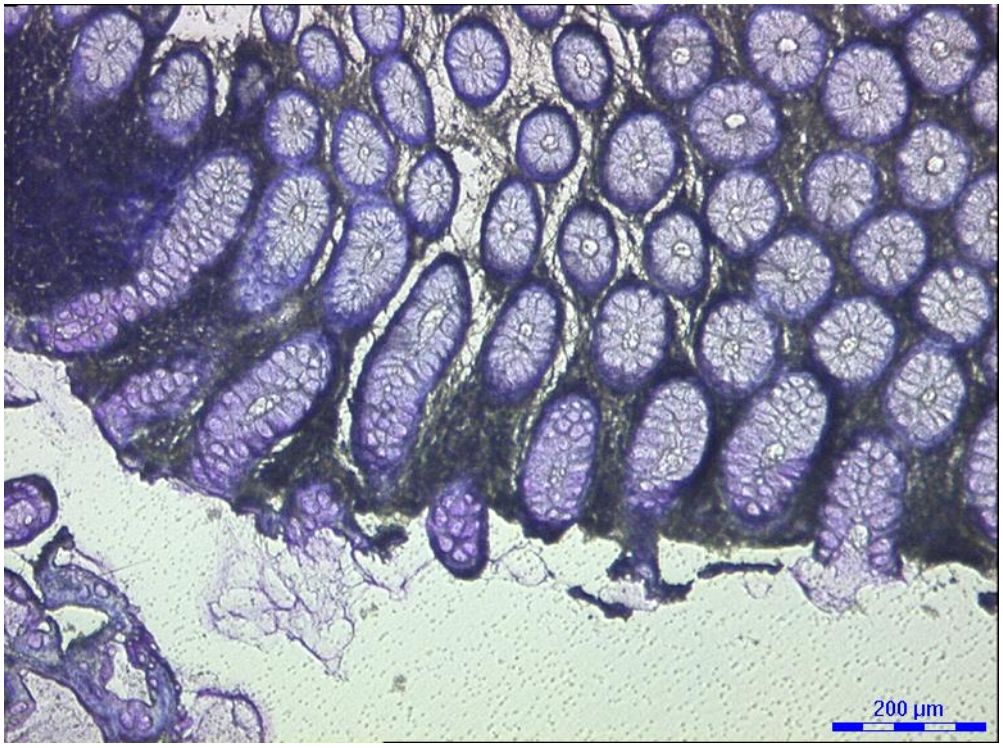


Figure 3.1: Colonic biopsy section prepared for laser microdissection and stained with cresyl violet.

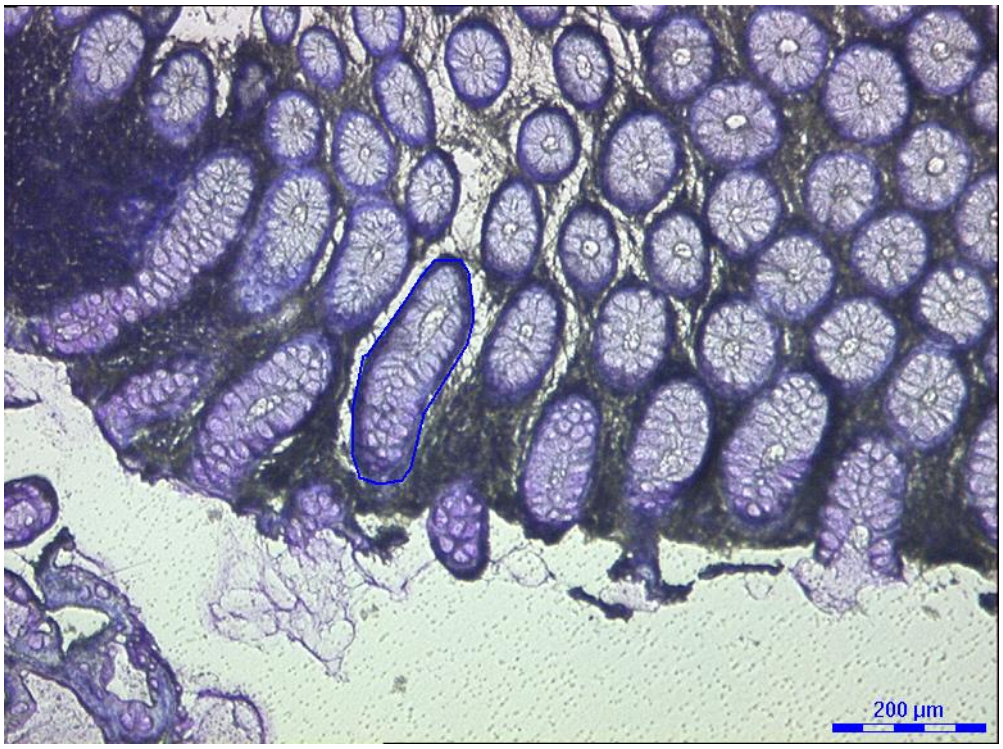


Figure 3.2: Selection of epithelial crypt for dissection

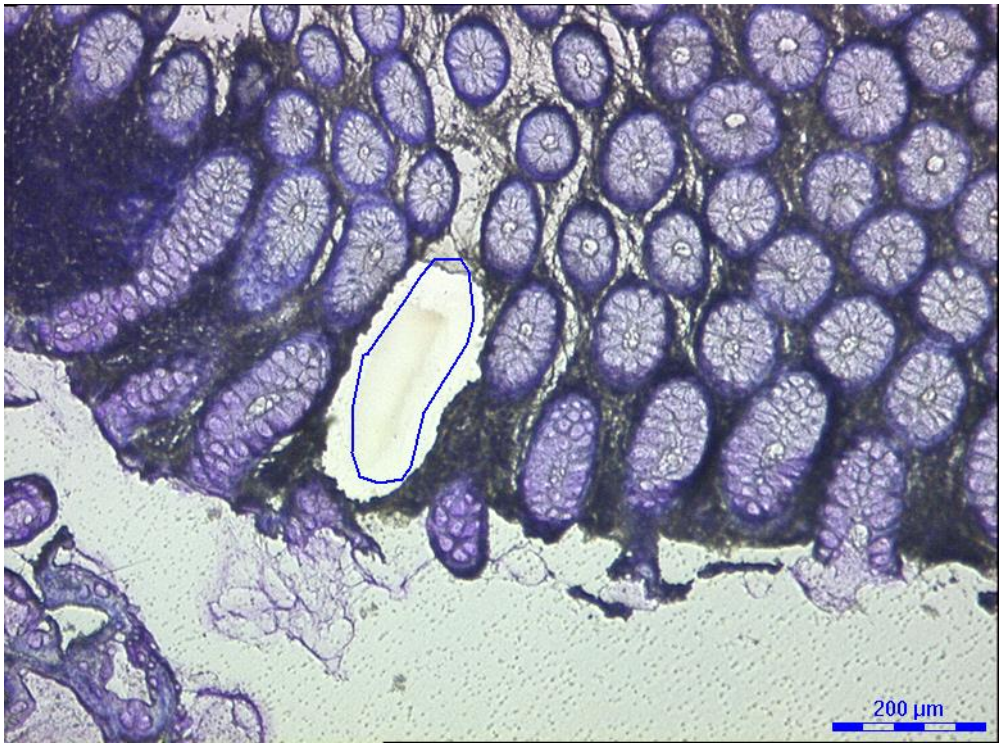


Figure 3.3: Dissection of identified crypt showing remaining intact tissue

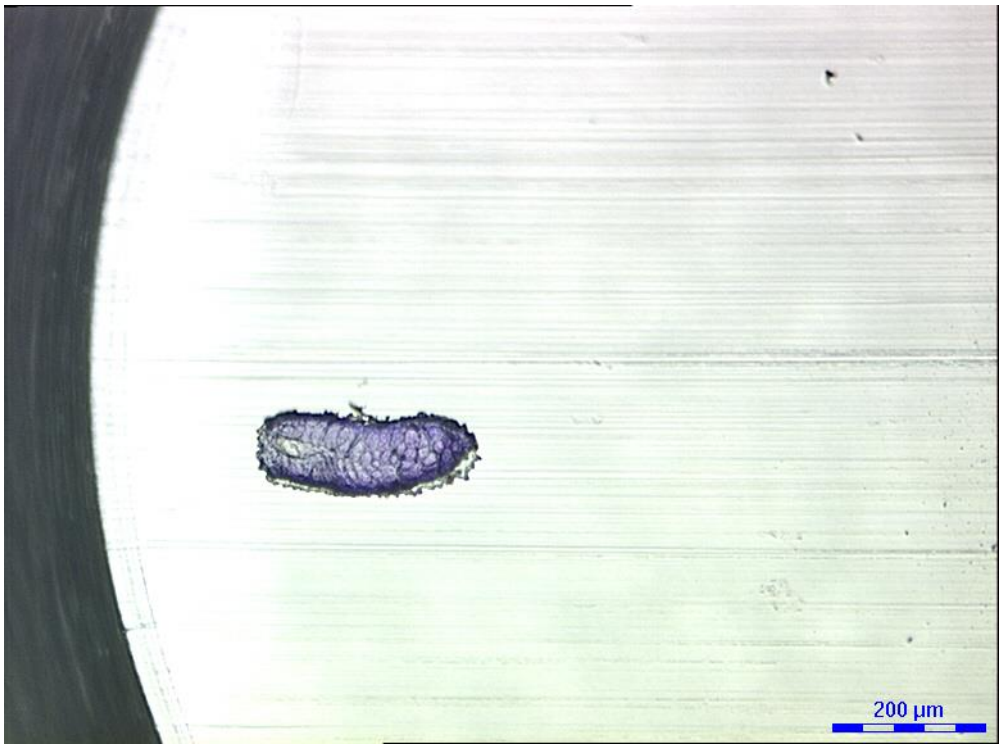


Figure 3.4: Cap image showing dissected crypt.

Laser capture microdissection was therefore found to be a viable way of selectively isolating epithelium from colonic biopsies. In both healthy tissue and samples from active IBD, gross cell morphology was maintained during the dissection process.

3.3 Isolation of total RNA from LCM epithelium

Following micro dissection total RNA was extracted from dissected epithelium using the Ambion RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE material. The extracted RNA was then retrieved, washed and eluted using the filters in the Ambion RNAqueous kit. Extractions took place on the same day as the epithelial isolation. RNA was eluted in 20ul of RNAse free water and frozen at -80°C until use. Prior to freezing, the concentration of RNA in each sample was determined by measuring the absorbance of light at wavelengths of 260 nm and 280nm. A typical readout is shown in Figure 3.5. Total RNA concentrations between 30 and 200 ng/µl were measured. This confirmed that RNA extraction from laser dissected colonic epithelium is both possible and yields concentrations which are suitable for downstream applications such as RT-qPCR.

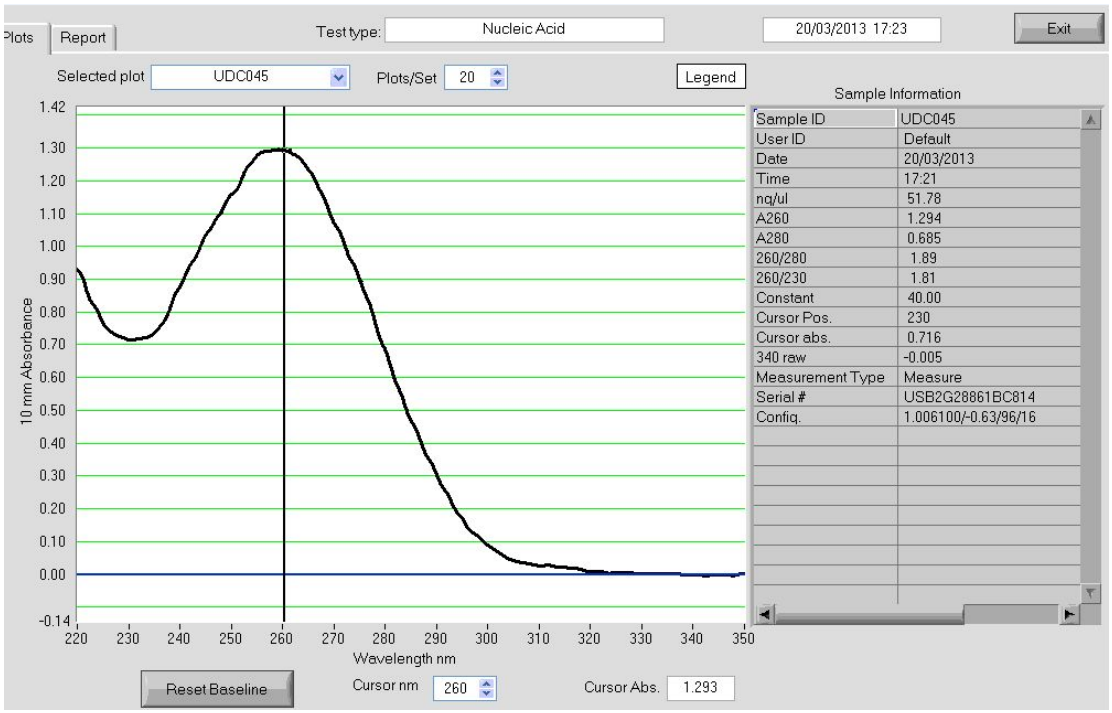


Figure 3.5: Representative Nanodrop readout.

Representative nanodrop spectrophotometer readout of a 1.5µl RNA sample showing a total yield of 51.78ng/µl and a 260/280 ratio of 1.89 indicating good sample purity.

3.4 RNU44 is a stably expressed in epithelium

In order to analyse miRNA expression data using the ΔΔcT method (see Materials and Methods), a stably expressed gene of reference for each sample must be used. This is known as the housekeeping gene. To determine the most appropriate housekeeping gene, 2 commonly used reference genes in our lab, RNU44 and miRNA1275 were measured in isolated colonic epithelia from 5 healthy patients. This data is shown in Figure 3.6.

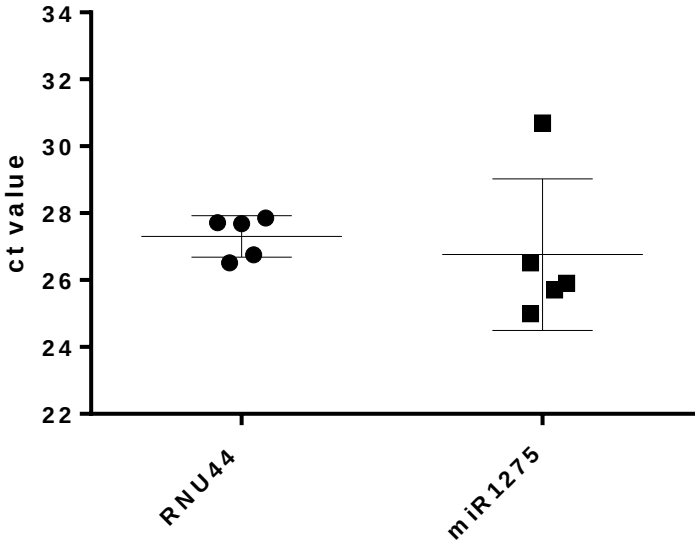


Figure 3.6: RNU44 and miRNA1275 expression from healthy colonic epithelium.

Expression of RNU44 and miRNA1275 in colonic epithelium from 5 healthy patients. Each point represents a single patient. Error bars show the standard deviation and the mean.

The cT values for RNU44 ranged from 26.52 to 27.86 with a standard deviation of 0.6189. The cT values for miRNA 1275 ranged from 24.99 to 30.70 with a standard deviation of 2.264. In this initial sample RNU44 was the most consistent in its expression and was therefore selected as the most appropriate reference gene for all future microRNA quantification. Healthy controls were used for this initial analysis so as to provide a stable baseline comparator for the disease groups.

3.5 Bioinformatic analysis and microRNA selection

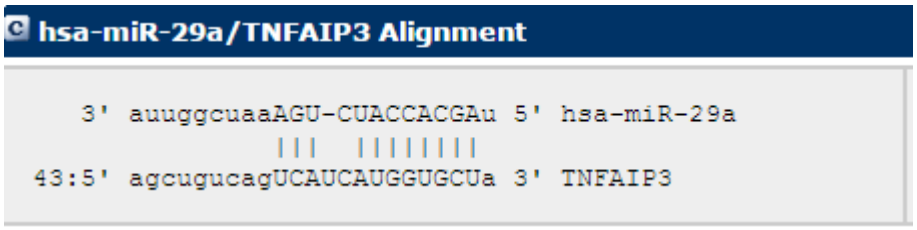
A previous miRNA microarray study in our lab, using an IBD T84 Transwell model was reviewed for microRNA showing altered expression with TNFα and IFNγ stimulation. This information from studies showing miRNA alterations in whole biopsies was also reviewed. Bioinformatic databases TargetScan (v6.2 June 2012) and microRNA.org (August 2010 release) were screened to identify miRNA with predicted molecular targets associated with control of epithelial tight junction or

immune regulation pathways. The TNFAIP3 gene was a particular focus due to its potential importance in NFκB regulation and possibly epithelial barrier stability, as discussed in chapter 1.

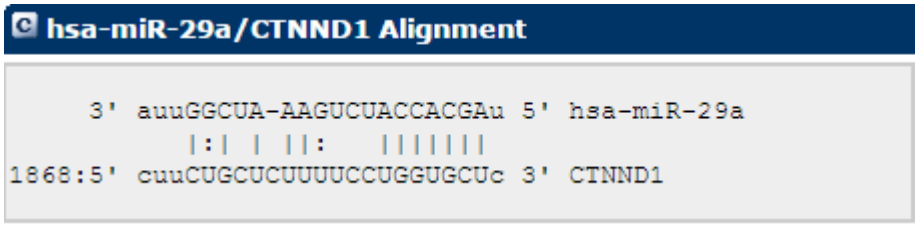
3.5.1 MiRNA29 family

Unpublished data from our lab suggested that members of the miRNA 29 family and miRNA429 were overexpressed in a T84 colonic epithelial barrier model in response to TNFα and IFNγ. MiRNA 29a has been shown to be overexpressed in intestinal tissue biopsies of irritable bowel syndrome (404) and in whole biopsies from IBD patients (419, 420). Predicted mRNA targets include TNFAIP3, p120ctn and claudin 1 as shown in Figures 3.7 – 3.9.

TNFAIP3



p120ctn



Claudin 1

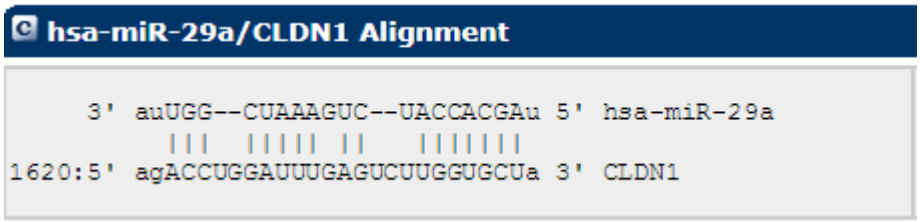
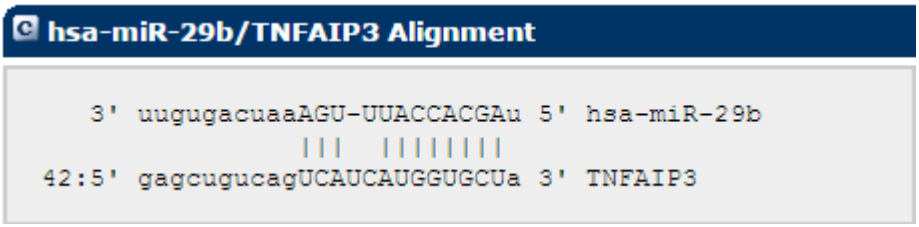
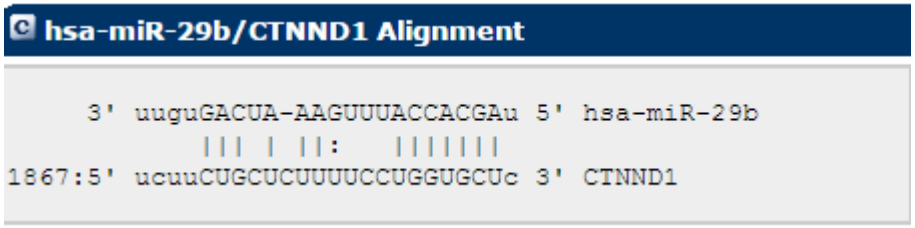


Figure 3.7: Bioinformatic target predictions for miRNA29a showing predicted junction protein mRNA targets

TNFAIP3



p120ctn



Claudin 1

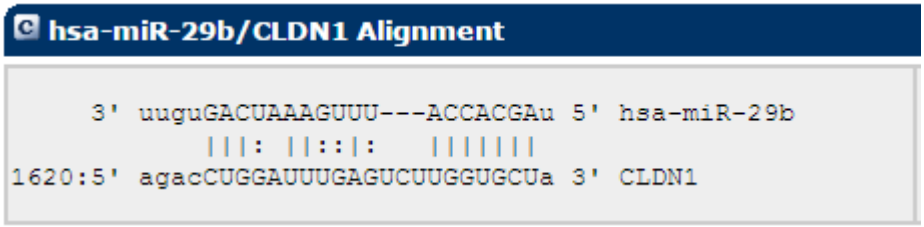
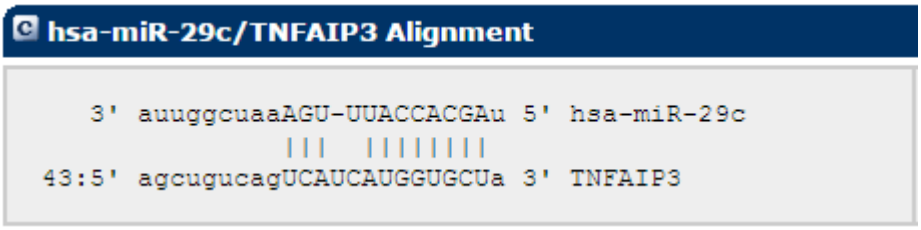
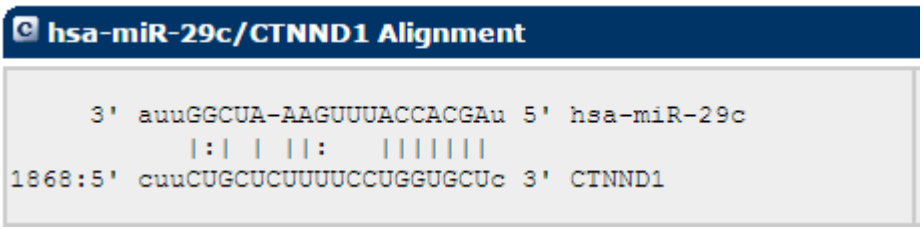


Figure 3.8: Bioinformatic target predictions for miRNA29b showing predicted junctional protein mRNA targets.

TNFAIP3



p120CTN



Claudin 1

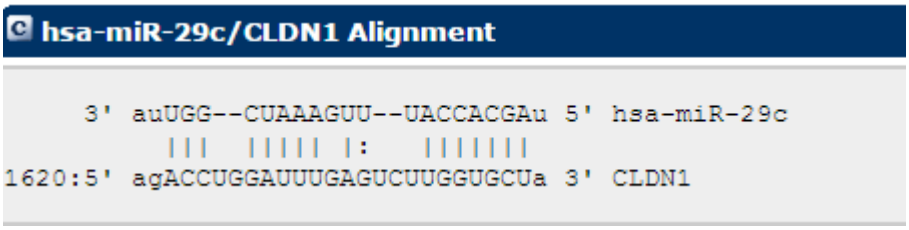


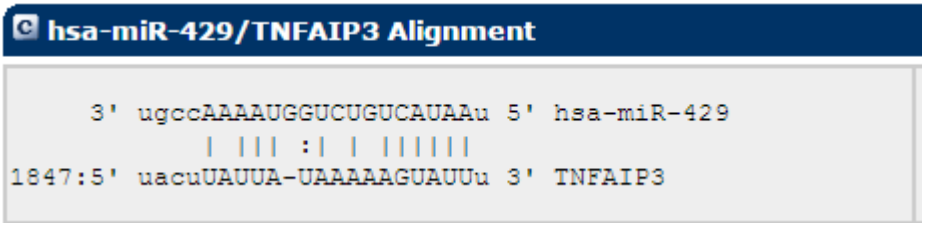
Figure 3.9: Bioinformatic target predictions for miRNA29c showing predicted junctional protein mRNA targets

This microRNA was therefore selected for further study.

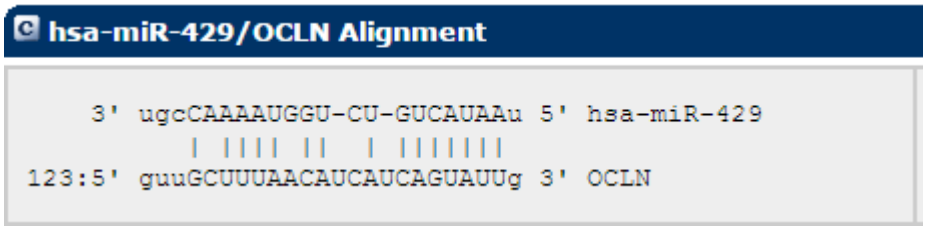
3.5.1.1 miRNA429

MicroRNA 429 belongs to the same family as miRNA200c. These are implicated in promoting epithelial to mesenchymal transition (EMT) (440). Tight junction disassembly is recognised as one of the initiating steps of EMT (441). As disruption of junctions also occurs in IBD (139) this suggest this microRNA may be relevant in IBD. Predicated mRNA targets include TNFAIP, occludin and Zinc finger E box-binding homeobox 1 (ZEB1). These are shown in Figure 3.10.

TNFAIP3



Occludin



ZEB1

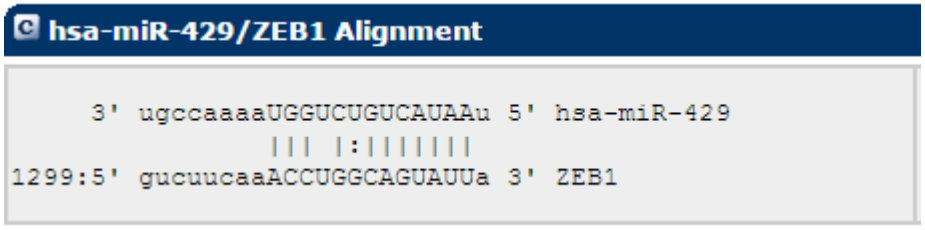
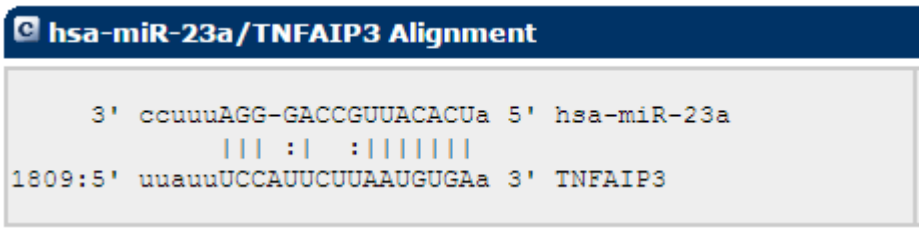


Figure 3.10: Bioinformatic target predictions for miRNA429 showing predicted junctional protein mRNA targets

3.5.1.2 MiRNA23a

Finally, previous work in our laboratory has shown that microRNA 23a is overexpressed in renal epithelium in response to TNFα and TGFβ (Geraint Dingley, unpublished, personal communication). The adherens junction protein E cadherin has been reported to be repressed by this microRNA (402, 442). In IBD studies miRNA23a has been shown to be differentially expressed in mucosal samples from UC patients(420). Its predicted targets include TNFAIP3 and E cadherin as shown in **figure 3.11.**

TNFAIP3



E-Cadherin

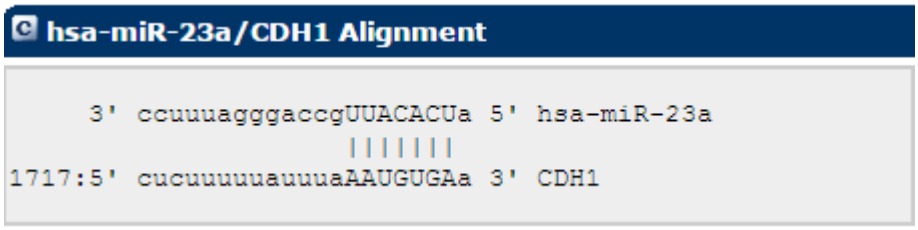


Figure 3.11: Bioinformatic target predictions for miRNA23a showing predicted junctional protein mRNA targets

3.5.1.3 Summary

MicroRNA 29a, 29b, 29c, 429 and 23a were therefore selected for quantitation in isolated colonic epithelium.

3.5.2 MicroRNA Expression – Preliminary Data

In an initial series of experiments biopsies were collected from 5 healthy controls, 5 patients with ulcerative colitis and 4 patients with Crohn's disease. Using RNU44 as a stably expressed internal control, microRNA expression was determined for each group. Results are reported as the mean fold change in expression for each disease group compared to the normal healthy controls. Results are compared with an unpaired two tailed t test.

3.5.2.1 MiRNA29a

The relative fold difference in expression of miRNA29a for CD was 4.06 compared to 2.079 for ulcerative colitis. Neither of these were significant when compared to the normal cohort (p =0.2115 CD and p=0.1283 UC).

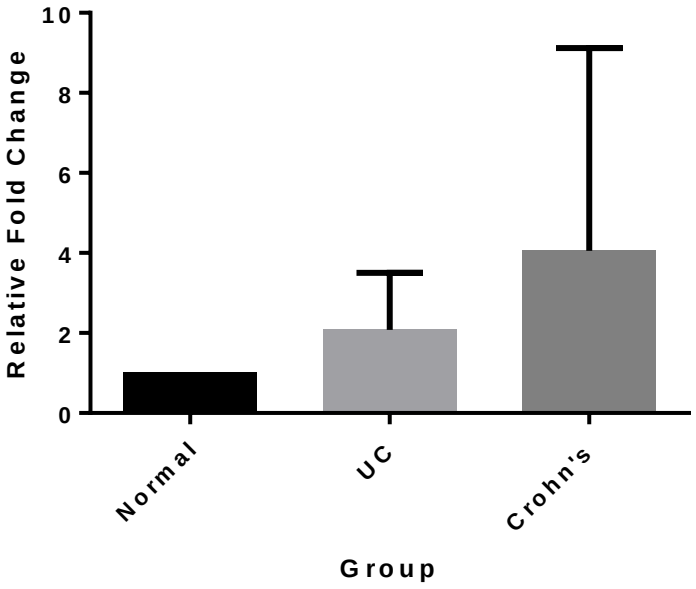


Figure 3.12: Expression of miRNA 29a in healthy epithelium, active UC and active CD. Data expressed as the mean of each group and standard deviation. n=5.

3.5.2.2 miRNA29b

The relative fold difference for Crohn's disease was 4.514 compared to 1.382 for ulcerative colitis. Neither of these was significant compared to healthy controls (p=0.267 CD and p=0.5046 UC).

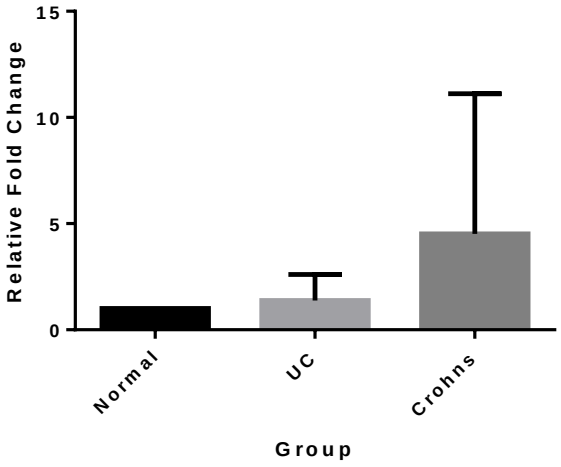


Figure 3.13: Expression of miRNA 29b in healthy epithelium, active UC and active CD. Data expressed as the mean of each group and standard deviation. n=5

3.5.2.3 miRNA29c

The relative fold change in expression for Crohn's disease was 5.117 compared to 2.711 for ulcerative colitis. Neither of these were significantly different when compared to healthy controls (p=0.0796 CD and p=0.0744 UC)

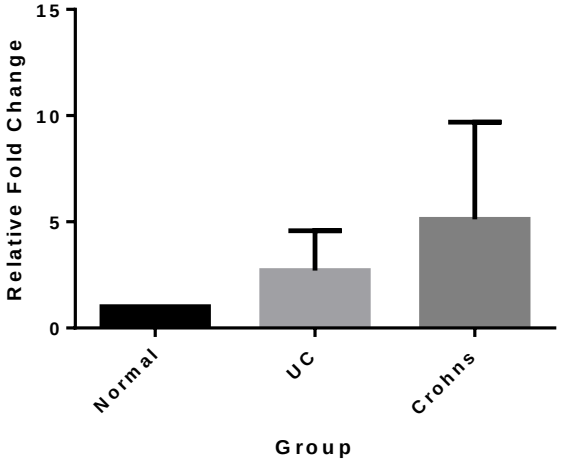


Figure 3.14: Expression of miRNA 29c in healthy epithelium, active UC and active CD. Data expressed as the mean of each group and standard deviation. n=5.

3.5.2.4 miRNA429

The relative fold difference in expression for Crohn's disease was 3.503 compared to 0.977 for ulcerative colitis. Neither of these were significant compared to healthy controls (p=0.2322 CD and p=0.9422 UC).

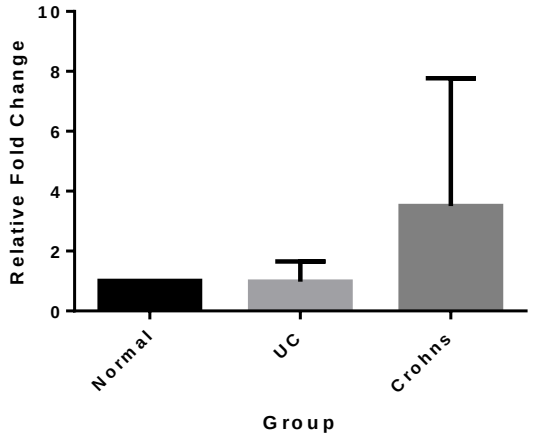


Figure 3.15: Expression of miRNA 429 in healthy epithelium, active UC and active CD. Data expressed as the mean of each group and standard deviation. n=5

3.5.2.5 miRNA23a

The relative fold difference in expression for Crohn's disease compared to healthy controls was significantly elevated at 6.70 (p=0.02). The fold difference for ulcerative colitis was not significantly different from controls at 2.199 (p=0.08

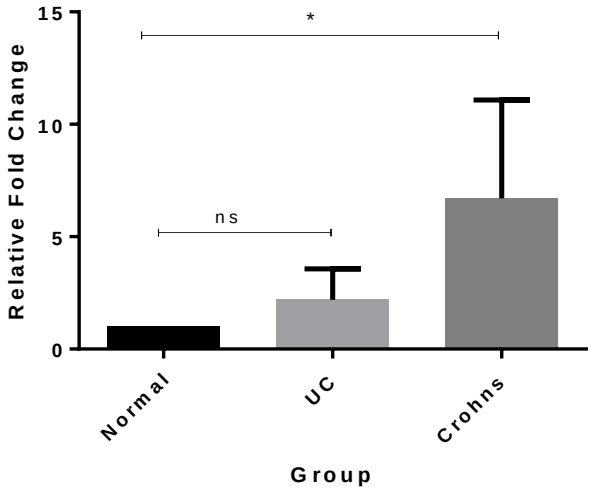


Figure 3.16: Expression of miRNA 23a in healthy epithelium, active UC and active CD. Data expressed as the mean of each group and standard deviation. P value <0.05 denoted with *. n=5

3.6 Discussion

The aim of this chapter was to gauge the technical feasibility of quantitating miRNA extracted from laser dissected colonic epithelium and to identify potential miRNA for further study.

Gene expression profiling over recent years has typically relied on microarray hybridisation techniques. Capable of generating transcription profiles for many thousands of genes in a tissue sample, this approach can be used to determine expression profiles between different tissues or disease phenotypes (443) and has been applied to diseases of the intestinal tract (444). Microarray techniques have also been used to determine microRNA transcription profiles in the intestinal mucosa of inflammatory bowel disease cohorts and have provided the first microRNA expression data for this disease (420, 434). However tissue samples are heterogeneous containing mixes of different cell types. The intestinal mucosa is no exception. In this study the aim was to address the heterogeneous nature of samples used in previous studies and undertake a focussed epithelial cell specific microRNA analysis. Laser capture microdissection was therefore chosen as it allows the precise isolation of specific cell populations from heterogeneous tissues (445).

The underlying principle of laser capture microscopy involves the use of a bright field microscope and a low energy pulsed laser typically in ultraviolet or infrared

wavelengths (446). Tissue samples are mounted onto polyethylene napthalate membrane slides. Polyethylene napathalate is heat sensitive and upon contact with the laser expands, attaching itself to the overlying cells (447). Upon cooling, the membrane contracts, separating from the rest of the sample allowing isolation of the target cells from the rest of the tissue. The isolated membrane and cells are then removed into a collection vessel for downstream processing and analytical applications (446-448). Of critical importance in LCM is sample preparation. The use of snap frozen sections is reported to offer the best preservation of nucleic acids, thus maximising the quality and quantity of RNA recovery (449). However this RNA quality is at the expense of the preservation of tissue morphology. Snap freezing of tissue can result in the loss of cellular morphology due to intracellular ice crystal formation (450). Frozen tissue sections were therefore not used in this study as future immunohistochemical studies on the same tissue used for LCM would have been compromised. Formalin fixed tissue is an alternative which preserves the structural integrity of tissues (451). The isolation of RNA has previously been described from formalin fixed tissue with adequate sample for analysis obtained from only 50 laser dissected cells (452). This was therefore adopted as the method of fixing tissue prior to LCM.

One of the concerns of formalin fixed tissue for protein and nucleic acid analysis is the potential loss of protein or RNA due to the formation of crosslinking bonds between nucleic or amino acids (453). Mono methyl groups are added to each of the 4 nucleotide bases during formalin fixation potentially impeding RNA recovery (453). This problem can be circumvented by incubating RNA during extraction at an increased temperature to disrupt the methyl bonds (453). This has been shown to be effective with no adverse effect on subsequent RNA amplification with RT-PCR (453) and was therefore included in the RNA extraction protocol used in this study. To further minimise the effects of formalin, patient samples were fixed in 10 % formalin for no more than 24 hours. This has been shown to be optimal with longer fixation times leading to reduced RNA recovery (454).

Another potential issue to be overcome was the use of an appropriate stain. For laser capture microscopy staining must be specific enough to allow identification of the relevant cell type within the tissue, in this case, the epithelium. However RNA integrity can be adversely affected by some of the commonly used staining agents (455, 456). Haematoxylin in particular, a common histological stain, has

been reported to cause a 33% reduction in RNA integrity (455). Aqueous staining methods also adversely affect RNA integrity with one series reporting a 20% reduction in RNA 30 minutes after an aqueous staining protocol (455). This same study reported 4.5 % cresyl violet to be the most effective stain for preservation of RNA with just 2.4% degradation reported(455). Cresyl violet was therefore adopted for use in this study. As shown in the results section, this enabled good definition of the epithelium /crypts for dissection with no significant disruptions to cellular morphology. The protocol that was followed, taking into account the modifications discussed above, resulted in good RNA yields from the epithelium with minimal contamination of the samples, as indicted by the 260/280 absorbance ratios.

Although RNA integrity can be an issue for LCM samples, microRNA have been successfully isolated from various tissue over the past few years. Within the colon, LCM has principally been used to isolate microRNA from tumour cell sets within normal tissue (457, 458). Epithelial isolation has been included as part of this work (458) However these tissues have been frozen or fixed in ethanol prior to LCM. The preliminary work presented in this chapter is the first use of LCM to isolate microRNA from the epithelium of inflamed tissue which has been formalin fixed.

Due to their small size microRNA are stable in tissue and the quality of microRNA recovery is reported to be similar to fresh frozen samples (459, 460). Recovery of intact microRNA has been possible from archived samples ranging in age from 10 to 28 years (460, 461). Tissue biopsies in my study were used within months of embedding. We have evidence in our group that long term storage over years can alter recovery RNA detection by PCR, indicating that age matching of control and diseases samples is desirable (Dingley and Collins – personal communication). Recovery has also been possible from fragile tissue of poor quality (462). The extraction of microRNA from inflamed tissue in this study supports this. Despite the inflammatory cell infiltrates of the lamina propria and crypt architectural distortion typically seen in IBD it was still possible to extract and quantitate microRNA from this epithelium.

The microRNA selected for this preliminary work exhibited differential expression compared to healthy controls. With the exception of miRNA429, overall microRNA expression was greater in both disease cohorts, compared to healthy controls, with the largest expression fold changes observed in Crohn's disease. However these were not statistically significant, accepting the limitation imposed by the

small sample size. Although the data needs to be interpreted with caution, the expression results for miRNA429 in particular highlight the importance of cell specific studies. This microRNA has previously been shown to be down regulated in the mucosa of active ulcerative colitis compared to healthy controls (435). The data presented in this chapter specifically from the epithelium suggests there is essentially no difference between healthy and ulcerative colitis. In Crohn's disease, the expression in the mucosa of active disease was reported to be reduced (463). However, in this study, increased expression was observed within the epithelium and the p value of 0.23 suggests that higher sample numbers may show a significant increase in miR429 in CD rather than a decrease. These findings emphasise the point made in the initial introduction; small changes in expression within particular cells of a tissue may be missed or masked if that tissue is analysed as a whole.

The only microRNA to show significant overexpression in this initial epithelium series was miRNA23a in active Crohn's disease. Very little is known about this miRNA in Crohn's disease and even less is known about its role in the epithelium. It is predicated to have complementary sequences with the 3'UTR of TNFAIP3 and given the potential importance of this protein in regulating TNFα driven NFκB responses and barrier integrity, this miRNA was therefore selected for further study. The remainder of this work will now focus on the epithelial expression and function of miRNA23a in Crohn's disease.

Chapter 4:

Expression of the MicroRNA23a-27a-24 Cluster in Crohn's Disease Colonic Epithelium

Chapter 4: Results

4.1 Introduction

The findings from preliminary work suggested that miRNA23a may show differential expression in CD as compared to healthy controls. This miRNA has previously been shown to be overexpressed in whole mucosal biopsies in UC (420). However, as discussed the major limitation of this approach has been the lack of a focussed study in a homogenous cell population. The expression of this miRNA in CD is unknown. Specifically expression in the epithelium remains uncharacterised. In addition, microRNA are grouped in clusters in the genome. MiRNA23a is part of the 23a-27a-24 cluster of miRNAs which showed differential expression in a number of diseases including ulcerative colitis (464).

Hypothesis:

MiR23a, 27a and 24 are specifically increased in the epithelium of colonic Crohn's Disease and the changes correlate with current clinical parameters of disease severity.

Aims

The aim of this section was to confirm the preliminary findings and characterise the expression of miRNA23a in the colonic epithelium of a larger cohort of patients with active or inactive Crohn's disease. The second aim of this section was therefore to define the epithelial expression profile of miRNAs in the 23a-27a-24 cluster in Crohn's disease epithelium.

Objectives

- Expand initial cohort of endoscopy patients
- Determine expression of microRNA23a in the epithelium of active and inactive Crohn's disease.
- Determine the expression of microRNA24 and 27a in the same epithelium.
- Correlate microRNA findings with clinical characteristics of disease severity in patients.

4.2 Patient Demographics

Sigmoid colon biopsies were collected from 16 patients with active Crohn's disease, 7 inactive Crohn's disease and 10 healthy controls. With fully informed consent, clinical and demographic information was also collected. Table 4.1 shows this data.

Table 4.1: Demographic and clinical data of patient cohorts

		Healthy	Active Crohn's	Inactive Crohn's
Number		10	16	7
Average Age (Range)		70 (49–86)	32 (17–66)	51 (26–80)
Sex	Male Female	3 7	7 9	4 3
Ethnicity	White Afro-Caribbean Asian	10 0 0	14 0 2	7 0 0
Smoking	Non smoker Smoker Ex-smoker	9 1 0	12 1 3	9 0 0
IBD medication	5 ASA Steroids Thiopurine TNF antagonist	0 0 0 0	1 3 3 2	1 0 3 3

		Normal	Active Crohn's	Inactive Crohn's
Rectal Bleeding	None	9	8	7
	<1/2 time	0	3	0
	>1/2 time	1	3	0
	All the time	0	2	0
Abdominal Pain	None	6	2	5
	Mild	2	7	1
	Moderate	2	2	1
	Severe	0	5	0
Well being	Well	8	3	4
	Slightly below	1	8	5
	Poor	1	3	0
	Very poor	0	1	0
	Terrible	0	1	0
Abdominal mass	Yes	0	0	0
	No	10	16	7
Laboratory Investigations	None	4	0	3
	Low Hb	n/a	3	0
	CRP >7.5	n/a	9	0
	Albumin <35	n/a	8	0

4.3 MiRNA23a is overexpressed in colonic epithelium in Crohn's disease

The epithelium was isolated from the colonic biopsies of 16 active CD, 7 inactive CD and 10 healthy controls using LCM. Total RNA was extracted as outlined in the methods. Expression of mirNA23a in the epithelium was quantitated using miRNA23a specific RT-qPCR primers. RNU44 was used as a stably expressed internal control. The expression of miRNA23a was determined for each sample using the ΔΔct method. All samples including healthy samples were compared to a single healthy control, whose cT values were closest to the mean of the group and therefore taken to be representative.

The mean fold change expression of miRNA23a was significantly elevated in the epithelium of both active and inactive Crohn's disease. The mean expression in active disease was 2.537 fold greater than healthy controls (p=0.0435). Inactive disease was similar with a mean expression 2.974 fold greater than healthy controls (p=0.0278). Data was tested for normality and analysed with the unpaired student's 2-tailed t test. This data is shown in Figure 4.1.

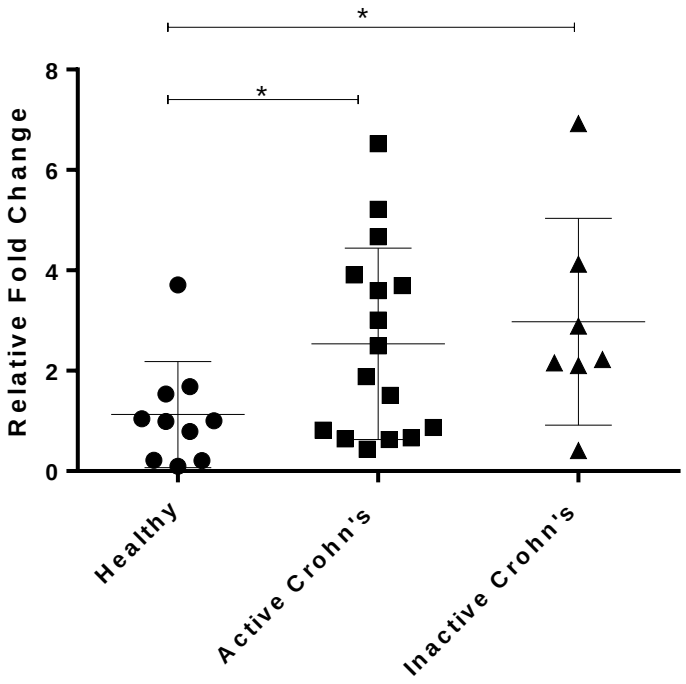


Figure 4.1: MiRNA23a expression in active and inactive Crohn's disease compared to healthy controls. Each dot represents an individual patient. Mean values are indicated with the standard deviation. Each patient compared to a single healthy control whose microRNA expression was set at 1. p value <0.05 assigned *

4.4 RNU44 is a suitable internal control

To ensure consistency in the analysis of the microRNA data the cTvalues of the internal control RNU44 were also compared across groups. RNU44 was initially chosen as it showed the least variation in the preliminary healthy patient cohort. Analysis of the cT values in the larger cohort of 10 healthy, 16 active Crohn's disease and 7 inactive Crohn's disease showed no significant differences in the expression across groups. The mean cT values were 26.07 for healthy, 25.9 for active CD and 26.3 for inactive CD. RNU44 was therefore confirmed as suitable as housekeeping gene.

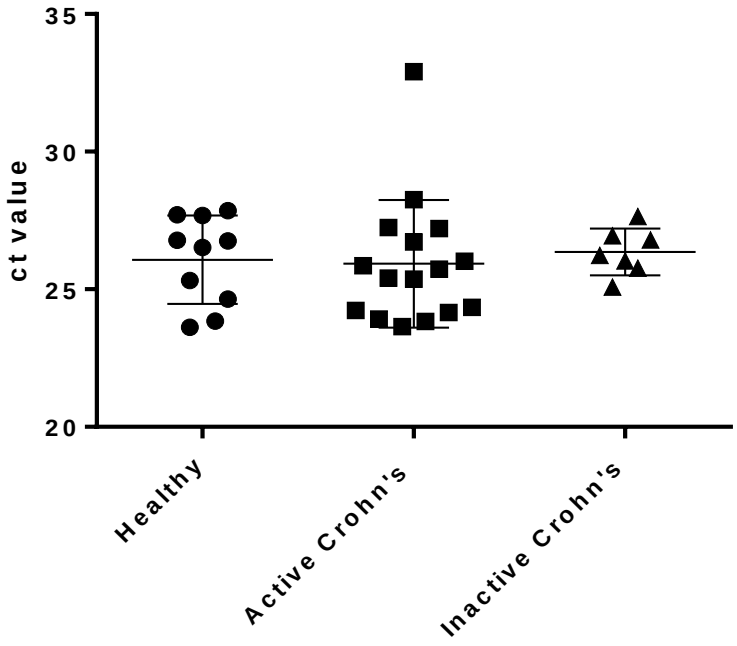


Figure 4.2: Expression data of the internal control RNU44 for healthy patients , active and inactive Crohn's Disease. Each dot represents an individual patient. Mean values are indicated with the standard deviation. Each patient compared to a single healthy control whose microRNA expression was set at 1.

4.5 Expression of miRNA24 and miRNA27a

Encouraged by the significant overexpression of miRNA23a it was decided to also investigate the expression of miRNA24 and miRNA27a. Together these form the 23a-27a-24 cluster. Expression of this cluster is altered in many diseases although the expression in Crohn's disease epithelium is not known (394).

All samples were compared to the same single healthy control used for miRNA23a analysis so as to provide a consistent comparator for expression of the cluster.

4.5.1 miRNA24

There was no significant difference in the expression of miRNA24 in both inactive and active disease compared to healthy controls. The mean fold difference in expression in active disease was 0.7162 compared to 0.34 for healthy (p=0.0968). The mean fold expression in inactive disease was 0.7883 (p=0.0590) as shown in figure 4.3.

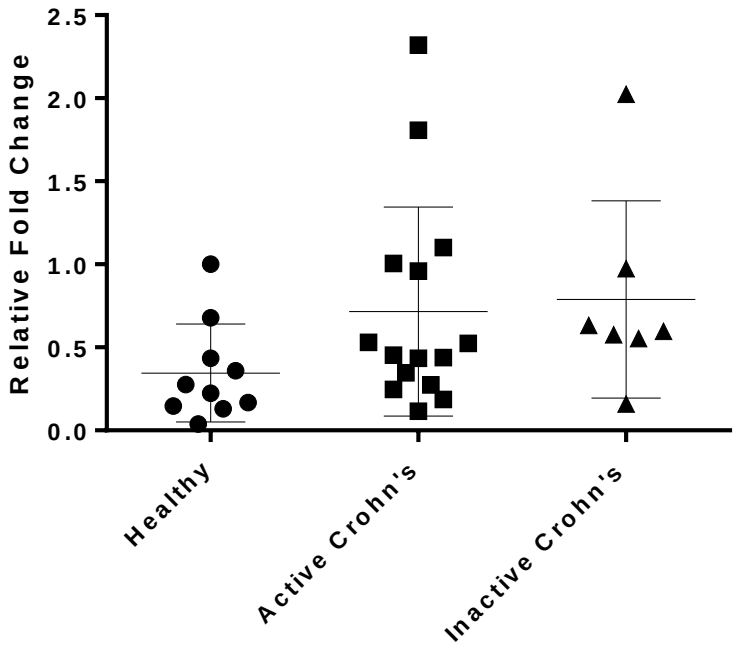


Figure 4.3: MiRNA24 expression in active and inactive Crohn's disease compared to healthy controls. Each dot represents an individual patient. Mean values are indicated with the standard deviation. Each patient compared to a single healthy control whose microRNA expression was set at 1.

4.5.2 miRNA27a

There was no significant difference in the expression of miRNA27a in both inactive disease and active disease compared to healthy controls. The mean fold difference in expression in active disease was 1.135 compared to 0.54 for healthy (p=0.1211). The mean fold expression in inactive disease was 1.131 (p=0.1878).

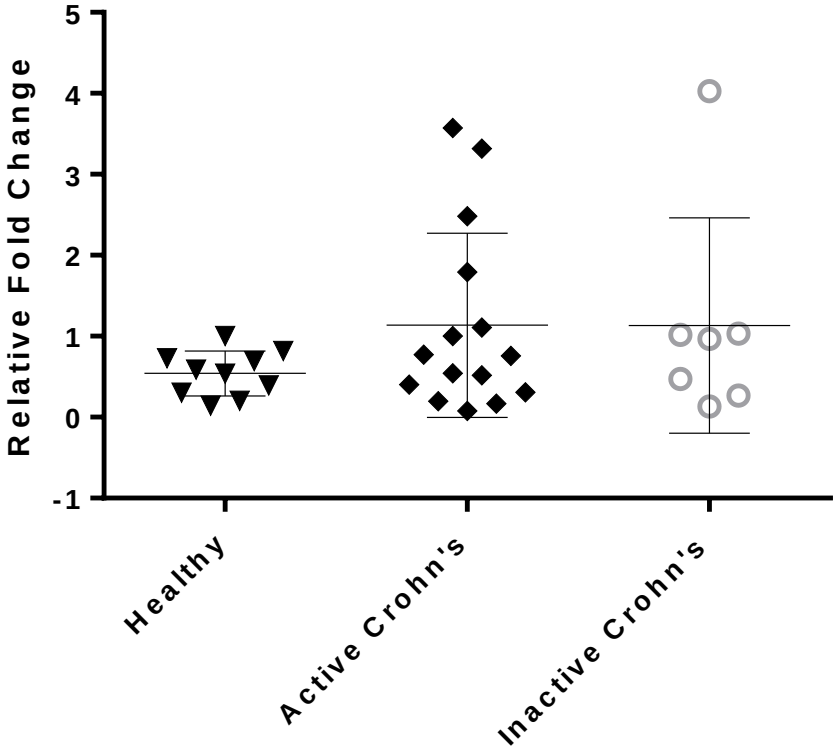


Figure 4.4: MiRNA27a expression in active and inactive Crohn's disease compared to healthy controls. Each dot represents an individual patient. Mean values are indicated with the standard deviation. Each patient compared to a single healthy control whose microRNA expression was set at 1.

In summary although there was a numerical trend towards increased expression, miRNA24 and 27a were not significantly different from healthy controls. These miRNA were therefore not investigated further.

4.6 miRNA23a correlates with clinical symptoms in active disease

Review of the miRNA23a expression data revealed that the spread of data in both the active Crohn's cohorts lent itself to being divided into 2 groups; those individuals with low/normal miRNA23a expression and those with expression greater than the mean of the control group. Figure 4.5 demonstrates this.

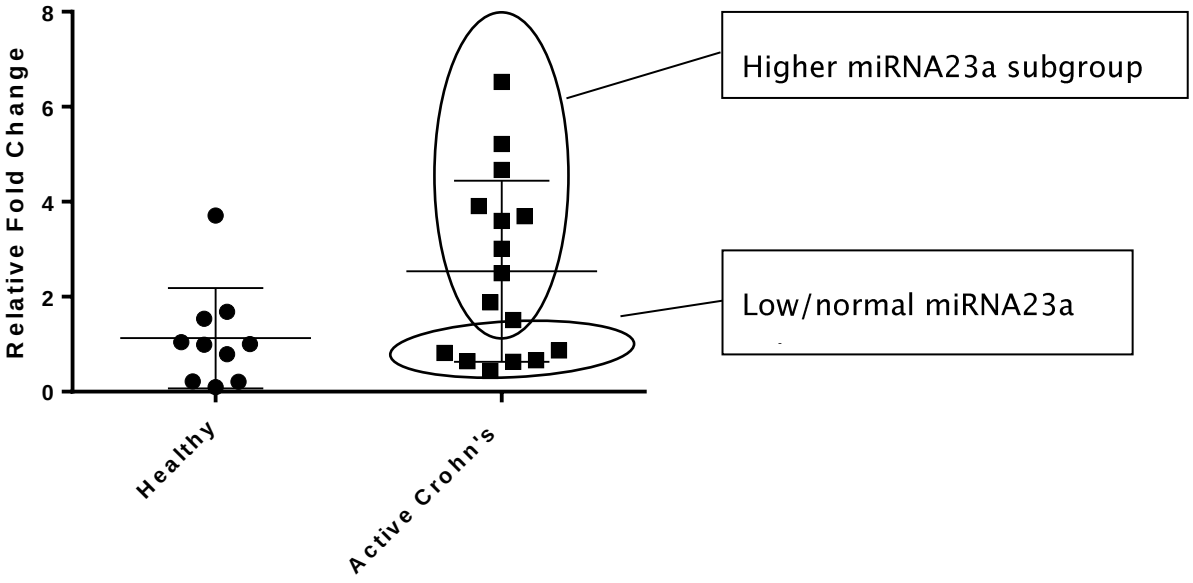


Figure 4.5: Separation of miRNA23a expression in active Crohn's disease.
Each dot represents an individual patient.

Every patient from whom biopsies were collected also provided written consent for the relevant demographic and clinical details about them to be recorded. This dataset was interrogated to evaluate if there were any correlations between miRNA23a expression and clinical features of the cohort focusing initially on stool frequency.

4.6.1 miRNA23a expression and stool frequency

All 16 active CD patients were grouped according to their reported daily frequency of loose stools and miRNA23a expression between groups compared. A loose stool was defined as a type 6 or 7 stool using the Bristol Stool Chart as a reference (465). This is provided in Appendix B.

As before, the same healthy patient was used as the baseline comparator representative of healthy and all patients compared to this. A significant positive correlation was observed between stool frequency and miRNA23a expression with a Pearson coefficient r=0.607 p=0 .001.

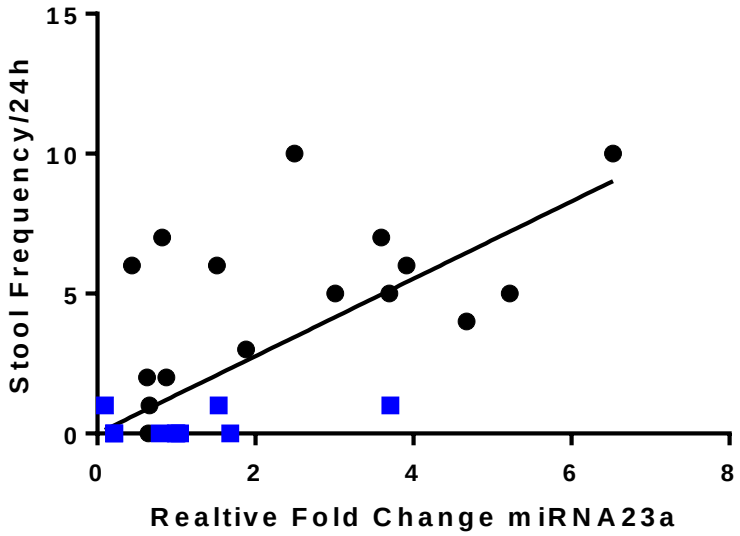


Figure 4.6: Correlation between stool frequency and miRNA23a in active Crohn's disease. MicroRNA23a expression data and stool frequency for each patient. Each point represents an individual patient. Blue squares represent healthy controls. Black circles represent active Crohn's disease patients.

It was noticed from this data that 5 Crohn's patients had a stool frequency <3 /day (bottom left corner of chart). This led to the hypothesis that there was a cut off value of 3 stools daily, above which the patients had on average the greatest miRNA expression. As shown in Figure 4.7, if patients were grouped according to this collective stool frequency, a significant difference was found in miRNA23a expression between those that have ≥4 loose stools per day and both those with ≤3 loose stools or normal formed stool. The relative expression of those with more severe diarrhoea was 3.263 fold greater, than patients with normal stool (p=0.0049), and those with ≤3 loose stools per day (p=0.017).

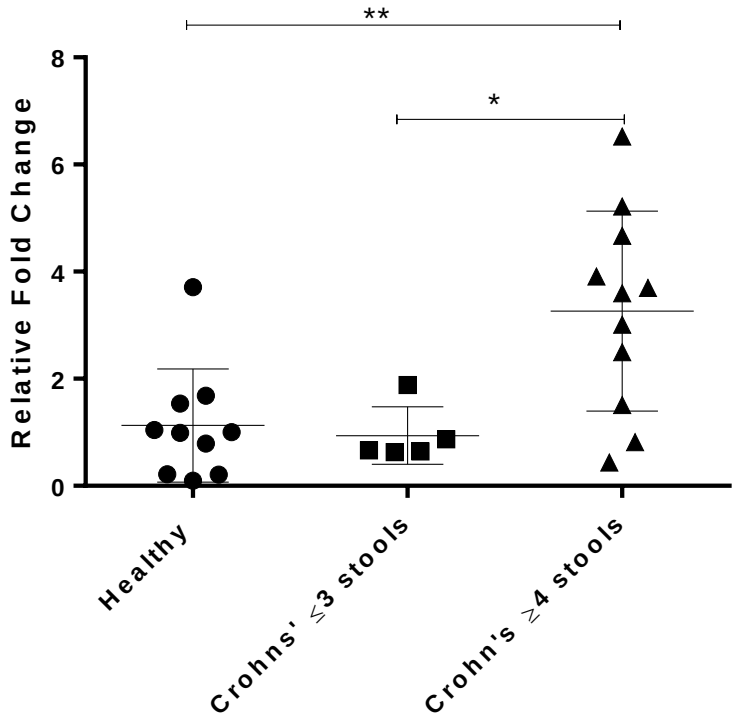


Figure 4.7: MiRNA23a stratified by patient reported stool frequency per day

Each dot represents an individual patient. Mean values are indicated with the standard deviation. Each patient compared to a single healthy control whose microRNA expression was set at 1. $p<0.05$ assigned *. $p<0.001$ assigned **

4.7 Association with clinical characteristics

Encouraged by the positive association with stool frequency the remaining patient characteristics were analysed. Associations were evaluated for significance using Fishers exact test with p,0.05 taken as significant at the 95% confidence interval as shown in table 4.2. With the exception of stool frequency as described above, no other positive associations were identified.

Table 4.2: Correlations between clinical characteristics of the active Crohn's disease cohort and miRNA23a expression

Characteristic	Number of patients		p value
	High 23a group	Low 23a group	
Male	4	3	
Female	6	3	1.0000
Smoker	0	1	
Non smoker	10	5	0.3750
≥4 loose stool/day	9	2	
≤ 3 loose stool/day	1	4	0.0357 *
Blood in stool	7	1	
No blood	3	5	0.1189
Abdominal pain	9	5	
No abdominal pain	1	1	1.0000
Well	2	1	
Unwell	8	5	1.0000
EI manifestations	3	2	
No EI manifestations	7	4	1.0000
Low Hb	3	0	
Normal Hb	7	6	0.2500
High CRP	5	4	
Normal CRP	5	2	0.6329
Low Albumin	5	3	
Normal Albumin	5	3	1.0000

EI = extra intestinal manifestations. CRP = C Reactive Protein. Normal Hb >13 g/dL for male and >11.5g/dL for females. Normal albumin > 35g/L. Normal CRP < 7.5mg/L

4.7.1 miRNA23a correlates with the Harvey Bradshaw Index in Active Disease

The Harvey Bradshaw Index (HBI) is a validated score of Crohn's disease activity with greater scores indicating more severe disease (466)(Appendix C). The HBI was calculated for each CD patient and compared to their miRNA23a result. Patients with greater expression of miRNA23A had a higher average HBI, than those with lower expression. The mean HBI in those in the highest miRNA23a group was 9.7 vs 5.5 in the lower miRNA23a group (p=0.0312).

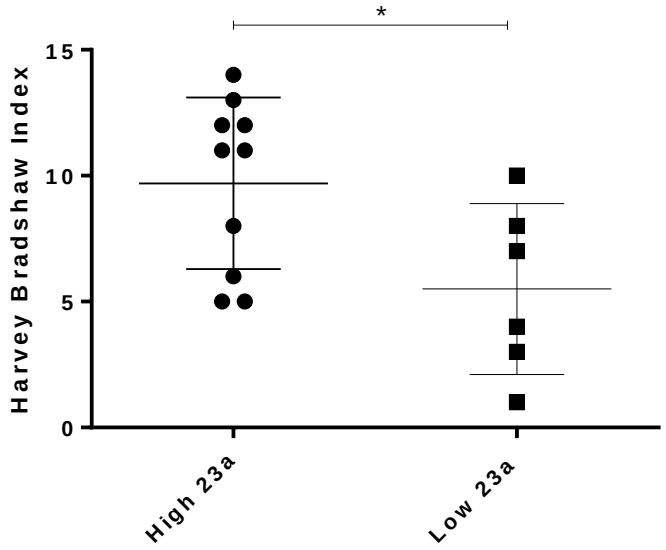


Figure 4.8: HBI score per patient stratified by miRNA23a expression in active disease. Each dot represents an individual patient. Mean values are indicated with the standard deviation. A p value of <0.05 assigned *.

4.8 miRNA23a and inactive disease

A similar distribution of miRNA23a seen in the active cohort was also observed is seen in the inactive group. It should be noted the sample size is smaller and should therefore be interpreted with caution. This is shown in Figure 4.9.

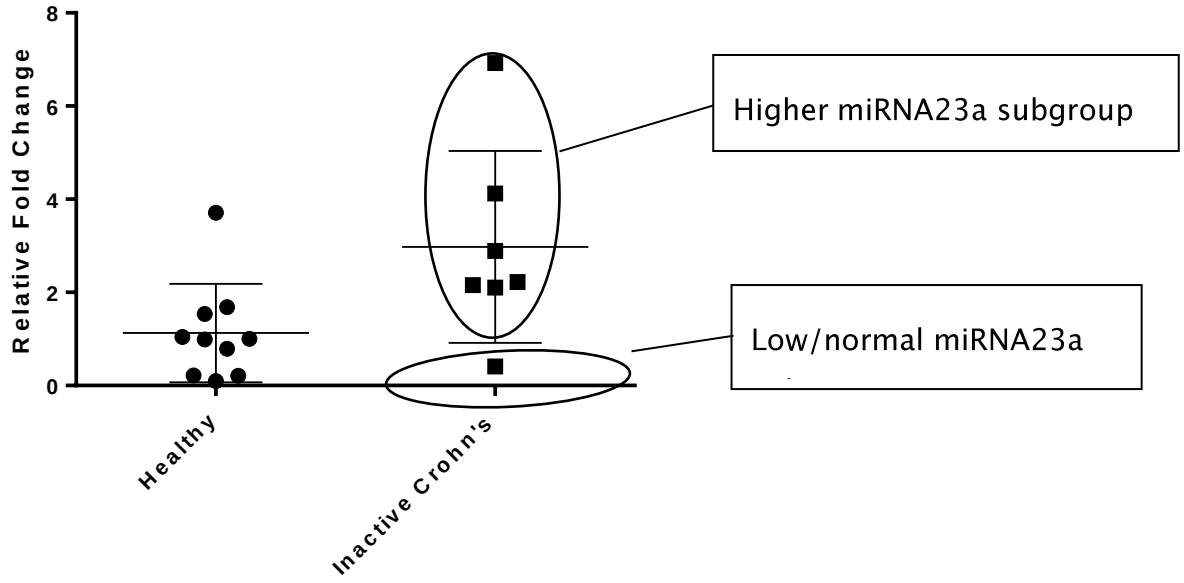


Figure 4.9: Separation of miRNA23a expression in inactive Crohn's disease.

Each dot represents an individual patient.

4.8.1 MiRNA23a does not correlate with clinical symptoms in inactive disease

The clinical dataset was interrogated to evaluate if there were any correlations between miRNA23a expression and clinical features of the inactive cohort. Associations were evaluated for significance using Fishers exact test with $p < 0.05$ taken as significant at the 95% confidence interval. Expression of miRNA23a showed no correlation to any of the clinical parameters.

Table 4.3: Correlations between clinical characteristics of the inactive Crohn's disease cohort and miRNA23a expression

Characteristic	Number of patients		p value
	High 23a group	Low 23a group	
Male	4	0	
Female	2	1	1.0000
Smoker	0	0	
Non smoker	6	1	1.0000
≥4 loose stool/day	1	0	
≤ 3 loose stool/day	5	1	1.0000
Blood in stool	0	0	
No blood	6	1	1.0000
Abdominal pain	2	0	
No abdominal pain	4	1	1.0000
Well	3	1	
Unwell	3	0	1.0000
EI manifestations	0	0	
No EI manifestations	6	1	1.0000
Low Hb	0	0	
Normal Hb	6	1	1.0000
High CRP	0	0	
Normal CRP	6	1	1.0000
Low Albumin	0	0	
Normal Albumin	6	1	1.0000

EI = extra intestinal manifestations. CRP = C Reactive Protein. Normal Hb >13 g/dL for male and >11.5g/dL for females. Normal albumin > 35g/L. Normal CRP < 7.5mg/L

4.8.2 MiRNA23A does not correlate with stool frequency in inactive disease

An analysis of microRNA expression and stool frequency was undertaken for the inactive CD patient biopsy cohort. No significant correlation was found with a Pearson correlation coefficient r = 0.2506 p=0.332

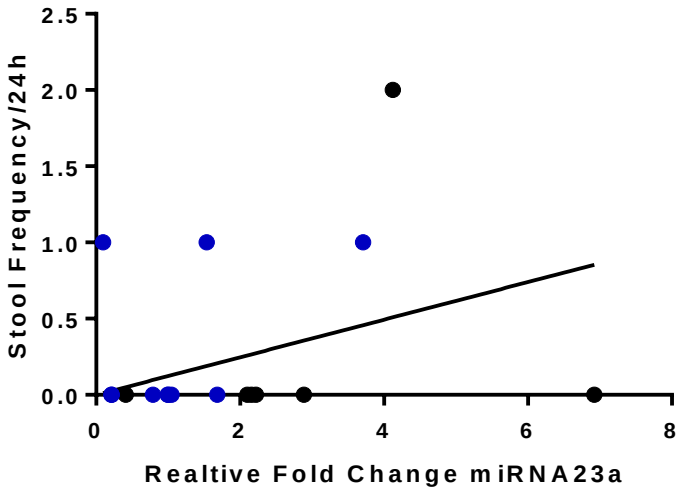


Figure 4.10: Correlation between stool frequency and miRNA23a in inactive Crohn's disease. MicroRNA23a expression data and stool frequency for each patient. Each point represents an individual patient. Blue circles represent healthy controls. Black circles represent inactive Crohn's disease patients.

4.8.3 MiRNA23a does not correlate with Harvey Bradshaw Index in inactive disease

The HBI was calculated for each inactive CD patient and compared to their miRNA23a result. This is shown on figure 4.14. As expected of an inactive disease cohort, all HBI scores were < 5, consistent with clinical remission. Therefore miRNA23A expression does not correlate with clinical indices or the HBI in an inactive disease cohort.

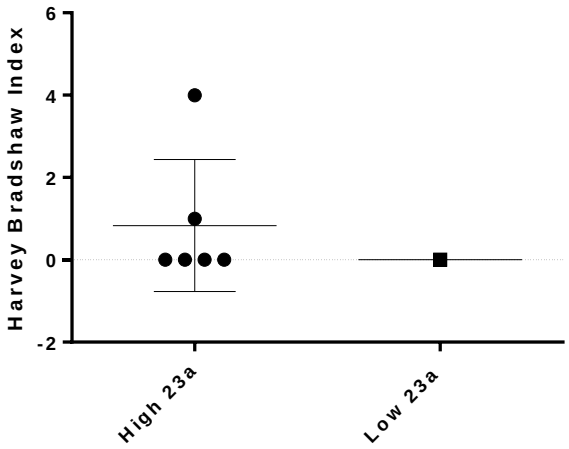


Figure 4.11: HBI score per patient stratified by miRNA23a expression in inactive disease. Each dot represents an individual patient. Mean values are indicated with the standard deviation

4.9 Medication use

Current medication use was collected for every patient. The microRNA expression data as shown in section 4.3 was then expressed with the current treatments displayed for each patient to visualise any potential effects of treatment on microRNA expression. As shown in Figure 4.12 treatment did not have any noticeable effect on the spread of the data.

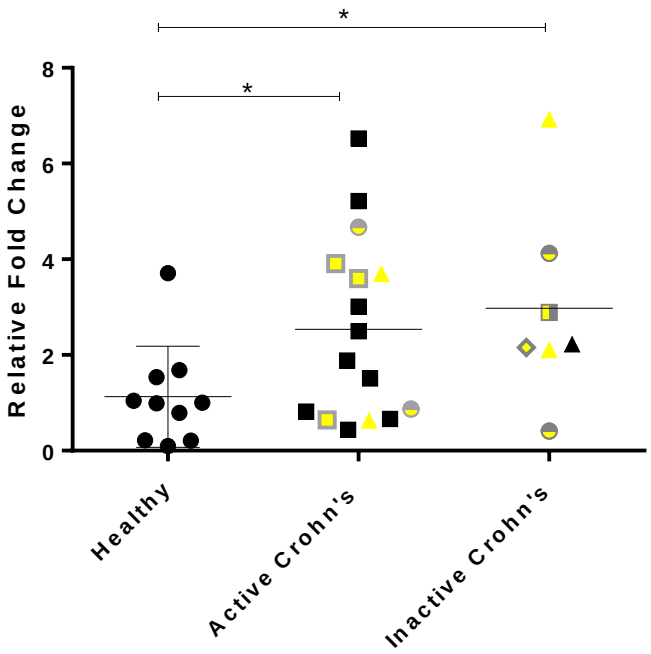


Figure 4.12: Medication usage in Crohn's Disease Cohort

MicroRNA 23a expression data with medication use highlighted. For clarity error bars have been removed. The medication symbol key is as follows

= Oral Steroid, = Thiopurine =TNFα anatagonist,

= Thiopurine/ TNFα antagonist combination, = 5ASA,

or = no treatment

4.10 Discussion

Following on from the preliminary data presented in chapter 3, this study has now characterised the expression profile of microRNA23a within the colonic epithelia of active and inactive Crohn's disease. It showed that miR23a is increased in the epithelium of active and inactive Crohn's Disease, but only in active Crohn's does this increase correlate with clinical disease severity in patients.

MicroRNA 23a has previously been reported to show expression changes in mucosal biopsies from ulcerative colitis cohorts (420). MicroRNA 23b has been reported to show overexpression in Crohn's colitis (434). MiRNA 23b is a

paralogue of miRNA23a differing by just 1 nucleotide (394). Increased serum expression of miRNA23a is also reported in active Crohn's disease (432). The source of expression in these samples is unclear. Macrophages which are abundant in colonic mucosa (467) are known to over express this microRNA and under normal circumstances reduce expression in response to non-pathogenic TLR stimulation (468, 469). It can therefore be reasonably inferred that a large proportion of the microRNA expression observed in the mucosa may be provided by innate immune cells.

Until now the only epithelial characterisation of this microRNA was in the mouse. In murine studies miRNA23a was found to be overexpressed specifically within inflamed colonic epithelial cells in a DSS colitis model (470). However this overexpression was relative to inflamed small intestinal epithelium. When compared to normal colonic mucosa no difference in expression was found (470). The data presented in this chapter therefore provides the first description of miRNA23a expression specifically in human colonic epithelium in Crohn's disease in both active and inactive disease.

Data about miRNA23a in Crohn's disease is lacking. Much of what is known about this microRNA has been provided by oncology studies in which increased tumour invasion and cellular migration have been observed (471-473). This suggest miRNA23a may be implicated in tissue barrier dysfunction. Direct evidence contributing to this comes from a lung cancer study in which miRNA23a indirectly via TGF-β, was shown to down regulate E cadherin in a lung cancer cell line contributing to cell migration and epithelial mesenchymal transition (402). Studies of miRNA23a function in macrophages suggest it is also important in the regulation of innate immune responses (468, 469). The role in IBD and Crohn's disease remains unknown.

It is interesting to note that the overexpression of miRNA23a in this study was found in both active and inactive colonic Crohn's disease patients. This raises the possibility that the increase in miRNA23a is an inherent feature of the disease itself and not solely related to disease activity. As miRNA23a is increased in both active and inactive disease, other factors driving the symptoms of disease may be operating in active Crohn's, in addition to those stimulating the increase in miR23a. This situation may then combine to promote or exacerbate inflammation in active Crohn's Disease, making the disease process more severe.

This raises the intriguing question of what is the functional consequence of the increase in miRNA23a? Active and inactive disease patients have differing clinical

features. Those with active disease have active mucosal inflammation, inflammatory cell infiltrates in the lamina propria (474) and clinical symptoms of diarrhoea. In contrast the inactive patients are essentially well. They report minimal if any loose stools and have little or no inflammatory activity. However both cohorts have a mean MiRNA23a expression that is greater than that of healthy controls.

It should be noted that although mean expression is raised, in both active and inactive cohorts the data is divergent, with a range of values obtained. In active disease expression of miRNA23a was found to correlate with stool frequency. Diarrhoea is a prominent feature of Crohn's disease and reflects underlying epithelia barrier dysfunction (224, 263). As discussed miRNA23a is associated with enhanced cell motility in cancer studies(471, 473) so it is plausible that this may also be a potential effect within the colonic epithelium. A positive correlation was also observed with the HBI clinical disease index. As expected this mirrors the stool frequency. As a single point is awarded in the score for every loose stool it follows that these with the greatest stool frequency are also more likely to have a greater HBI. To provide an insight into the functional relevance of the microRNA change further studies investigating predicted miRNA23a targets will be needed. This is addressed in the next chapter.

Recent work has suggested that microRNA expression is modified by the immunomodulatory drugs used to treat Crohn's disease and may therefore confound microRNA expression data from patient cohorts (475). To take account of this, clinical treatment details were carefully collected for each patient in this study. No clear treatment effect was seen on the epithelial expression of miRNA23a.

Current treatments are directed against adaptive or innate immune signalling pathways. It is possible that in whole mucosal samples global effects on microRNA expression are observed due to the relative abundance of inflammatory cells within the sample. These changes may be from the immune cells and due to their relative abundance any signal from the epithelium may be overwhelmed and undetectable. Our data is obtained from samples highly enriched in epithelial cells, thus reducing the possibility of immune cell 'contamination' of samples. Based on the data in this study it would appear that miRNA23a expression within the epithelium is unaffected by immunomodulation.

MiRNA 23a is part of the intergenic miRNA23a-27a-24 cluster on chromosome 19 q 22 (394). The transcript spans a 2000 base pair region of the chromosome and codes for microRNAs 23a 27a and 24(394). In Crohn's disease epithelium, although a trend towards increased expression of miRNA24 and 27a was seen for both active and inactive disease this was not significantly different from healthy controls. It is recognised that although microRNAs are in close proximity in clusters on the genome, formation of the primary transcript does not always translate into formation of fully mature microRNA (464). This is observed in a wide variety of conditions specifically with this microRNA cluster (394). The expression of parts of this cluster has also been reported to be increased in the intestinal mucosa in ulcerative colitis (394, 420). In the context of Crohn's disease this is the first time differential expression of components of the cluster has been reported. It is interesting to note that although the expression of 24 and 27a is different to healthy it is only 23a which is significantly elevated. Differences in post transcriptional processing may account for this.

As mentioned previously, to further unpick the role of miRNA23a in epithelia, focussed studies are needed with predicted mRNA targets of this microRNA. One of the predicted mRNA targets of miRNA23a is TNFAIP3, a homeostatic negative regulator of NFκB and an important pro inflammatory target in IBD, as reviewed in the Introduction. This protein has immune regulatory and barrier stabilising functions and is therefore an intriguing target for study, particularly given the miRNA changes described. The focus of the next chapter is therefore the potential relationship between miRNA23a and TNFAIP3 expression in colonic epithelia.

Chapter 5:

TNFAIP3 and microRNA23a

Chapter 5: Results

5.1 Introduction

In human samples, TNFAIP3 mRNA has been reported to be reduced in the colonic mucosa of Crohn's disease patients (331). The reason for this decrease is not known. Although reduced expression of the mRNA is seen it is not known if this translates into reduced A20 protein expression. A major limitation of previous work has been the use of whole mucosal biopsies. Similar to miRNA expression, due to the heterogeneous cellular nature of these samples it is difficult to interpret the results in any meaningful way. TNFAIP3 is expressed in cells of the innate and adaptive immune system (476, 477), which will be abundant in inflamed colonic tissue. It is therefore unclear which cell is predominantly responsible for the TNFAIP3 mRNA changes reported in Arnescue's study.

Cell specific expression of A20 within colonic epithelia remains unknown. In the previous chapter, miRNA23A was shown to be overexpressed in the epithelium in Crohn's disease. Bioinformatic data analysis, as discussed in chapter 3, indicated that the miRNA23a seed sequence shows partial complementarity to a sequence within the 3'UTR of TNFAIP3, which infers that an miRNA mediated mechanism of translational repression may be involved in regulation of A20 expression.

Hypotheses:

MiRNA23a reduces A20 protein levels via its seed target sequence in the 3'UTR of TNFAIP3.

A20 protein encoded by the TNFAIP3 gene is reduced in the epithelium of Crohn's disease biopsies compared to biopsy sections from healthy patients

Aims

The aim of this section was therefore to determine if miRNA23a is able to repress the expression of luciferase protein when the 3' UTR of TNFAIP3 is linked to luciferase mRNA. Also to assay A20 protein expression in the same colonic

Crohn's disease biopsies from clinically characterised patients that were defined in Chapter 4 as overexpressing miRNA23a.

Objectives

- To confirm if exogenous transfection of an miRNA23a expressing plasmid represses luciferase activity, via the TNFAIP3 3'UTR with miRNA23a target seed, in a reporter assay.
- To immunohistochemically localise TNFAIP3/A20 protein in colonic epithelium from Crohn's disease and healthy patient samples, using adjacent sections from the same clinically characterised patient biopsies used for miRNA23a quantification.
- To quantify TNFAIP3/A20 immunohistochemical localisation within the colonic epithelium in the sections above.

5.2 MiRNA23a represses translation of TNFAIP3

To determine if TNFAIP3 is a valid target of miRA23a, a luciferase reporter experiment was undertaken. As described in the methods the pre-Mir of 23a was cloned in the pcDNA3.1 mammalian expression vector and transfected in to Hela cells. The 3'UTR of TNFAIP3, or a 3'UTR with the miRNA23a target site mutated, were cloned into pRLTK reporter vectors 3' to *Renilla luciferase* and transfected into Hela cells. These constructs were pre-prepared in our lab and had been stored as DNA at -20^{o}C (Geraint Dingley unpublished). Due to the length of time these had been stored it was decided not to use this DNA directly. Instead Dr Dingley's original TNFAIP3 constructs were re transformed into competent cells and fresh DNA maxi-preparations were made. These were then analysed with a restriction enzyme digest and sequence analysis to ensure the construct sequences were as expected.

5.2.1 Restriction Enzyme Digest Analysis

The pRLTK plasmid contains a Not1 restriction site at position 1978 (see section 2.11 in the Materials and Methods). This allows the intact plasmid to be linearised using a Not1 restriction enzyme. As only one Not1 site exists in this plasmid this would be expected to produce a single band when run on an agarose gel. An initial digest with both the TNFAIP3 wild type (wt) and TNFAIP3 mutant plasmids confirmed this was the case and confirmed the presence of DNA in the preps at

the expected molecular weight (plasmid is 5629bps with the insert) as shown in Figure 5.1.

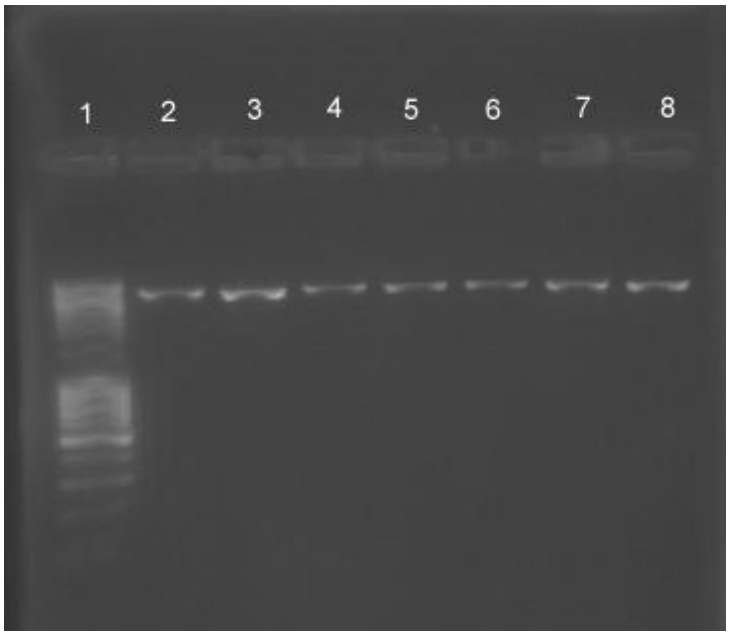


Figure 5.1: Restriction digest with Not1.

Lane 1 Ladder. Lane's 2-5 TNFAIP3wt NOT1 digest Lane's 6-8 TNFAIP3 mutant NOT1 digest. The single band in lane 2 to 8 indicates a linearised plasmid cut with Not1 producing a single fragment.

The mutational changes in the miRNA23a binding site engineered an EcoR1 restriction site into the 3'UTR of TNFAIP3. This is in addition to the EcoR1 sequence which already exists within the pRLTK plasmid. Each construct was therefore incubated with an EcoR1 restriction enzyme. As the wild type plasmid has only one EcoR1 site only a single cut would be expected and therefore a single band when run on a gel. The mutated TNFAIP3 construct has an additional site, therefore the restriction enzyme would be expected to cut twice, resulting in 2 distinct bands when run on a gel.

As shown in Figure 5.2, the mutated 3'UTR produced 2 separate fragments indicating that the plasmid was cut at 2 EcoR1 sites. When the same construct was incubated with a Not1 restriction enzyme, as only one Not1 site is present in the plasmid, only a single cut and thus a single band is produced. The wild type plasmid produced only a single band when cut with EcoR1 confirming that it does

not have a second site. Together these results suggest that the TNFAIP3 mutant plasmid remains intact.

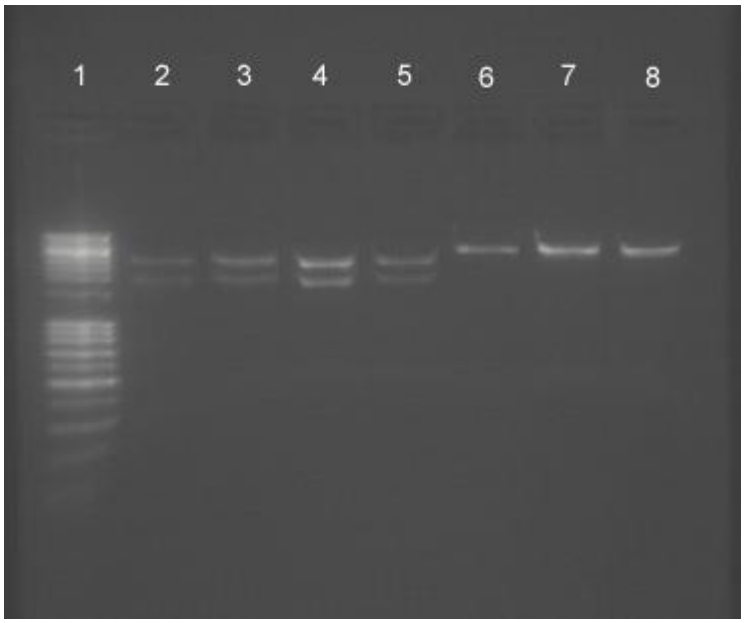


Figure 5.2: Restriction digest with Not1 or EcoR1.

Lane 1 ladder. Lane 2-5 TNFAIP3mut EcoR1 digest showing 2 distinct bands indicating 2 EcoR1 sites within the construct. Lane 6 TNFAIP3mut Not 1 digest showing a single band, Lane 7-8 TNFAIP3wt EcoR1 digest showing a single band.

5.2.2 Sequence Analysis

To confirm the suggestion from the restriction enzyme analysis that the TNFAIP3 constructs were intact, with the mutated miRNA23a site remaining in place, samples of the TNFAIP3 wild type and TNFAIP3 mutant constructs were sent for sequence analysis. The resulting sequences were them compared to the original cloning sequences for each construct using the BLAST nucleotide align algorithm. This confirmed that the microRNA23a binding site was still mutated as before in the TNFAIP3 construct and the binding site was intact and correct in the wild type construct. These constructs were therefore used for transfection experiments in Hela cells.

5.2.3 TNFAIP3 Luciferase Reporter Assay

Combined data from 5 independent experiments (transfection performed in duplicate for each experiment) showed a significant reduction in luciferase

activity when miRNA23a is transfected in the presence of the 3'UTR of TNFAIP3. Relative expression of luciferase 23a/TNFAIP3 was 0.69 (p=0.0256). This recovers when the microRNA binding site is mutated. Relative expression of luciferase 23a/TNFAIP3 mutant was 1.04 (p=0.8904). These results confirm that miRNA23a is able to repress the translation of TNFAIP3 via its 3'UTR. For final confirmation, transfection of miRNA23a in the absence of the 3'UTR of TNFAIP3 was performed. This had no effect on the translation of the luciferase gene. Relative expression of luciferase 23a/pRLTK was 1.13 (p=0.1904). Data was analysed with the non-parametric Wilcoxon signed rank matched pairs test.

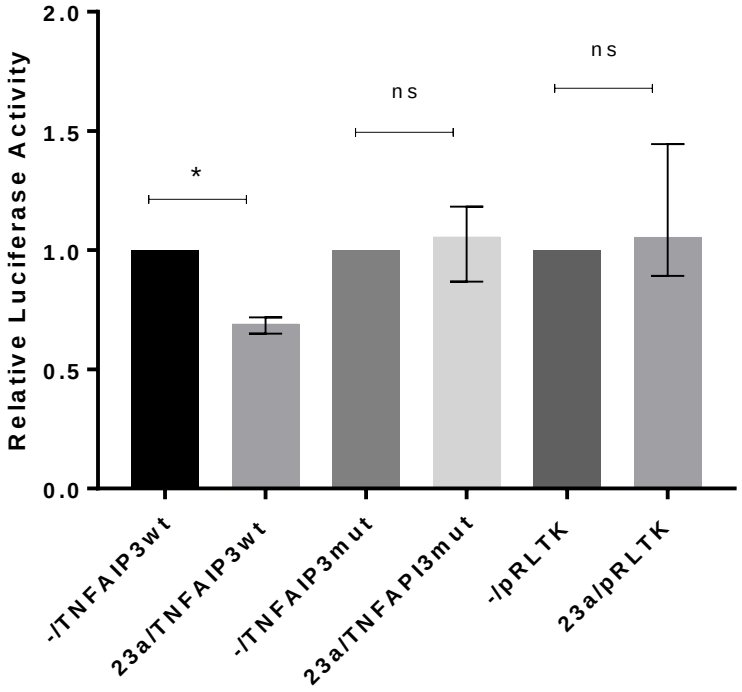


Figure 5.3: Relative luciferase activity in Hela cells transfected with pRLTK/TNFAIP3 3'UTR and pcDNA3.1/miR23a with appropriate comparisons and controls as labelled.

Empty pRLTK denoted -. pRLTK/TNFAIP3 wild type denoted TNFAIP3wt. pRLTK/TNFAIP3 with the microRNA seed sequence site mutated denoted TNFAIP3mut. pcDNA3.1 microRNA23a expression vector denoted 23a. Data is expressed as the median with range. p value <0.05 is represented by *

5.3 TNFAIP3 mRNA

Encouraged by the results of the luciferase reporter assay, TNFAIP3 mRNA expression was investigated in the colonic epithelium of the same patients used

for the miRNA analysis. Expression of the mRNA was normalised to GAPDH as internal control. All samples were compared to a single healthy control, whose cT values were closest to the mean of the group and therefore taken to be representative.

As can be seen in Figure 5.4 there was no significant difference in TNFAIP3 mRNA expression between the Crohn's disease cohorts and healthy control. The relative expression of active disease compared to healthy was 1.155 (p=0.333). For inactive disease relative expression was 0.3703 (p=0.2941). There was no significant difference when both disease groups were compared (p=0.0656).

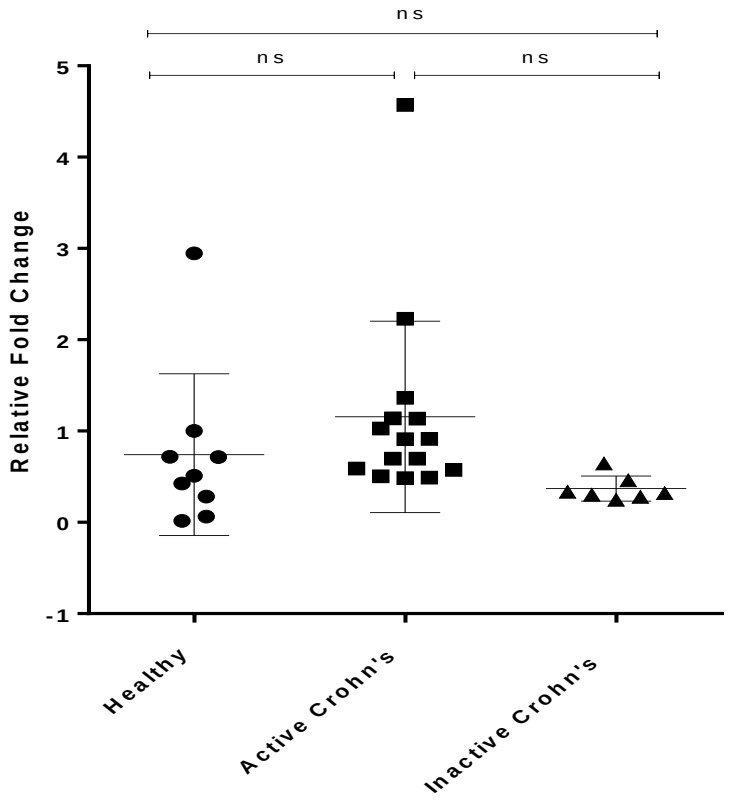


Figure 5.4: mRNA expression for TNFAIP3.

Mean values with standard deviation shown. Each data point represents an individual patient. Healthy n=9 Active CD n=15 Inactive CD n=7

5.4 TNFAIP3 Immunohistochemistry

Having demonstrated that miRNA23a is able to repress translation of TNFAIP3, the next aim was to investigate the expression of the TNFAIP3 protein (A20) in colonic epithelium.

Serial sections of the same biopsies used for the miRNA23a quantification were cut into 4um sections and mounted on plain glass slides. These were incubated overnight at 4°C with a rabbit polyclonal antibody against TNFAIP3 as described the methods chapter. Initial antibody titration experiments were undertaken using normal colonic biopsies to determine the optimum antibody concentration. This was determined to be 1:2000 (500ng/ml). All sections were examined using plain white light microscopy at 400x magnification.

5.4.1 Active Crohn's Disease

In biopsies from healthy controls, strong TNFAIP3 staining was seen at apical regions of epithelial cells, with particular densities of staining at the apicolateral cell junctions of similar location to tight junctions. In biopsies from active Crohn's disease there was an observed loss of staining from these regions. In other sections staining was still present but reduced in intensity or patchy in nature. Representative images are shown.

Healthy control 1

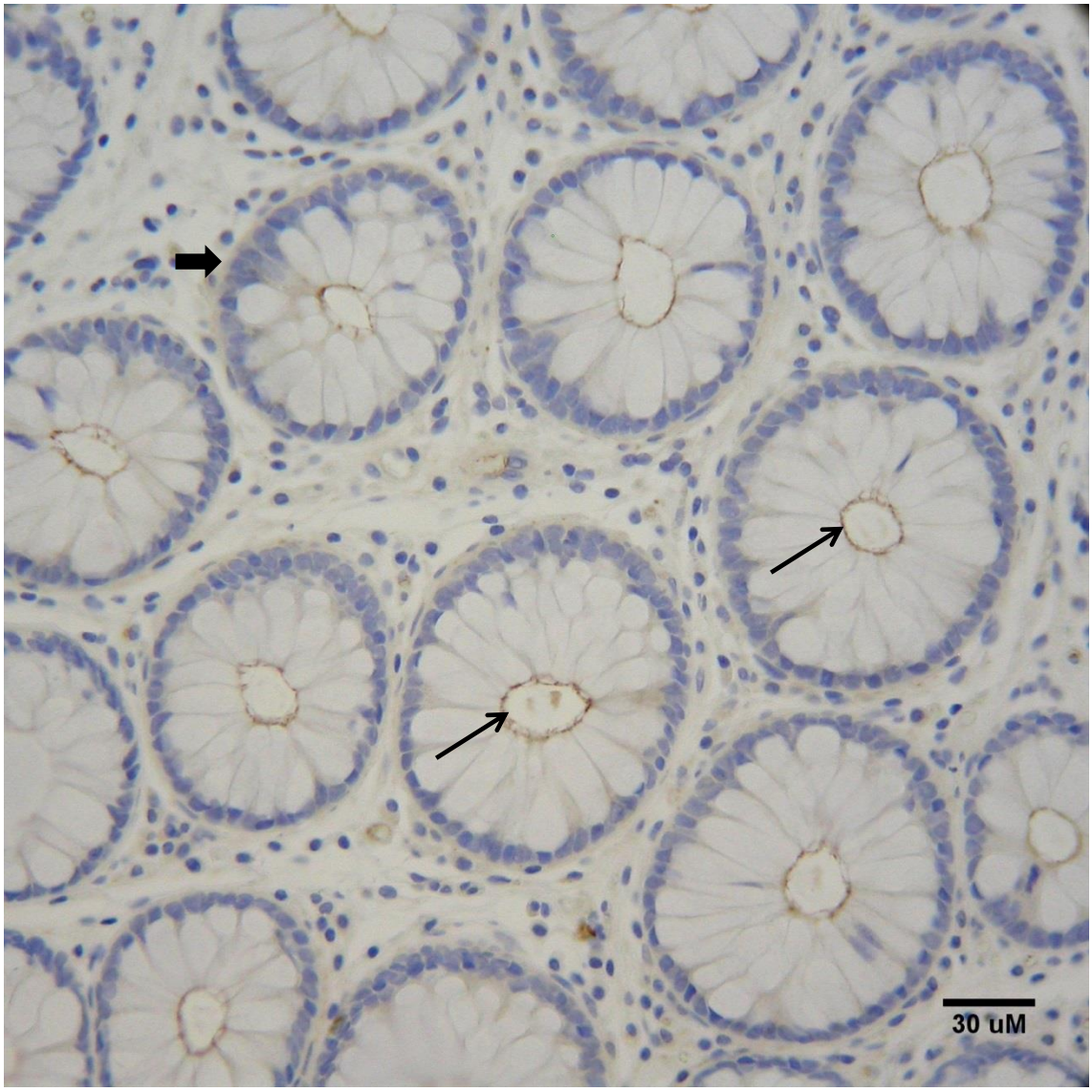


Figure 5.5: Representative images of healthy control colonic biopsy sections stained with a polyclonal anti-TNFAP3 antibody with a HRP labelled secondary antibody detection system. Strong apical and apicolateral densities of staining is seen (thin arrow), together with some diffuse basal cytoplasmic localisation (thick arrow).

Healthy control 2

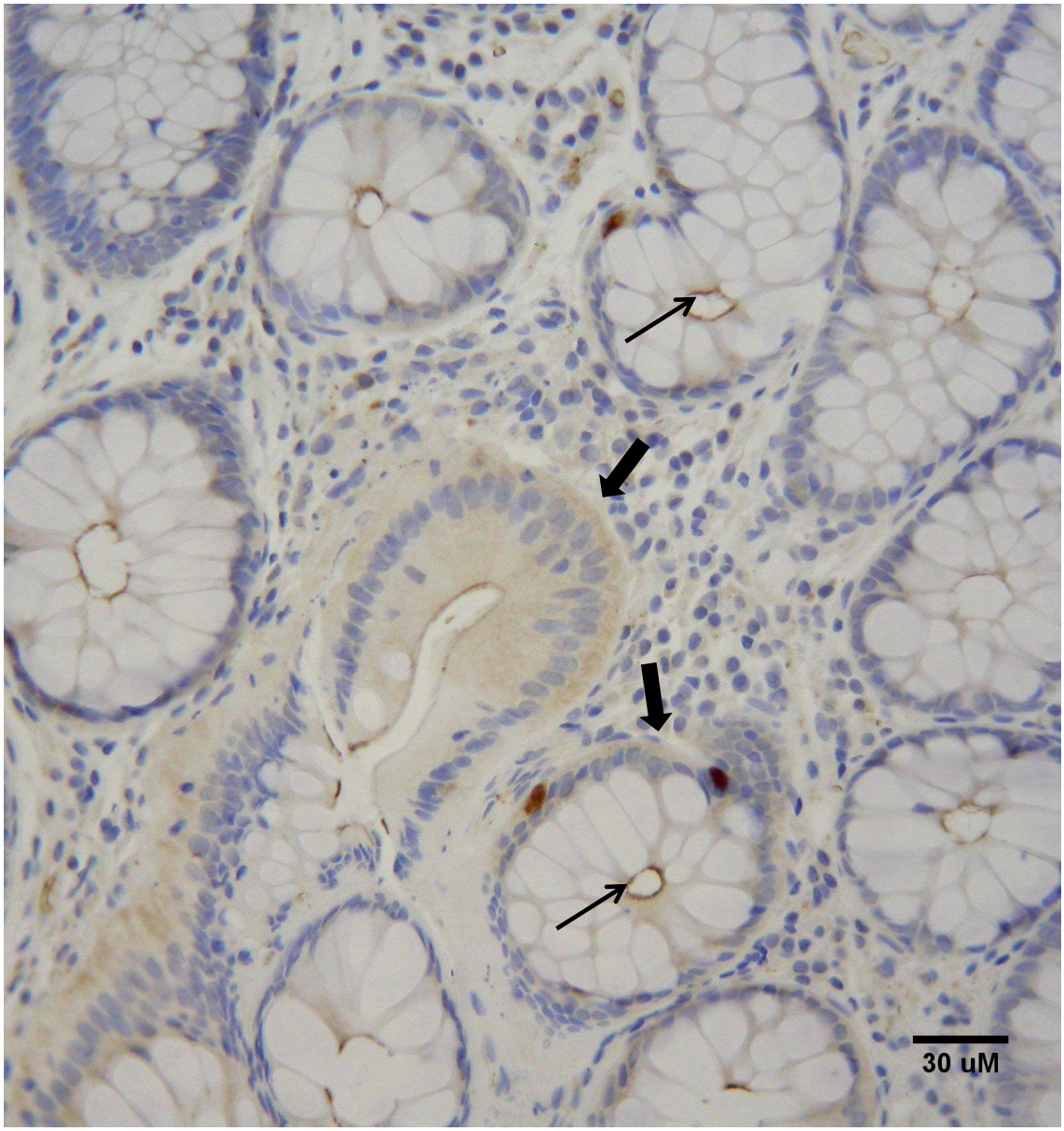


Figure 5.6: Representative images of healthy control colonic biopsy sections stained with a polyclonal anti-TNFAP3 antibody with a HRP labelled secondary antibody detection system. Strong apical and apicolateral densities of staining is again seen (thin arrows), together with more basal cytoplasmic localisation (thick arrow).

TBS control

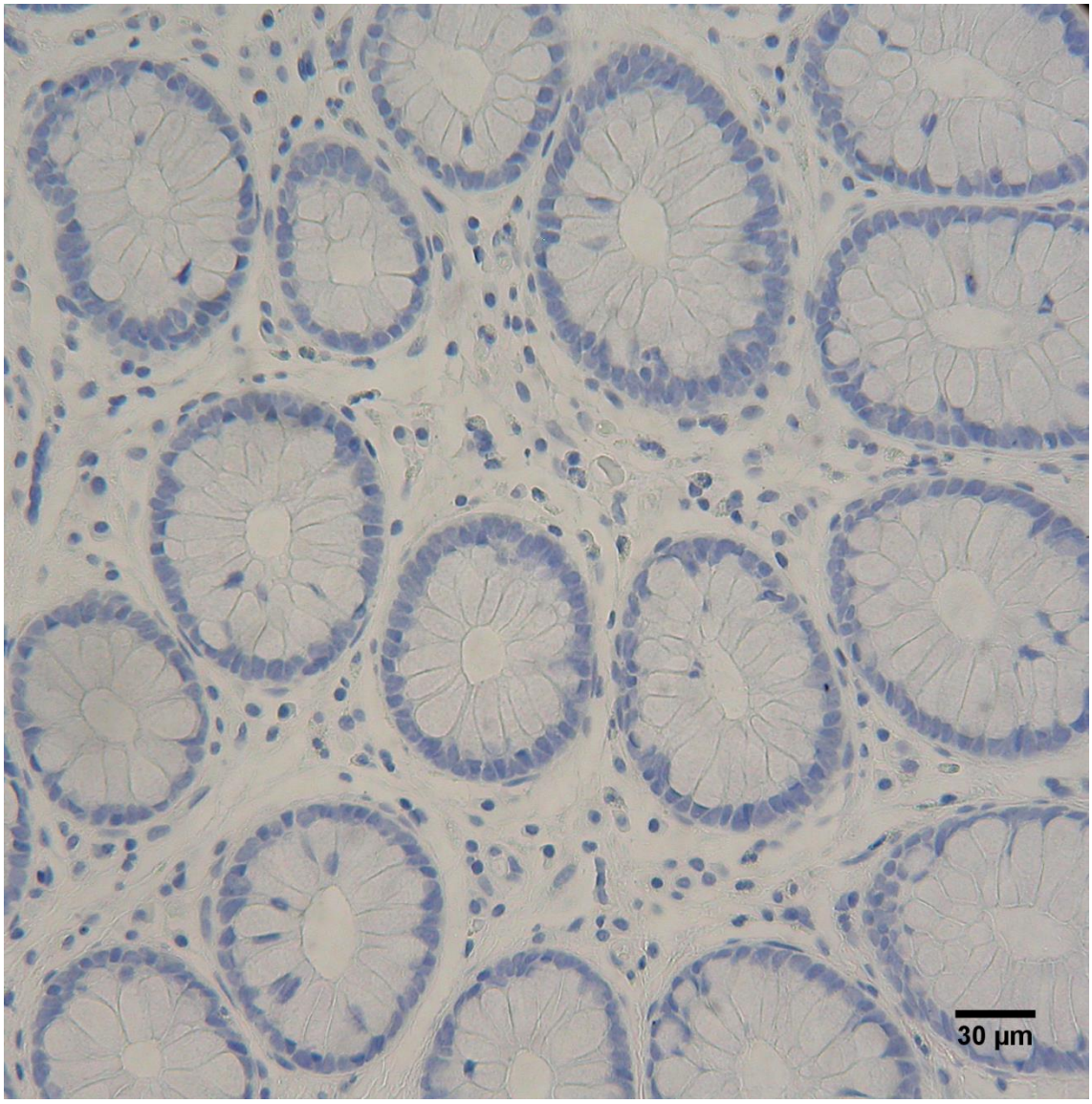


Figure 5.7: Representative images of healthy control colonic biopsy section stained with a secondary antibody HRP labelled detection system only. A total absence of staining was observed, confirming that the secondary antibody is not contributing to the staining seen with the primary antibody.

Active Crohn's Disease 1

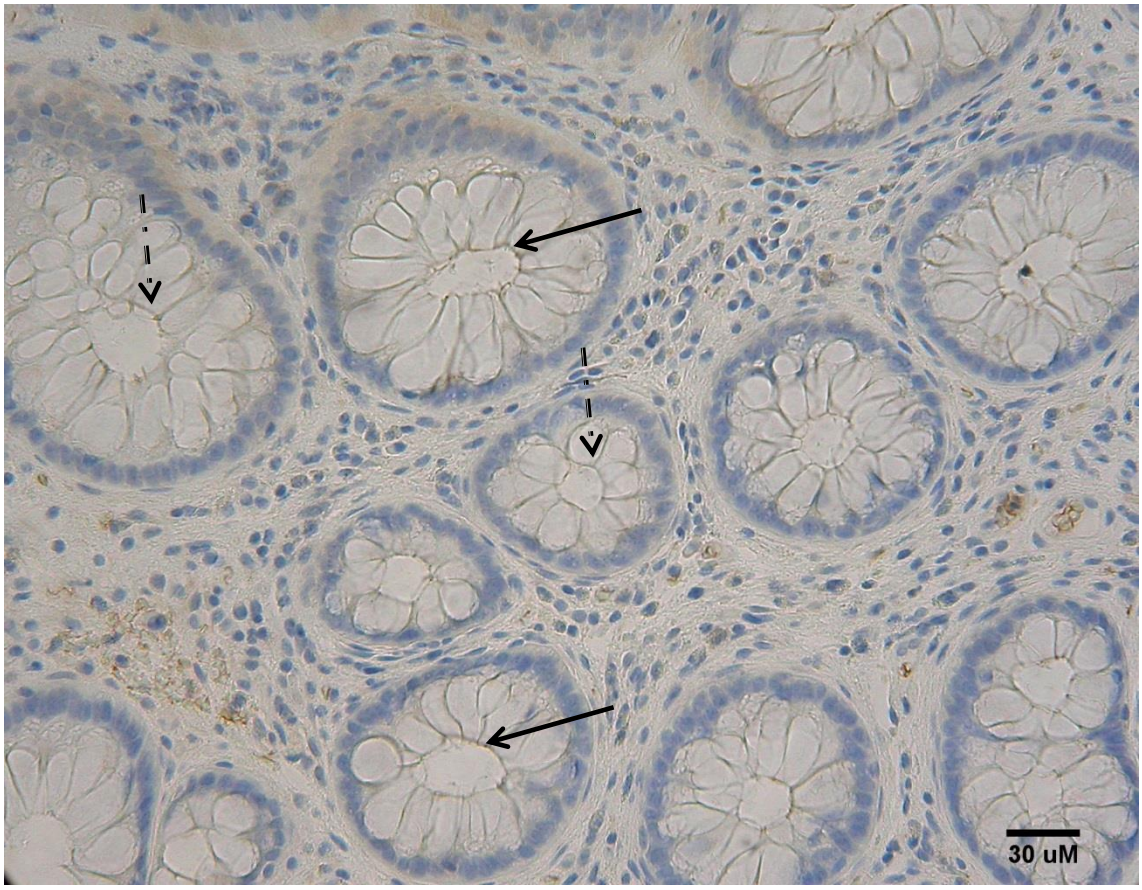


Figure 5.8: Representative images of active Crohn's disease colonic biopsy sections stained with a polyclonal anti-TNFAP3 antibody with a HRP labelled secondary antibody detection system. Although still present within the apical and apicolateral regions, TNFAIP3 staining is reduced in intensity (solid arrows). In other areas staining is lost (dash arrows). Basolateral staining is also absent.

Active Crohn's disease 1

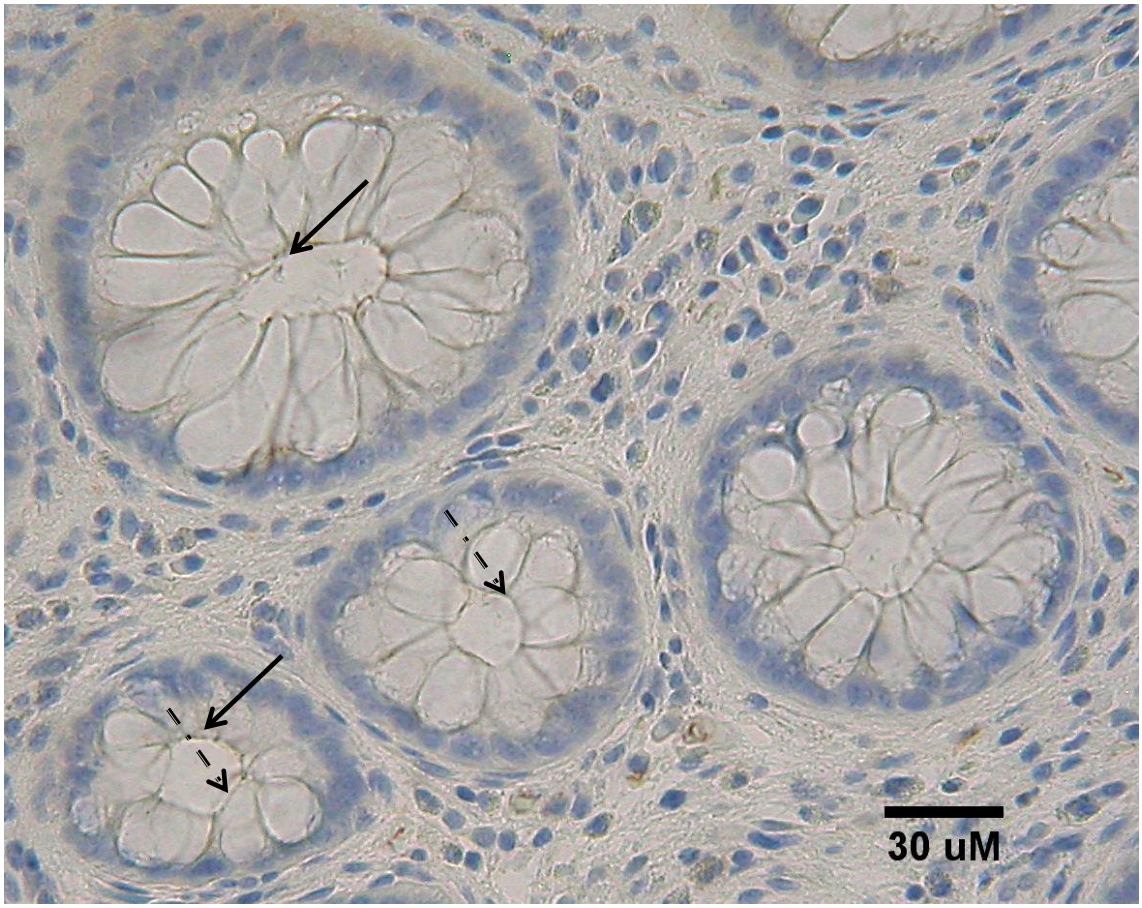


Figure 5.9: Representative images of active Crohn's disease colonic biopsy sections stained with a polyclonal anti-TNFAP3 antibody with a HRP labelled secondary antibody detection system. Cropped enlarged image from figure 5.8, showing almost total loss of TNFAIP3 from apical surface (dash arrow). Discrete of foci of weak apicolateral positive staining are also seen (solid arrow).

Active Crohn's Disease 2

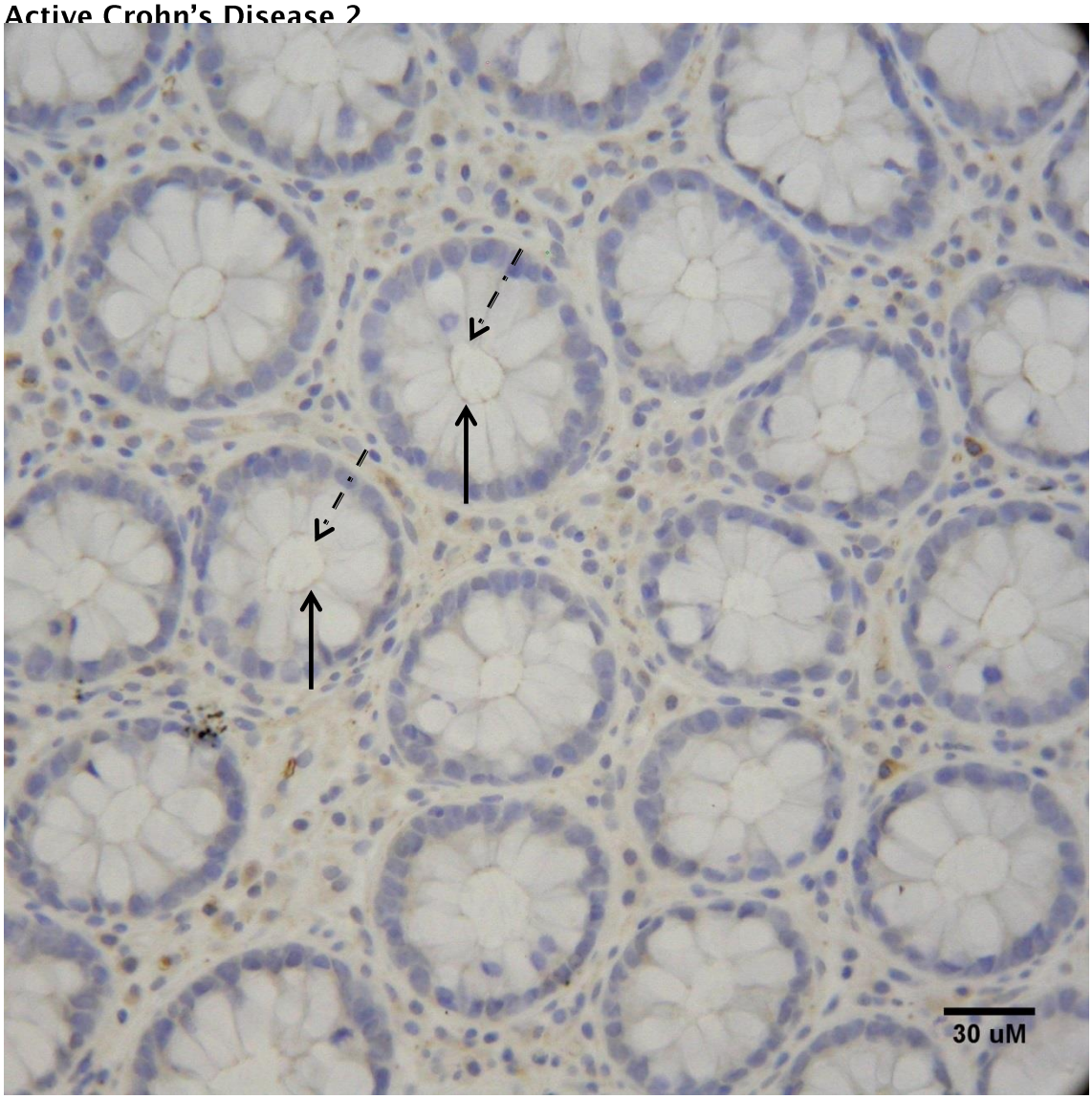


Figure 5.10: Representative images of active Crohn's disease colonic biopsy sections stained with a polyclonal anti-TNFAP3 antibody with a HRP labelled secondary antibody detection system. Discreet areas of apical TNFAIP3 staining (solid arrow) with areas of staining loss (dash arrow)

Active Crohn's Disease 3

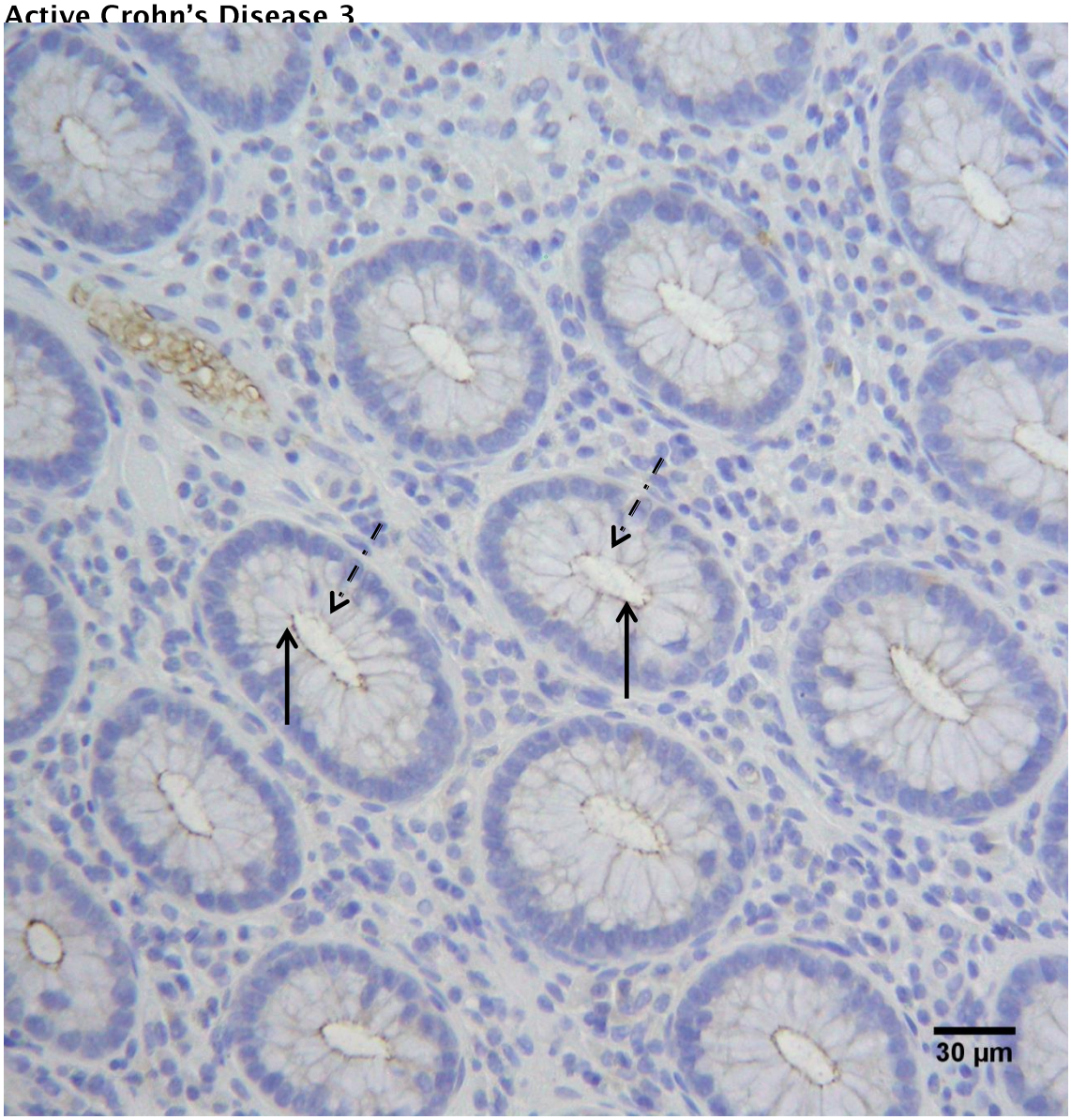


Figure 5.11: Representative images of active Crohn's disease colonic biopsy sections stained with a polyclonal anti-TNFAP3 antibody with a HRP labelled secondary antibody detection system. Discreet areas of apical and apicolateral TNFAIP3 staining (solid arrow) with areas of staining loss (dash arrow)

TBS

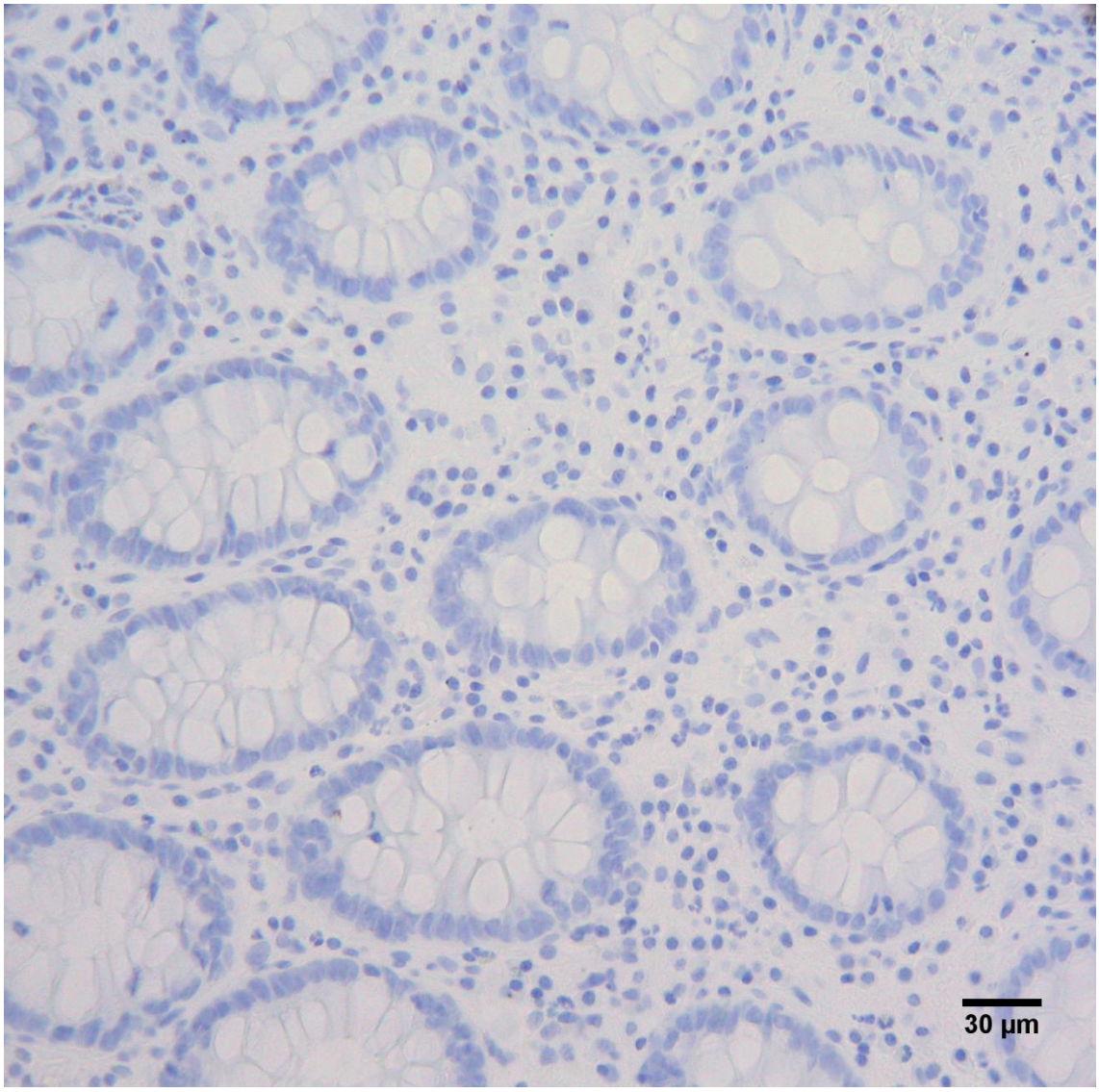


Figure 5.12: Representative control section from active Crohn's patient stained with secondary antibody HRP labelled detection system only. A total absence of staining was observed confirming that the secondary antibody is not contributing to the staining seen with the primary antibody.

5.4.2 Active Crohn's Disease Quantification

Using the immunohistochemical staining scoring system described in the Materials and Methods section 2.9, a subset of biopsy sections from of 5 healthy patients and 9 active Crohn's disease patients were quantitated for staining amount and intensity. The remaining biopsies (5 healthy, 7 active CD) were unsuitable for analysis. This was due to poor architectural preservation of the tissue following antigen retrieval or folded tissue sections on the slide making them unusable. Using the system outlined each patient had a total of 25 randomly selected epithelial crypts evaluated. The maximum score per epithelial crypt was 28. The maximum available score per patient for all 25 crypts was therefore 700.

Epithelial sections from active Crohn's disease patients had a significantly lower staining score than healthy controls. The median score for the healthy patients was 566 compared to 145.5 in the Crohn's patients (p= <0.0001).

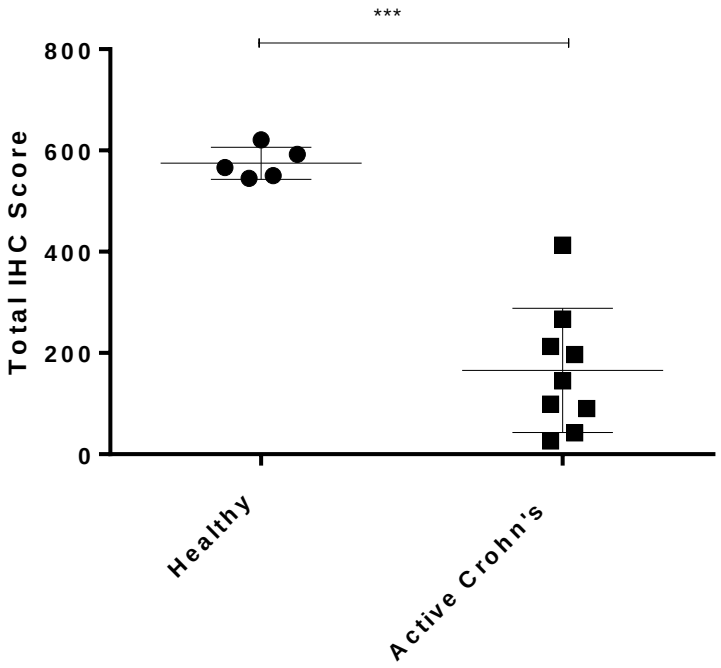


Figure 5.13: A20 immunohistochemistry staining score in active Crohn's disease. Data presented as the median score with range. Each data point represents an individual patient. P value <0.001 assigned ***

5.4.3 Inactive Crohn's Disease

Using the same healthy patient sections for comparison, similar reductions in TNFAIP3 staining, as those seen in active disease were observed in the inactive disease cohort. Representative images are shown.

Inactive disease 1

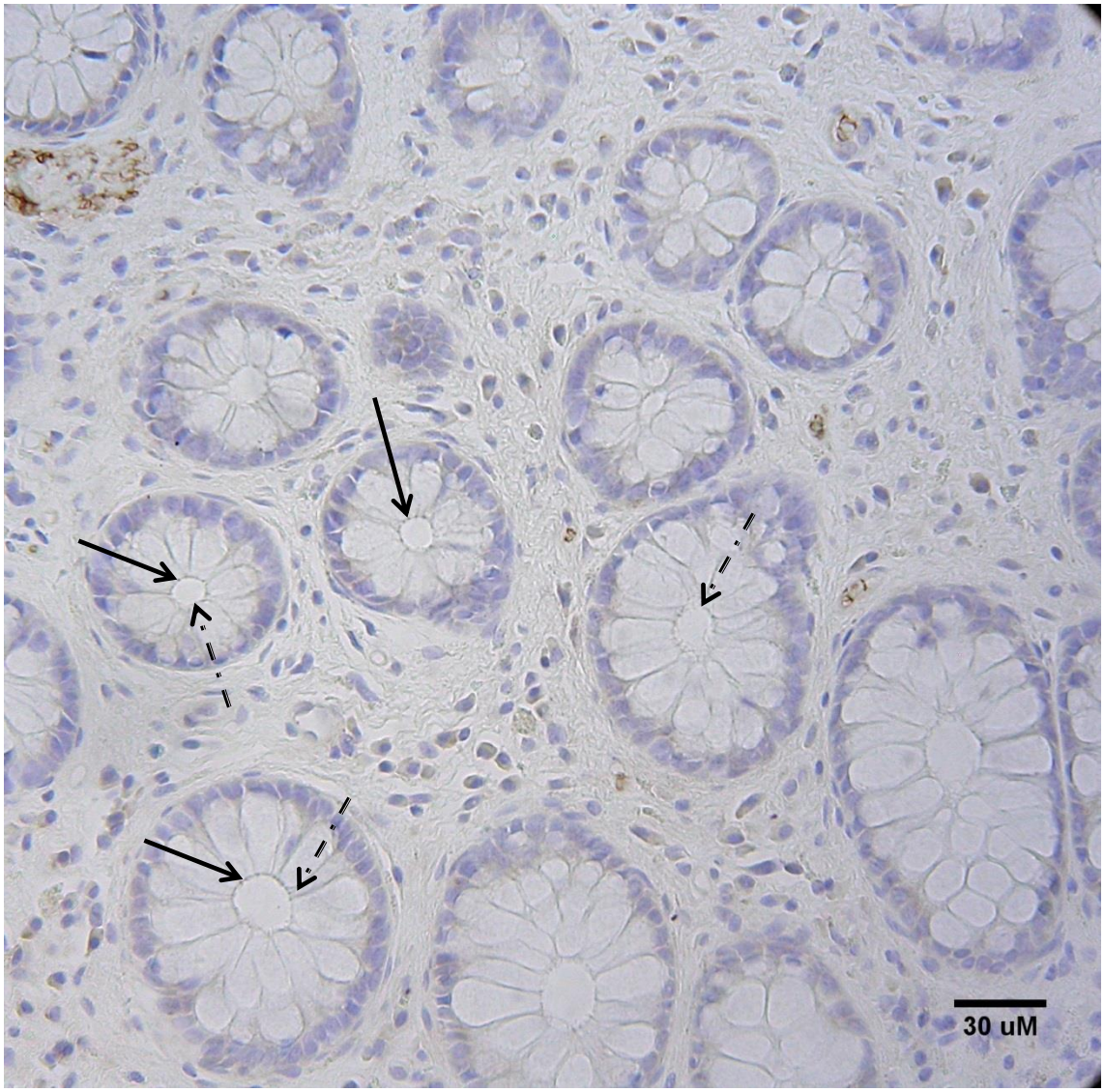


Figure 5.14: Representative images of TNFAIP3 staining in inactive Crohn's disease colonic biopsy sections stained with a polyclonal anti-TNFAP3 antibody with a HRP labelled secondary antibody detection system. Weak discreet areas of TNFAIP3 are seen (solid arrows). Large areas of apical staining loss are also observed (dash arrow).

Inactive disease 1

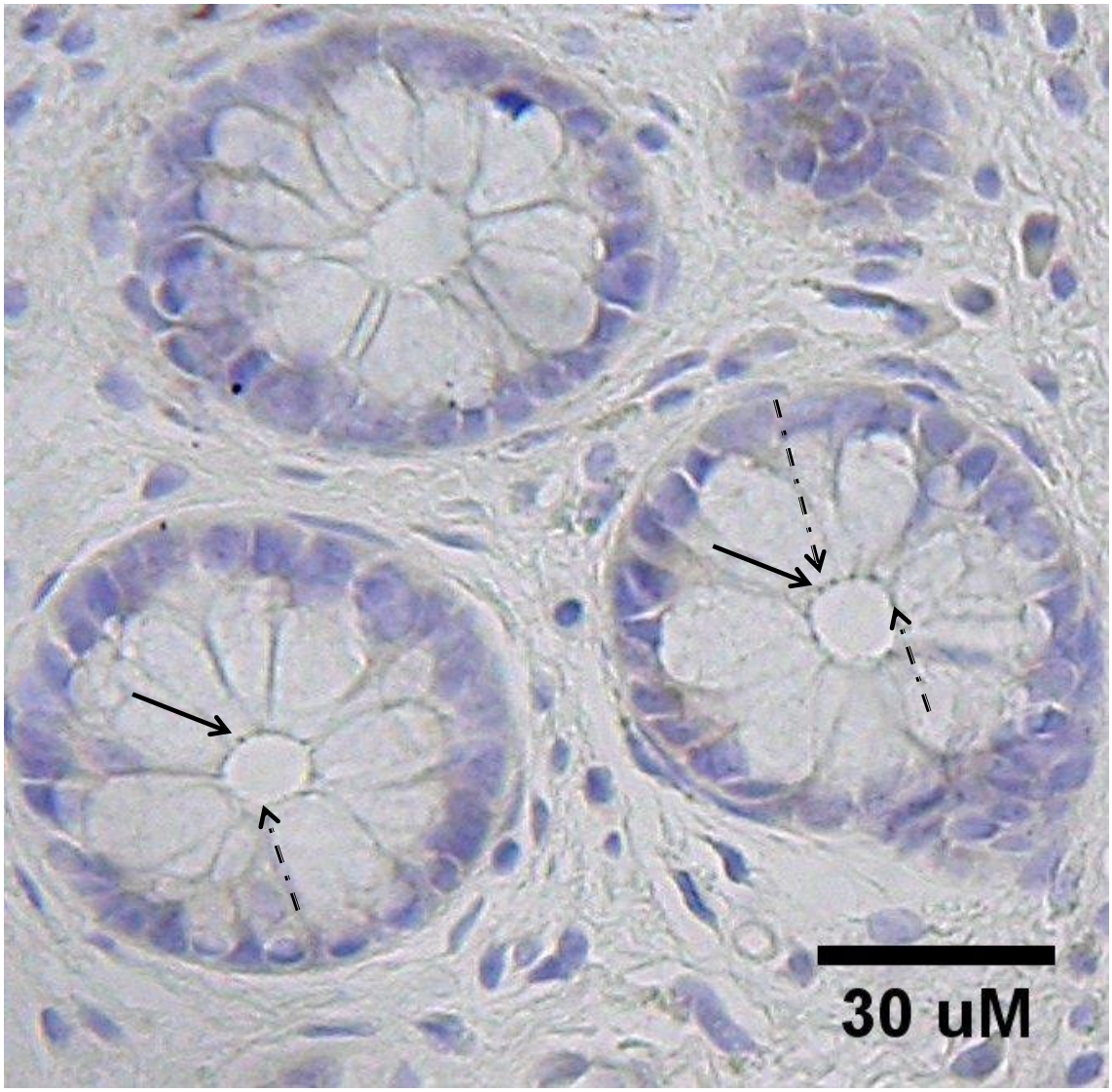


Figure 5.15: Representative images of TNFAIP3 staining in inactive Crohn's disease colonic biopsy sections stained with a polyclonal anti-TNFAP3 antibody with a HRP labelled secondary antibody detection system. Cropped enlarged image of figure 5.14showing discreet areas of weak TNFAIP3 staining (solid arrow) and areas of apical and apicolateral staining loss (dash arrow).

Inactive Crohn's disease 2

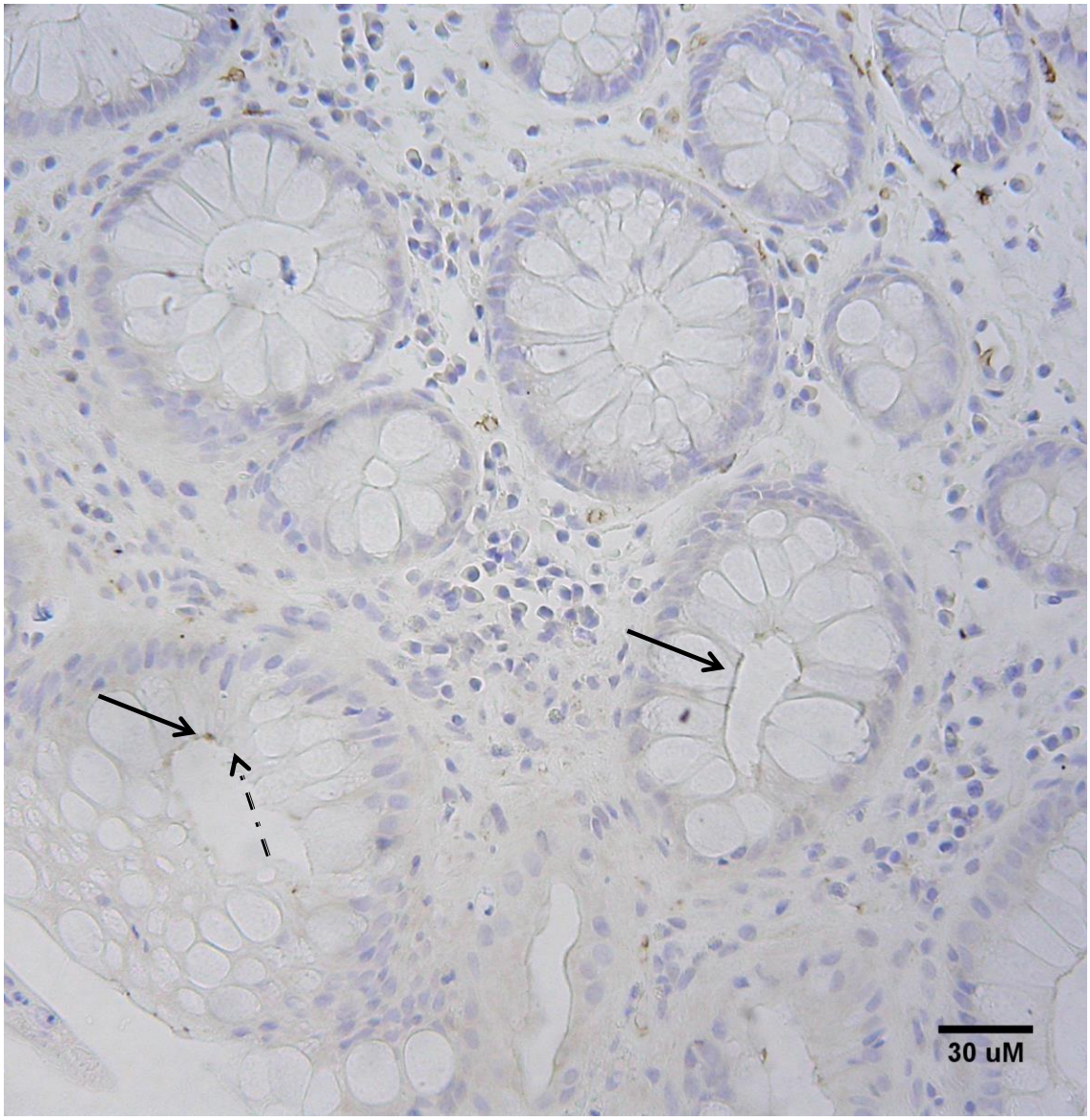


Figure 5.16: Representative images of TNFAIP3 staining in inactive Crohn's disease colonic biopsy sections stained with a polyclonal anti-TNFAP3 antibody with a HRP labelled secondary antibody detection system. Weak discreet areas of TNFAIP3 are seen (solid arrows). Large areas of apical staining loss are also observed (dash arrow).

Inactive Crohn's disease 3

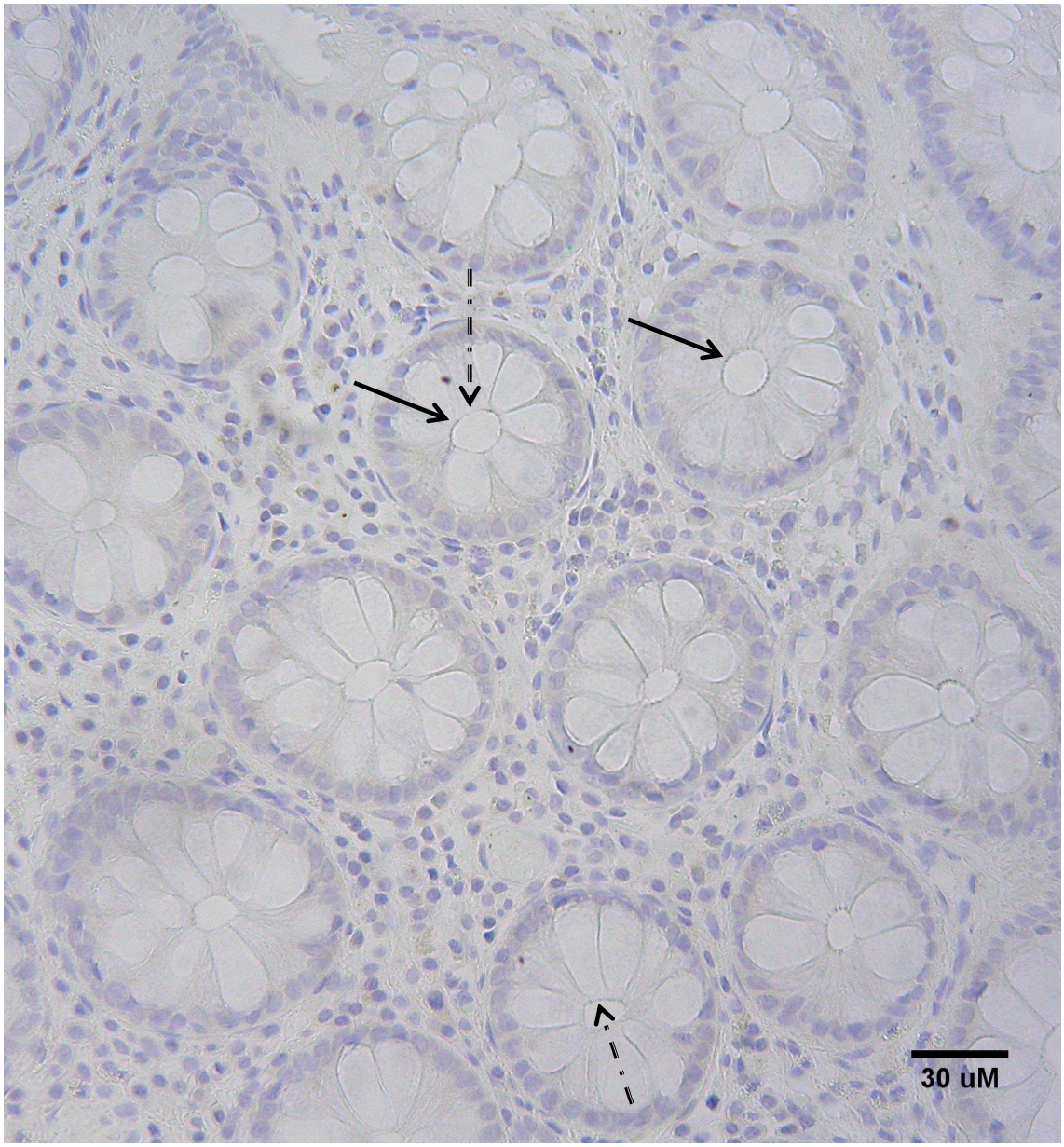


Figure 5.17: Representative images of TNFAIP3 staining in inactive Crohn's disease colonic biopsy sections stained with a polyclonal anti-TNFAP3 antibody with a HRP labelled secondary antibody detection system. Weak discreet areas of TNFAIP3 are seen (solid arrows). Large areas of apical and apicolateral staining loss are also observed (dash arrow).

TBS

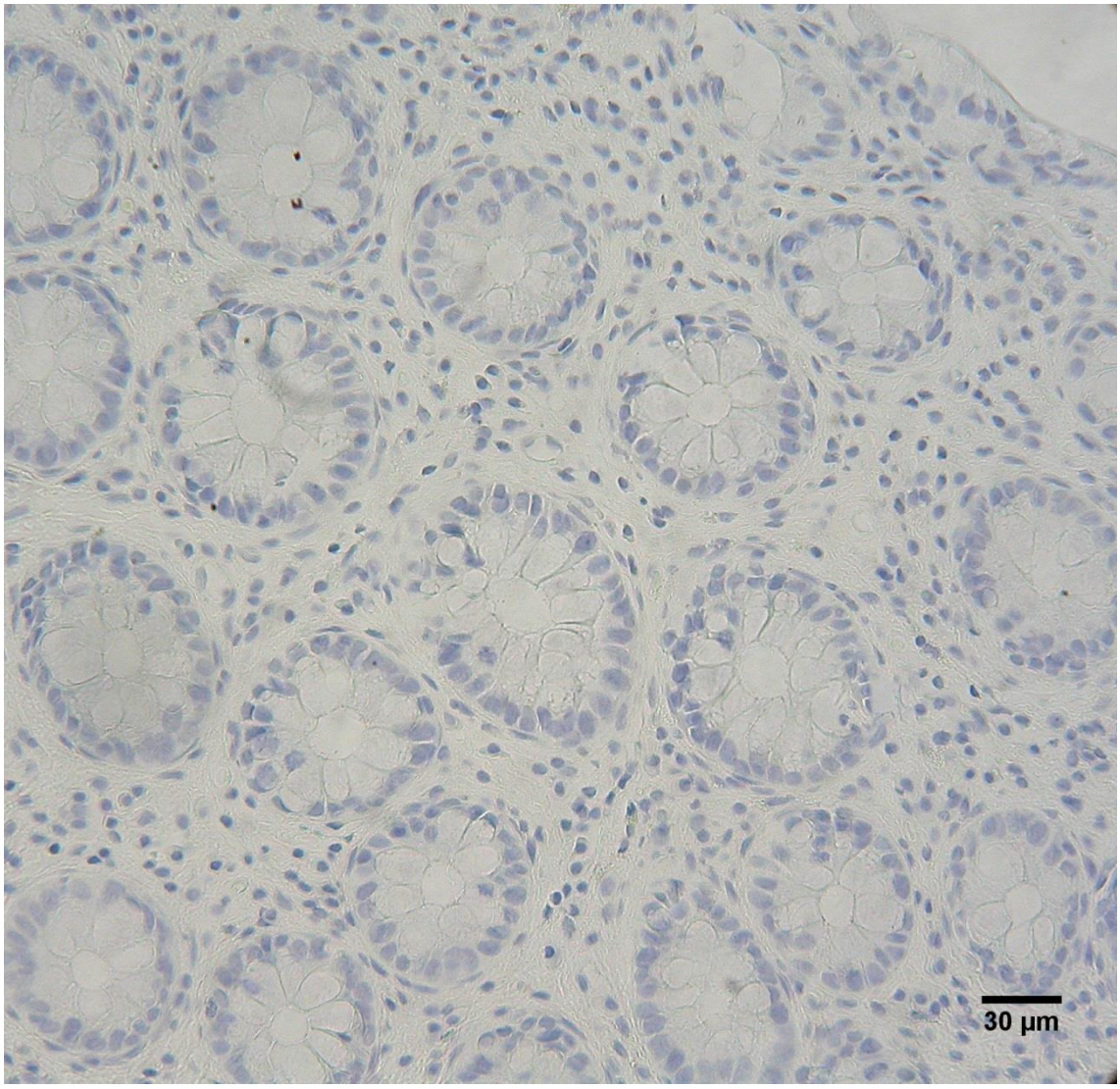


Figure 5.18: Representative control section from inactive Crohn's disease patient biopsy stained with secondary antibody HRP labelled detection system only. A total absence of staining was observed, confirming that the secondary antibody is not contributing to the staining seen with the primary antibody.

5.4.4 Inactive Crohn's Disease Quantification

Using the same scoring system, a score was calculated for each of the 7 inactive Crohn's disease patient and compared to the healthy cohort. A significant decrease in A20 staining was found. The median score for the inactive Crohn's patients was 204 compared to 566 in the healthy cohort (p=0.0025). This data is shown in Figure 5.19.

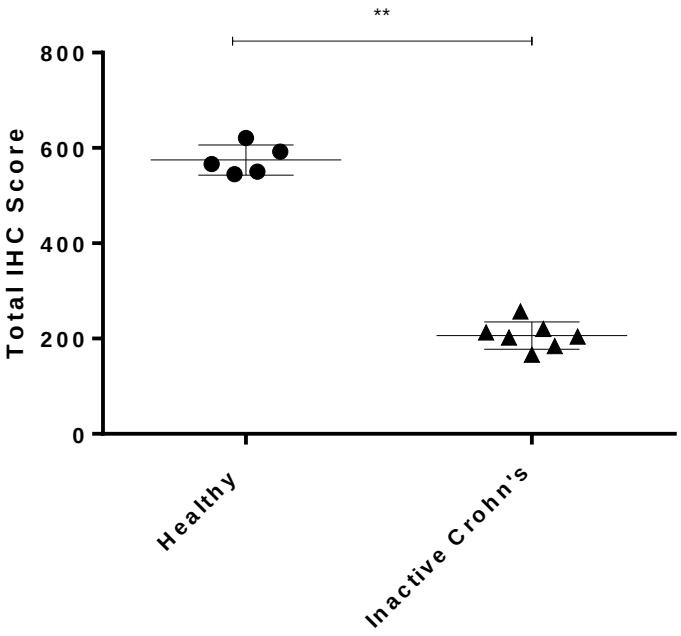


Figure 5.19: A20 immunohistochemistry staining score in inactive Crohn's disease. Data presented as median score with range. Each data point represents an individual patient. P value <0.01 assigned **

As can be seen from this data both the active and inactive cohort had significantly reduced A20 staining scores compared to healthy controls. Although several scores were lower in the active Crohn's disease group, when compared as a group to those with inactive Crohn's disease, there was no significant difference between the two Crohn's cohorts as shown in Figure 5.20.

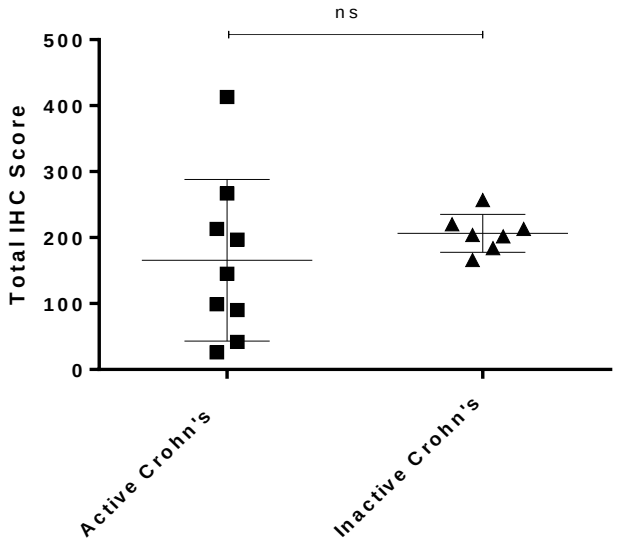


Figure 5.20: A20 immunohistochemistry staining score in active and inactive Crohn's disease. Data presented as median score with range. Each data point represents an individual patient.

5.5 Discussion

Bioinformatic sequence data from two independent databases (Target Scan and MicroRNA.org) suggest that the 3'UTR of TNFAIP3 contains a nucleotide sequence with partial complementarity to miRNA23a. This implies that TNFAIP3 may be a target for miRNA23a and was confirmed with the results of the luciferase reporter assay. Hela cells are established as one of the oldest cell lines used in biomedical research. Although not intestinal in origin they have been previously used to investigate microRNA effects on mRNA and protein outputs and were therefore considered a suitable transfection host (361).

It has previously been reported that the expression of TNFAIP3 mRNA within the colonic mucosa of Crohn's disease patients shows no difference between active or inactive disease (332). However both are reduced when compared to healthy controls (331, 332). In contradiction to this, mRNA expression within the colonic epithelium in the patients in my study showed no difference from healthy controls with median expression across all three groups broadly similar. This discrepancy may reflect the nature of the samples used in each study. The Arsenecu et al and Bruno et al cohorts utilised whole mucosal biopsies. Dendritic cells, macrophages, lymphocytes all of which are found in the mucosa, express TNFAIP3 and are therefore potentially susceptible to miRNA23a. (476, 478-480).

Global analysis of this tissue may mask individual cell specific changes as highlighted by my study.

The effects of miRNA on mRNA levels are varied and complex with both translational repression and mRNA degradation described (344, 376, 380). It has been suggested that observed changes in mRNA may reflect the degree to which a protein target is repressed (361). Translation repression by more than a third has been observed to result in mRNA degradation, with lesser degrees of repression leading to changes in protein expression but not mRNA expression (361). It is therefore possible that the difference in mRNA expression between studies reflects differential mRNA repression between cell phenotypes. The target prediction algorithm TargetScan suggests 11 microRNA have conserved seed sequence complementarity with TNFAIP3. Other microRNA may therefore also be contributing to the TNFAIP3 repression in these cells, leading to mRNA degradation and reduced mRNA levels.

The luciferase reporter assay can be considered the gold standard of the *in vitro* reporter assays. However a limitation of this approach is that it only answers one question; does this particular microRNA interact with this particular mRNA sequence under these experimental conditions? By its very nature this experimental setup is artificial. The concentration of microRNA or mRNA transfected may not reflect endogenous in vivo levels and although a microRNA mRNA interaction can be confirmed, it cannot necessarily be confirmed that the same happens in vivo. Taken in isolation luciferase results provide an answer to a specific question in essentially an artificial set of circumstances. However if these results are interpreted in conjunction with expression data of the protein produced by the mRNA in question, the results become more meaningful. It was for this reason that I also chose to look for the expression of the TNFAIP3 protein in the Crohn's disease patient biopsies.

Work by Kolodziej etal suggests that TNFAIP3 protein plays an important role in the stabilisation of the epithelial tight junction and helps to maintain barrier integrity. In an epithelial TNFAIP3$^{-/-}$ mouse model loss of occludin was observed from the apical surface of the epithelium, which was not seen in wild type animals. This was associated with increased intestinal permeability, evaluated with FITC-dextran permeability assays in explanted intestinal loops, suggesting that TNFAIP3 protein may be important in preserving barrier integrity. TNFα is known to cause tight junction disruption via the endocytosis of occludin. To test whether the barrier disruption was due to the loss of TNFAIP3 directly or the

effect of uncontrolled inflammation, TNFAIP3 was overexpressed in both the mouse intestinal epithelium and the epithelial cell line HTC116. TNFAIP3 overexpression was protective of barrier disruption in response to TNFα stimulation, compared to wild type, both in the mouse and the HCT116 epithelial cell line. Further study by this group showed that TNAFIP3 protein maintained occludin localisation at the apical membrane via its deubiquitination, uncovering an important role for this protein in barrier stability (191).

It is established that increased intestinal permeability is an important feature of Crohn's disease, and often present before the onset of clinical symptoms(224, 228, 231, 481). It is also known that TNFα is a leading driver of inflammation in Crohn's disease and has direct detrimental effects on the barrier(259, 260, 263). The work of Kolodziej offers a potential mechanism by which the TNF regulatory protein A20 (TNFAIP3) is able to support the barrier, maintaining its integrity.

The cellular location of TNFAIP3 protein within human colonic epithelium is however, unreported. My studies have now shown that this protein is localised to the apical and apicolateral junctional regions of the epithelial cell in support of the findings from Kolodziej etal. I have also shown that this protein is reduced in Crohn's disease. Taken together with the results presented in Chapters 3 and 4, a picture is emerging where miRNA23a is overexpressed in the colonic epithelium of Crohn's disease patients and this results in reduced expression of TNFAIP3 protein in this epithelium. The fundamental question now becomes what is the functional consequence of this? The relationship between miRNA induced reductions in A20 and intestinal barrier permeability are the focus of the next chapter.

Chapter 6:

Functional Studies of MicroRNA23a

Chapter 6: Results

6.1 Introduction

With a few exceptions, a major limitation of microRNA studies in IBD been the quantitative nature of the data reported. As discussed in chapter 1, due to partial sequence complementarity, each single microRNA may potentially repress many hundreds of messenger RNAs (376). This makes it difficult to determine what the functional significance of any observed microRNA changes are.

Epithelial barrier function is known to be impaired in inflammatory bowel disease. A recent study suggested that TNFAIP3 stabilises occludin at the tight junction, thus contributing to the maintenance of epithelial barrier integrity (191). The microRNA and TNFAIP3 data presented in previous chapters display similar expression changes in both active and inactive disease. This leads to the question of what is the functional effect of this microRNA in gut epithelial cells?

Hypotheses:

In a T84 cell model of colonic epithelium, increased expression of miRNA23a reduces the level of A20 protein, increases barrier permeability and promotes activation of the proinflammatory transcription factor NFκB.

Primary human colonic biopsies from healthy donors with experimentally increased microRNA23a, release increased levels of NFκB dependent proinflammatory cytokines,

Aims

The aim of this section was to define the functional consequence of increased expression of miRNA23a on TNFAIP3/A20 levels and the immune and barrier properties of epithelial cells.

Objectives

- To confirm that microRNA23a overexpression reduces TNFAIP3 protein in T84 cells.
- To investigate the effect of microRNA23a overexpression on the barrier properties of the epithelium using a T84 transwell culture model.

- To investigate the effect of microRNA23a expression on NFκB transcription utilising an NFκB dependent luciferase reporter assay system.
- To overexpress microRNA 23a in healthy human colonic epithelium in an ex vivo biopsy culture system, by transfection with exogenous microRNA23a preMir to increase mature microRNA23a and determine the effect on epithelial pro inflammatory cytokines, TNFα, IL-6 and IL-8 release.

6.2 Transfection of T84 cells

Epithelial cell lines derived from the intestine are cultured and used as a model of intestinal function. T84 cells are a widely used cell line. They were derived from a colonic adenocarcinoma. They were of value, because they retain the ability to differentiate in confluent monolayers on filters, with well-defined tight junctions, resembling mature colonic epithelium (482, 483). They are therefore suitable for studies of barrier function.

Prior to any functional microRNA studies, preliminary work was performed to confirm that T84 cells could be successfully transfected to overexpress miRNA23a. Previous microRNA transfection studies in our lab had used 100nM of miRNA preMir (Marcus Gwiggner, personal communication). In preparatory work for these experiments, transfection attempts with superfect and Interferrin were unsuccessful. T84 cells could however be successfully transfected with Hipperfect, which was therefore used for these experiments. Using Hiperfect according to the manufactures instructions T84 cells were therefore transfected with 100nM of preMir23a and RT-qPCR used to determine expression

The mean cycle threshold for miRNA23 in both untransfected cells and cells transfected with empty vector was 25. Cells transfected with preMir23a had a lower cycle threshold of 18. This confirmed that the transfection with miRNA23a was successful. These results were consistently replicated in 3 separate experiments as shown in Figure 6.1.

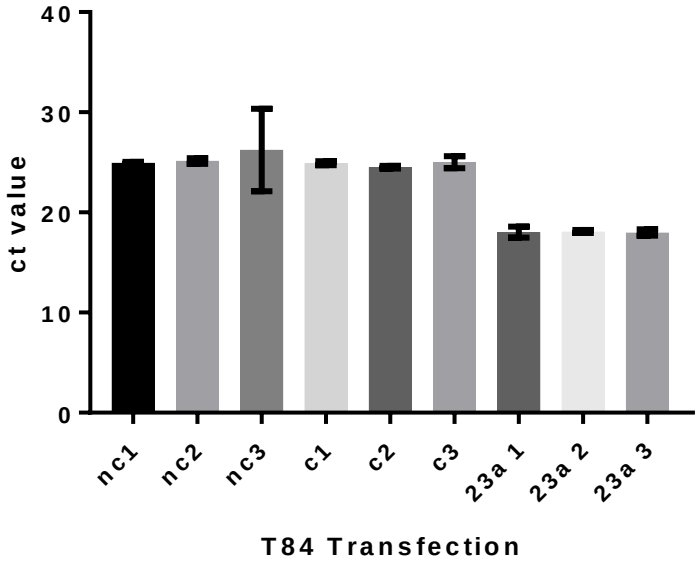


Figure 6.1: Cycle threshold values for miRNA23A in T84 cells

Cells transfected with empty vector (nc), left untransfected (c) or transfected with premiR23a (23a). Each transfection was performed 3 times. Triplicate cT values measured for each experiment. Each bar represents an individual experiment with data presented as the mean and standard deviation.

6.3 TNFAIP3 protein expression in T84 cells was reduced by overexpression of miRNA23a.

We have demonstrated that TNFAIP3 protein was reduced in human colonic epithelial cells in Crohn's disease, compared to healthy donor epithelium and that these same cells overexpress miRNA23a. Our luciferase reporter assays confirmed that miRNA23a caused repression of luciferase mRNA translation via the 3'UTR of TNFAIP3 mRNA (Chapter 5). To confirm that miRNA23a mediated repression of A20 protein occurred in T84 cells and they were therefore a suitable cell line for microRNA23a barrier studies, T84 cells were transfected with the premiR of 23a to increase mature miRNA23a levels. Cells were incubated for 24 hours. Total cell lysates were then probed for TNFAIP3/A20 protein as described in the methods (Chapter 2).

Transfection of T84 cells to overexpress miRNA23a resulted in a reduction of TNFAIP3/A20 protein levels as determined by western blot. To confirm that this

was due to the effect of the microRNA and not toxicity of the transfection media, cells were also transfected with either empty vector or left untransfected. In both these conditions, no reduction in TNFAIP3 was seen confirming the TNFAIP3 reduction was due to the effect of the microRNA.

Each blot was then stripped of the TNFAIP3 antibody and re-probed for a stably expressed internal control protein. Initially this was beta actin. However we also observed reductions in beta actin in the cells over expressing microRNA23a. Similar results were obtained with cytokeratin 19 and GAPDH. Toxicity of the transfection media was again considered as a possible cause for this. However comparison to empty vector transfected and un-transfected cells were similar with no reductions seen in any of these proteins. This suggested that the observed reductions in beta actin, cytokeratin 19 and GAPDH were not due to cell toxicity or the transfection media and may possibly be due to the microRNA itself. It therefore was not possible to find a stably expressed housekeeping protein in this system.

Representative blot from the same cell lysates are shown in Figure 6.2.

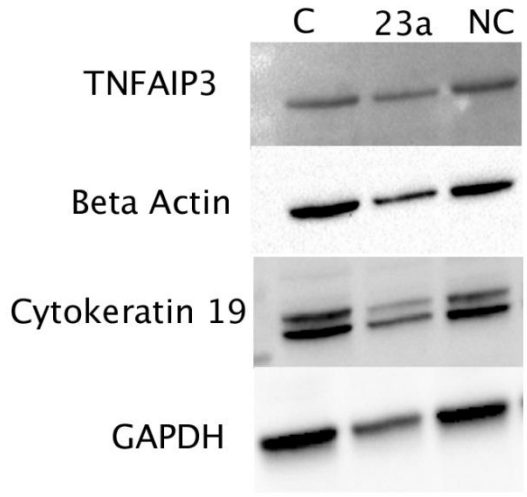


Figure 6.2: Representative western blot of total cell lysates from T84 cells transfected to overexpress miRNA23a, empty vector or non-transfected and probed for TNFAIP, Beta actin, cytokeratin 19, and GAPDH using a HRP linked detection system.

Lysates are from untransfected cells (C), cells transfected with preMir23a to overexpress miRNA23a (23a) and cell transfected with empty preMir vector

(NC). Reductions in band intensity of TNFAIP3, Beta Actin, Cytokeratin 19 and GAPDH are seen in cells transfected to overexpress miRNA23a.

The TNFAIP3 band intensity from three individual experiments was quantitated using BioRad Image Lab software. Although comparison with a stable reference protein was not possible, a direct comparison was made between miRNA23a transfected and empty vector transfected cells. This confirmed there was a significant reduction in TNFAIP3 protein band intensity in miRNA23a transfected cells. The mean intensity was 3.92×10^6 compared to 5.37×10^6 in the empty vector transfected cells ($p=0.04$). This is show in Figure 6.3.

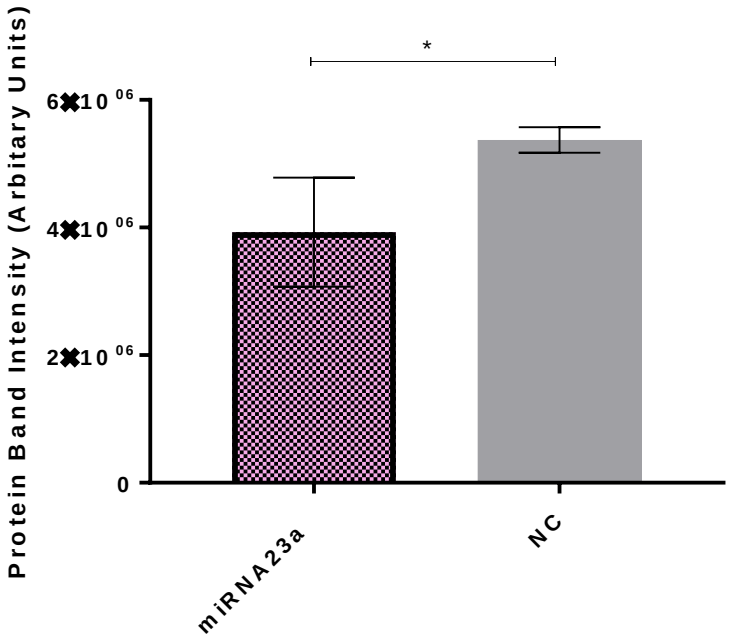


Figure 6.3: TNFAIP3 protein band intensity in T84 cells transfected to overexpress miRNA23A

Protein band densities in miRNA23a transfected and empty vector (NC) transfected T84 cells. N =3. Mean values presented with standard deviation . * $p<0.05$

To determine if antagonism of miRNA23a could restore TNFAIP3 expression and if the reductions in beta actin were indeed due to the microRNA, cells were then transfected with 100nm of an antiMir directed against miRNA23a. Cells were incubated for 48 hours. Total cell lysates were then probed for TNFAIP3 protein as described in the methods.

Antagonising mirNA23a prevented a reduction in TNFAIP3 expression as determined by western blot. Expression of beta actin was also preserved. The TNFAIP3 and beta actin protein band intensity from 3 individual experiments was quantitated with BioRad Image Lab Software. There was no significant difference in protein expression for either TNFAIP3 (p=0.292) or Beta actin (p=0.4697) compared to empty vector control as shown in Figures 6.4 -6.6.

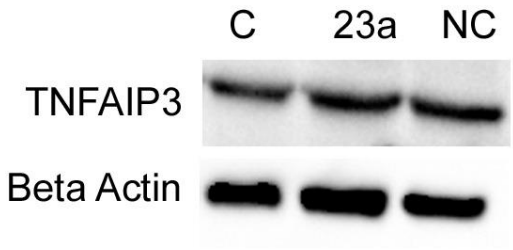


Figure 6.4:Representative western blot of total cell lysates from T84 cells transfected with anti-miRN23a, empty vector or left untransfected and probed for TNFAIP, Beta actin, cytokeratin 19, GAPDH using a HRP linked detection system.

Lysates are from untransfeced cells (C), cells transfected with anti-Mir23a to inhibit miRNA23a (23a) and cell transfected with antiMir negative contorl (NC).

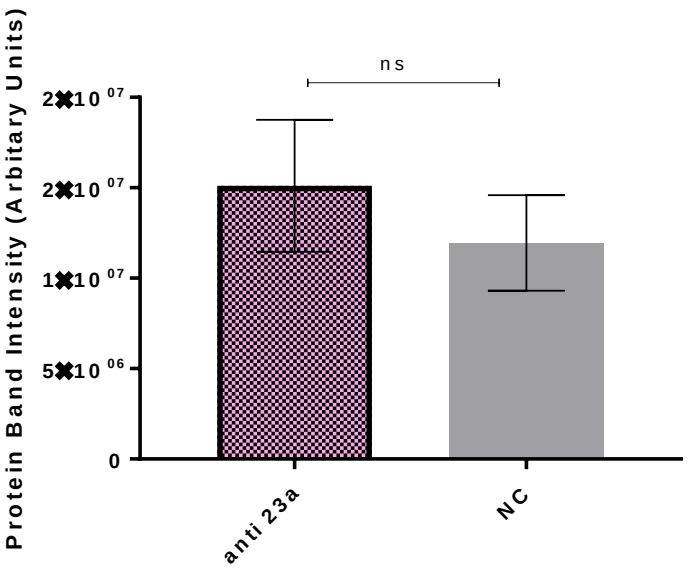


Figure 6.5: TNFAIP3 protein band intensity after anti-miRNA23a transfection

Protein band densities in anti-miRNA23a transfected and empty vector transfected (NC) T84 cells. N=3 Mean values presented with standard deviation.

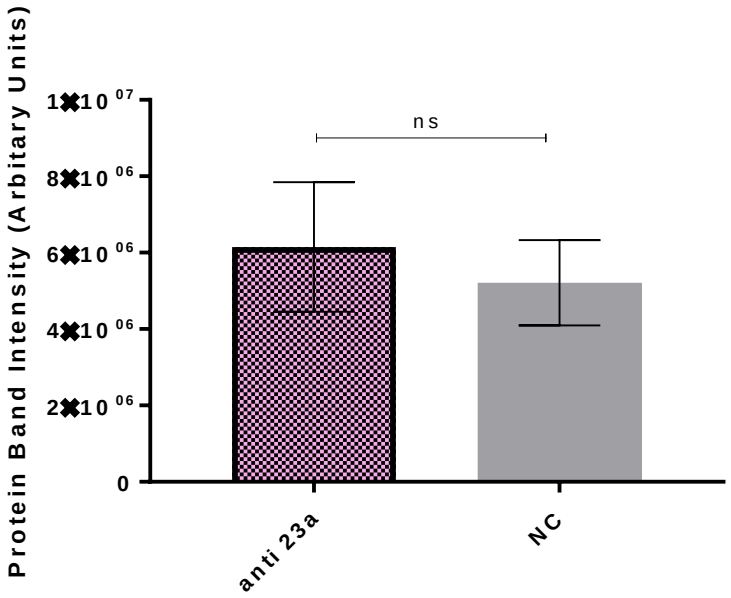


Figure 6.6: Beta Actin protein band intensity after anti-miRNA23a transfection

Protein band densities in anti-miRNA23a transfected and empty vector transfected (NC) T84 cells. N=3Mean values presented with standard deviation.

6.4 Barrier properties of the T84 colonic cell line

Having established that miRNA23a leads to reductions in TNFAIP3 protein, we next sought to understand the effect of this reduction on the integrity of the epithelial barrier using the T84 cell model.

It was established that the tight junction barrier of T84 cells is minimally responsive to TNFα stimulation (151). As this original data was published some 17 years ago, preliminary work was undertaken to confirm these results were still applicable in the cell clones we had available in our liquid nitrogen stores. T84 cells were grown to confluence on collagen coated transwell filters and exposed to TNFα and IFN, either singly or in combination, as described in the methods. Ionic barrier properties of these cells were then measured with transepithelial resistance over 3 successive days. The larger macromolecular pore pathway was evaluated with a FITC 4 kDa dextran assay at day 3.

6.4.1 Transepithelial resistance

Addition of 1ng/ml of TNFα to the tissue culture medium above and below the filters, had no detrimental effect on the transepithelial resistance compared to untreated cells. There was no significant difference in the median TER values for each group until day 3. At this time point the TER of the TNFα exposed cells displayed a slight increase compared to untreated cells with a median TER in TNFα 560 Ω/cm^2 compared to 498.5Ω/cm^2 in the untreated group ($p=0.0264$).

When cells were treated with 100ng/ml of IFNγ daily, significant reductions in TER were observed compared to untreated cells on each the 3 days measured. At day 3 the median TER in IFNγ exposed cells was 161.5Ω/cm^2 compared to 498.5Ω/cm^2 in the untreated group ($p<0.0001$). Similar significant reductions were also observed when TNFα and IFNγ were added in combination with the TER drop almost identical to that seen with IFNγ alone. As TNFα was demonstrated to have no negative effect the reduction in TER with the cytokine combination was attributed to the effect of IFN.

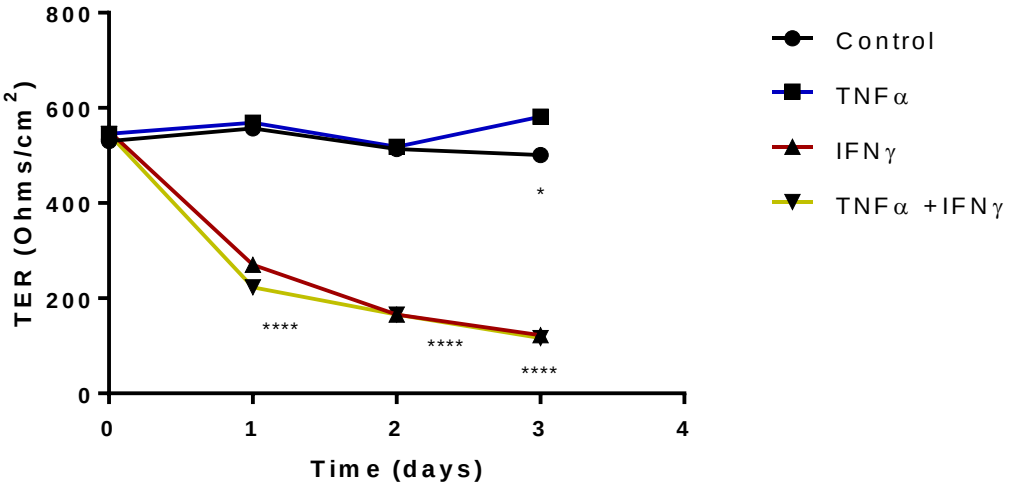


Figure 6.7: TER measurements of filter grown T84 monolayers treated with proinflammatory cytokines.

TER measurements of filter grown T84 cells cultures with TNFα, IFNγ either singly or in combination. For control cells were cultured without the addition of cytokines. Mean TER values presented from triplicate experiments. (n=3)
* p=≤0.05 **** p≤0.0001

6.4.2 FITC Dextran permeability

Addition of TNFα did not significantly affect FITC dextran flux across the T84 monolayer. The median concentration of FITC dextran which had moved through the barrier into the basolateral media was 0.699mcg/ml in the untreated cells compared to 0.882mcg/ml in the TNFα treated cells (p=0.889). When exposed to IFNγ this increased to 3.18 mcg/ml (p=0.001) and 3.51mcg/ml (p=0.0004) when TNFα and IFN γ were added.

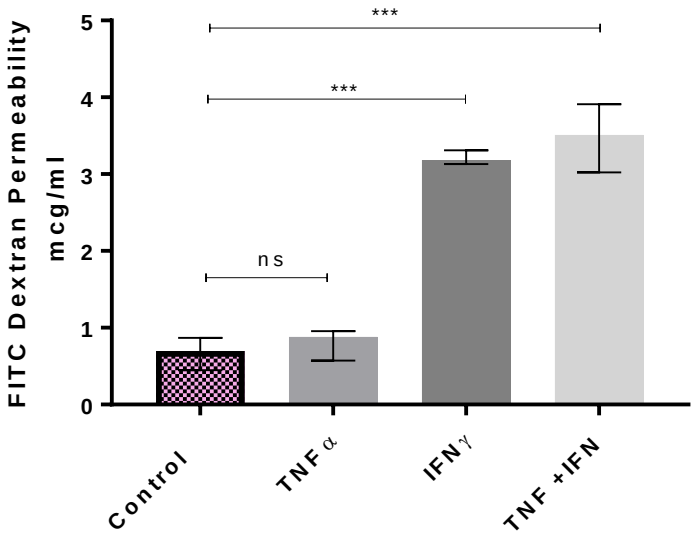


Figure 6.8: FITC Dextran Permeability measurements of filter grown T84 monolayers treated with pro inflammatory cytokines.

FITC dextran permeability measurement of T84 cells grown on filters for 3 days in the presence of TNFα, IFNγ either singly or in combination. For control cells were cultured without the addition of cytokines. Data presented as median with range from triplicate experiments (n=3)Non parametric comparisons between groups with Kruskal Wallis. *** p≤0.001

Collectively these results confirm the reported findings by Fish etal (151). When cultured in a monolayer and treated with TNFα, T84 cells maintain similar barrier integrity as untreated cells. This confirmed the suitability of the T84 cell line for studies investigating microRNA23a effects on barrier integrity in an IBD model.

6.5 Overexpression of microRNA23a impairs epithelial barrier integrity.

Utilising the T84 barrier model validated above, T84 cells were then transfected with the preMir of microRNA23a and plated on transwell filters. Barrier integrity was evaluated with TER measurements daily for 5 days and FITC dextran permeability measured on day 5. To mimic the inflammatory environment present in Crohn's disease each experiment was performed with and without the addition of 1ng/ml of TNFα daily.

6.5.1 MicroRNA23 overexpression in the absence of TNFα has no effect on the barrier

In the absence of an inflammatory stimulus there was no difference in the integrity of the epithelial barrier between cells overexpressing microRNA23a and control. Both the ionic and macromolecule paracellular transport pathways showed similar integrity as assessed by trans-epithelial resistance and FITC dextran measurements respectively.

MicroRNA transfected and negative control empty vector transfected cells showed similar daily increases in TER measurement. At day 5 the TER in 23a transfected cells was 868 Ω/cm^2 compared to 763 Ω/cm^2 in the negative control (p=0.121).

The macromolecular permeability pathway was found to be less permeable in cells transfected with miRNA23a. However this was not significantly different from cells transfected with empty vector. Median FITC permeability was 0.16 mcg/ml in miRNA23a transfected cells and 0.43mcg/ml in the control group (p=0.870).This data is summarised in Figures 6.9 and 6.10.

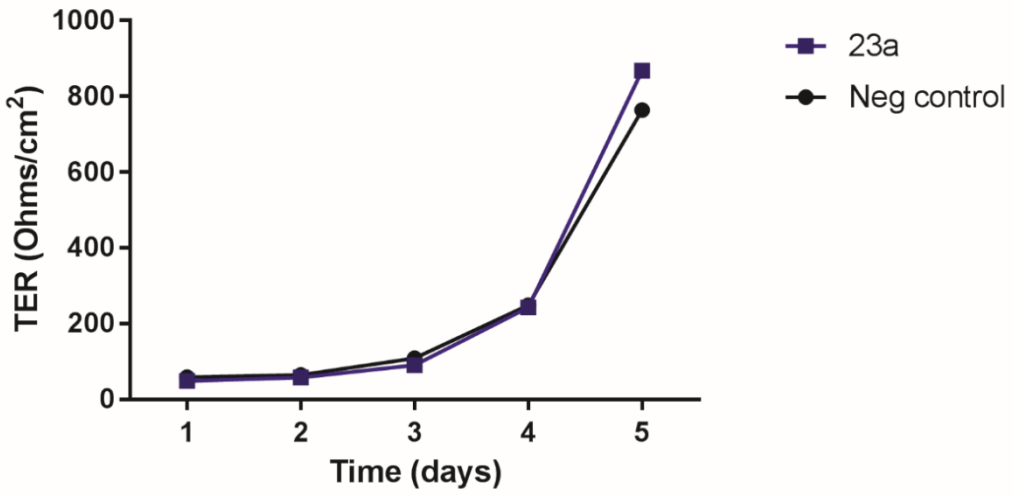


Figure 6.9: TER measurements in filter grown T84 monolayers transfected to overexpress miRNA23a cultured without TNFα

T84 cells transfected with preMir23a to overexpress miRNA23a (23a) or with empty vector as control (neg control). Cells cultures on filters without TNF. Data presented as mean from n=3.

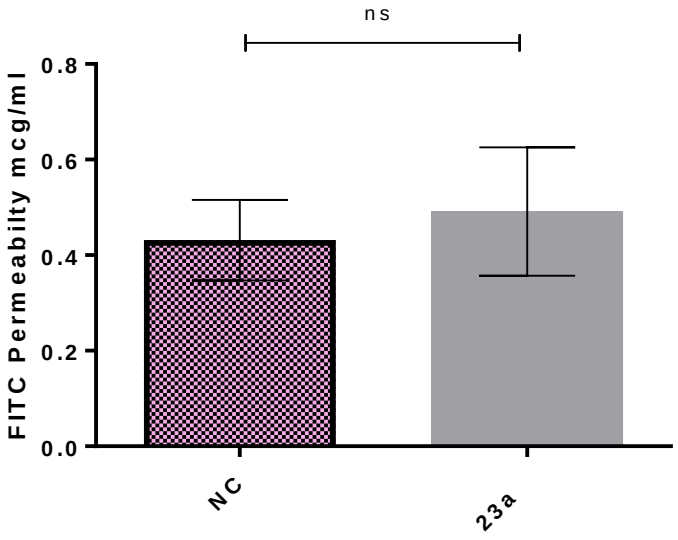


Figure 6.10:FITC Dextran permeability in filter grown T84 monolayers transfected to overexpress miRNA23a and cultured without TNFα

FITC dextran permeability measurement at day 3 of T84 cells transfected with preMir23a to overexpress miRNA23a (23a) or with empty vector as control (NC). transfected cells grown on filters for 3 days without TNFα. Data presented as median with range from triplicate experiments (n=3)Non parametric comparisons between groups with Wilcoxon matched pairs.

6.5.2 MicroRNA23 overexpression in the presence of TNFα has an adverse effect on the macromolecular barrier.

In the presence of TNFα, there was no significant difference in the integrity of ionic permeability as measured by TER. Daily measurements were almost identical for microRNA23a and empty vector transfected cells. At day 5 there was again a small increase in TER in miRNA23a transfected cells but this was not significant. The median TER at day 5 was 718Ω/cm^2 compared to 655Ω/cm^2 (p =0.4385). This data is shown in Figure 6.11.

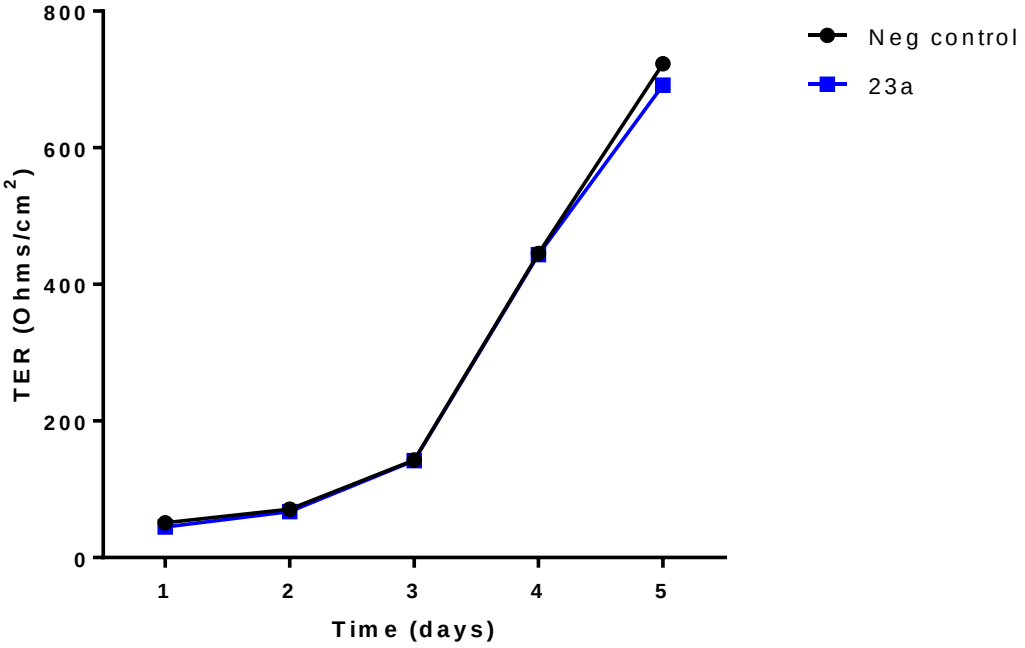


Figure 6.11: TER measurements in filter grown T84 monolayers transfected to overexpress miRNA23a cultured with TNFα

T84 cells transfected with preMir23a to overexpress miRNA23a (23a) or with empty vector as control (neg control). Cells cultures on filters without TNF. Data presented as mean from n=3.

There was however an increase in macromolecule permeability observed in microRNA23a transfected cells. The median FITC permeability in empty vector control cells was 0.68 mcg/ml compared to 1.11mcg/ml in miRNA 23a transfected cells (p=0.0009). This data is shown in Figure 6.12.

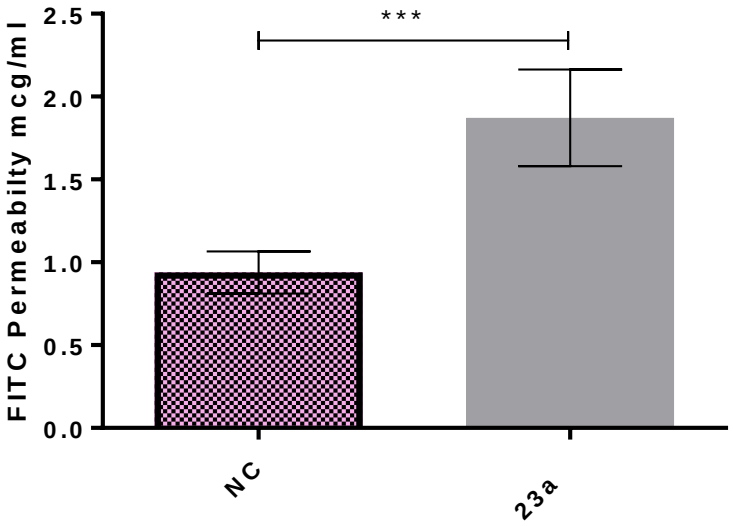


Figure 6.12: FITC Dextran permeability in filter grown T84 monolayers transfected to overexpress miRNA23a and cultured with TNFα

FITC dextran permeability measurement at day 3 of T84 cells transfected with preMir23a to overexpress miRNA23a (23a) or with empty vector as control (NC). transfected cells grown on filters for 3 days without TNFα. Data presented as median with range from triplicate experiments (n=3)Non parametric comparisons between groups with Wilcoxon matched pairs.

Collectively these data suggest that microRNA23a overexpression had no adverse effect on the epithelial barrier in a T84 model, as long as there was no inflammatory stimulus. However in the presence of TNFα although the ionic pore pathway remained intact, there was an increase in the large molecule pore pathway.

6.6 MicroRNA23a increases NFκB transcription

TNFAIP3 is known to negatively regulate NFκB activity (320, 484). Our data has shown that TNFAIP3 is itself regulated by microRNA23a with microRNA overexpression causing a reduction in TNFAIP3 protein level. We therefore wanted to investigate if microRNA23a overexpression resulted in an increase in NFKB transcriptional activity.

As described in the methods section, a vector containing three NFκB transcriptional response elements 5 prime to a basal TATA box promoter

activating transcription of a firefly luciferase reporter was transfected along with the pcDNA3.1-23a expression vector. Empty vectors for each were transfected as controls. Stimulation of NFκB transcription was provided by adding 1 ng/ml of TNFα 2 hours prior to assay.

Combined data from 5 independent experiments (transfection performed in duplicate for each experiment) showed no difference in basal NFκB transcription, as measured by luciferase reporter activity, when miRNA23a is transfected with the NFκB reporter, possibly because it is so low under these conditions. The relative luciferase activity of NFκB/23a transfected cells compared to NFκB alone was 1.08 ($p=0.1602$). As anticipated, no luciferase activity was detectable in the absence of the NFκB reporter. When NFκB transcription was stimulated by the addition of TNFα, a significantly greater increase in transcription was observed in empty vector transfected cells, confirming the effect of TNFα on NFκB activity in the system. Notably, when cells were transfected with miRNA23a, in combination with TNFα, a significantly greater increase in transcription confirming the effect of miRNA23a on NFκB activity in the system. The relative luciferase expression with TNFα compared to baseline (no TNFα) in stimulated cells without miRNA23a was 12.89. In miRNA23a transfected cells the relative luciferase expression was 17.43 ($p=0.0098$). This suggests that miR23a expression leads to an increase in NFκB activity in the presence of TNFα.

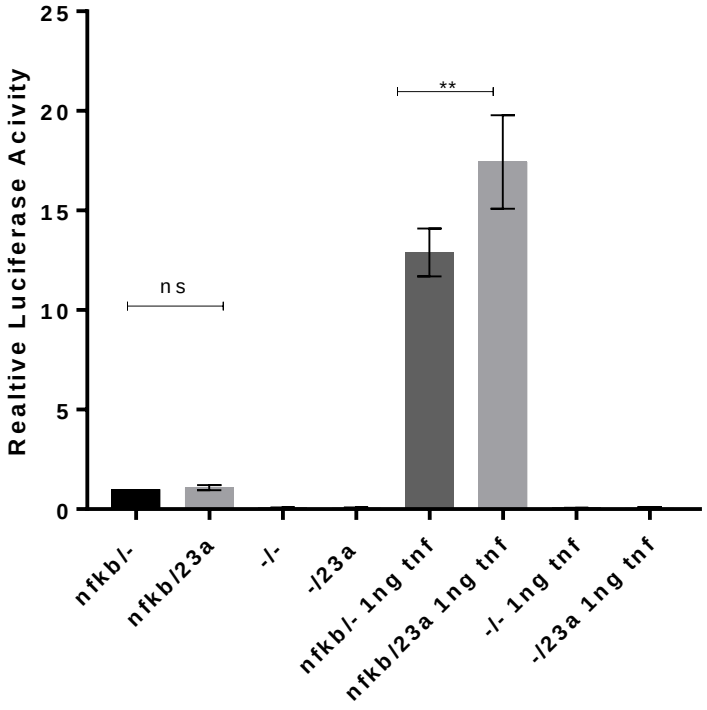


Figure 6.13: Relative NFkB Luciferase activity in Hela cells transfected to overexpress MiRNA23

Cells transfected with NFkB luciferase reporter (NFκB) or control (-). Cells also transfected with pcDNA3.1-23a (23a) to overexpress miRNA23a or pcDNA3.1 as control (-). TNFα was added to stimulate NFκB where indicated by TNF . Data presented rom 5 experiments, each performed in duplicate. p <0.01 **. Median and range presented,

6.7 MicroRNA23a overexpression in an ex vivo colonic biopsy culture model

Having shown that increased miR23a appears to influence NFkB activity and I have measured an increase in miR23a in Crohn's biopsies ex vivo, I decided to explore the effect of increasing expression of miR23a in healthy patient biopsies. Initially the feasibility of transfecting cultured explant biopsies was studied. The use of Crohn's biopsies was eliminated from study due to the fragile nature and variability of these biopsies in explant culture (data not shown). Early attempts at ex vivo culture used Aquix® culture media. However RPMI1640 was found to be superior, with better preservation of biopsy integrity and was therefore used for all culture experiments presented here. Media volumes of 500µl and 1ml per biopsy were tried, with 500µl found to be optimal for biopsy preservation.

Between 2 and 4 sigmoid colonic biopsies were collected from 8 healthy patient controls. Each patient had no macroscopic inflammation at the time of biopsy. Biopsies were not included from patients who reported a change in bowel habit to loose stool. Biopsies from each patient were transfected with either the preMir for microRNA23a or empty vector as described in the methods (Section 2.15) and cultured *ex vivo* for 24 hours. After this time, culture supernatants were collected and frozen at -20°C until use. Pro inflammatory cytokines, IL-6, IL-8 and TNFα in this media were assayed, with Meso Scale Discovery high sensitivity cytokine assays.

To confirm that the colonic epithelium had been transfected, 4 individual biopsies from the same patient were transfected with the preMir of 23a or non-specific PreMir control, cultured for 24 hours before being formalin fixed and paraffin embedded. Laser capture microdissection was used to isolate the epithelium from these biopsies and microRNA23a expression quantitated with RT qPCR.

Cycle threshold values for miRNA23a in the preMiR23a transfected biopsies were lower than the nonspecific preMir control biopsies, as shown in Figure 6.14, indicating that the transfection method was successful and resulted in transfected epithelium over expressing microRNA23a.

The 23a cT value for the nonspecific preMir control transfections were 29.84 In the biopsies transfected with preMir23a the cT values were 27.82 As part of this initial work 2 biopsies had 1ng/ml TNFα added to them 2 hours before culture was terminated. However, at laser dissection it was noted that this caused some degradation of the biopsy, (evidenced by cell necrosis) and was therefore not pursued further.

As a further validation, the cT values for miRNA23a, from premiR negative controls obtained via transfection, were compared to the expression data obtained from the original healthy patient cohorts presented in chapter 4. The mean cT value in this healthy cohort was 30.2. This compared very favourably with the cT value obtained in the negative control transfection experiment and indicated that the transfection process was not independently altering miRNA23a expression.

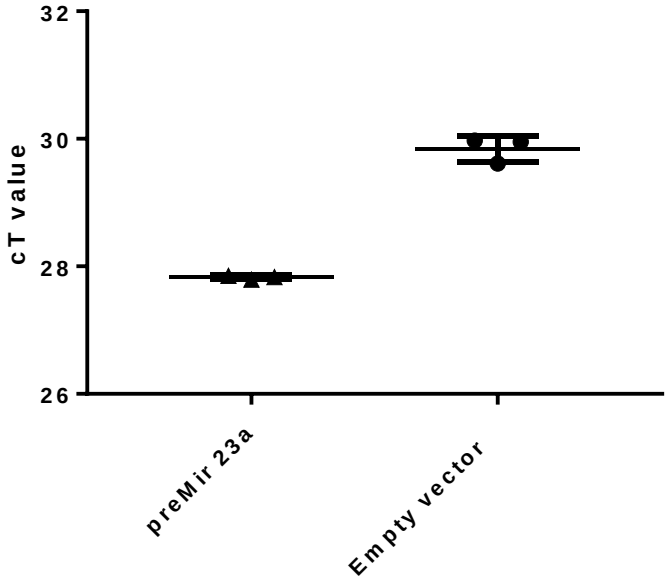


Figure 6.14: cT values of laser-dissected epithelium from transfected explant healthy colonic biopsies

Raw cT values of healthy colonic explant biopsies transfected with preMir23a to overexpress microRNA23a or transfected with nonspecific preMir negative control (Empty vector). Mean presented n=3.

Raw cT values have little value in determining expression changes due to potential variations in cells/ assay conditions affecting results. They must therefore be normalised to an internal control. As in chapter 3, the internal control used was RNU44. The cT values for miRNA23a were then normalised to RNU44 as an internal control which I had previously shown to be a stably expressed internal comparator. Using the empty vector transfected biopsy as the reference a fold change was calculate for the other biopsies using the ΔΔct method. In keeping with the raw cT values, this confirmed overexpression of miRNA23a in the biopsies transfected with the preMiRNA construct with a 4.59 fold increased expression compared to the control transfected biopsy.

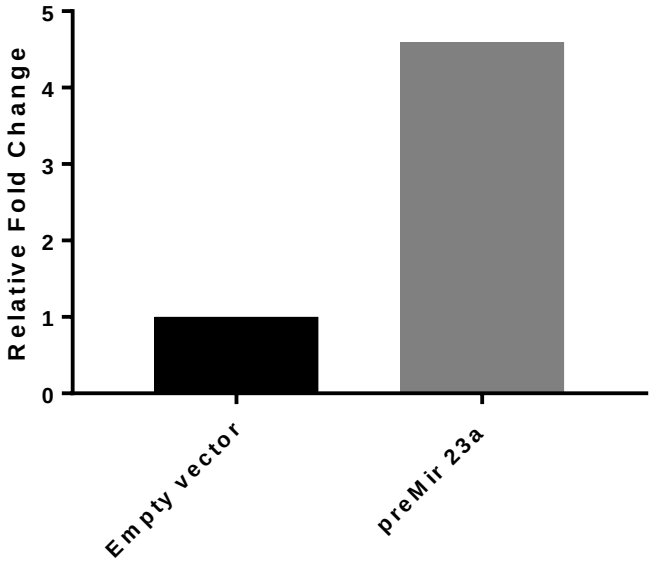


Figure 6.15: Relative fold change microRNA23a expression

6.7.1 Pro inflammatory Cytokine Release

Having confirmed that the colonic epithelium had been transfected to over express microRNA23a, inflammatory cytokine concentrations were measured in the 24-hour tissue culture supernatants of each biopsy. Concentrations of TNFα were significantly greater in the supernatant of miRNA23a transfected epithelium compared to the empty vector transfection control. The median concentration in the miRNA23 overexpression biopsy was 42.26pg/ml compared to 14.37pg/ml in the control (p=0.039)

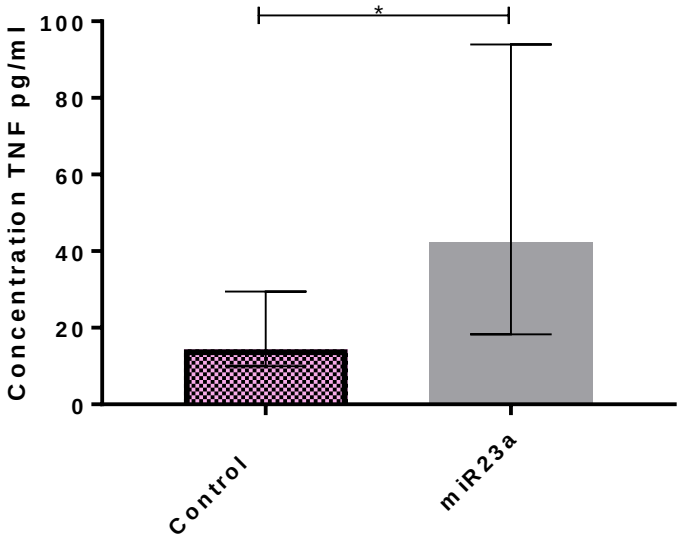


Figure 6.16: Concentration of TNFα in 24 hour explant tissue culture supernatants. Explant culture of healthy colonic biopsies transfected with RNA preMir23a to overexpress miRNA23a (miR23a) or nonspecific preMir negative control vector (control). n =8 . Median with range presented. $p<0.05$ assigned *

The concentrations of IL-1β, IL6 and IL8 all showed increased expression in the microRNA23a overexpression biopsy; however, these did not attain statistical significance when compared to the empty vector control. The median concentration of IL-1β was 42.81pg/ml in the miRNA23a overexpression sample and 35.51pg/ml in control ($p>0.999$). The median concentration of IL6 was 161.28 in the miRNA23a overexpression sample and 137.27pg/ml in control ($p=0.6322$). The concentration of IL8 in the miRNA23a overexpression sample was 3383.28pg/ml compared to 2439.08pg/ml in control ($p=0.7057$). This data is shown in figure x and suggests that the presence of increased miR23a in the biopsy epithelium promoted increased release of TNFα, a key cytokine raised in Crohn's Disease.

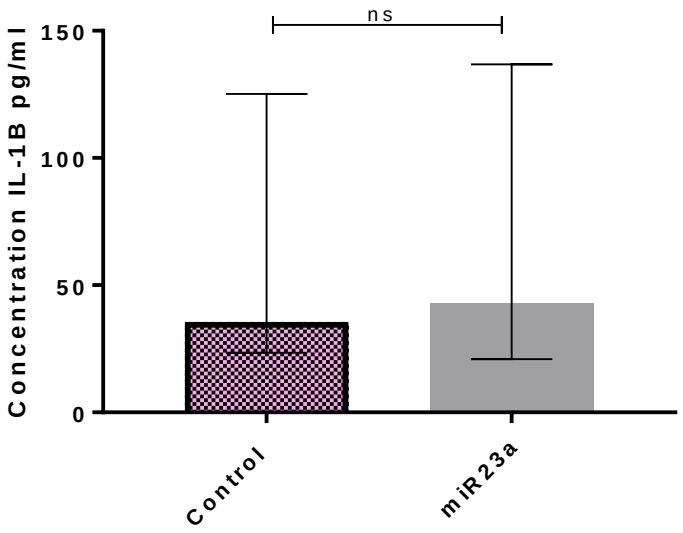


Figure 6.17: Concentration of IL-1β in 24 hour explant tissue culture supernatants. Explant culture of healthy colonic biopsies transfected with RNA preMir23a to overexpress miRNA23a (miR23a) or nonspecific preMir negative control vector (control). n =8 . Median with range presented.

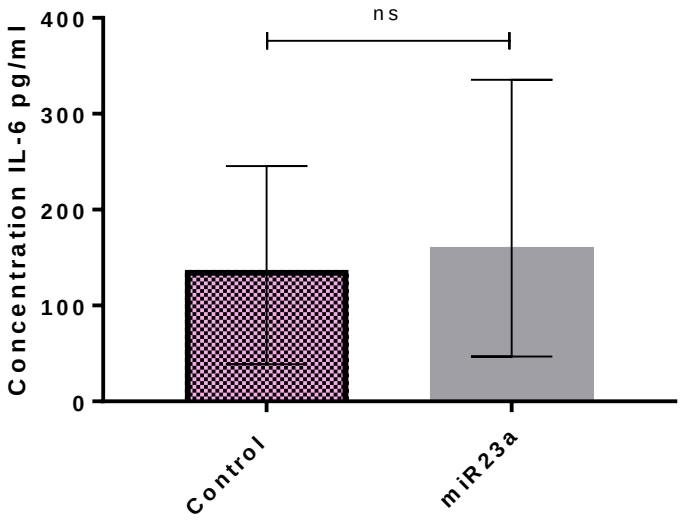


Figure 6.18: Concentration of IL-6 in 24 hour explant tissue culture supernatants. Explant culture of healthy colonic biopsies transfected with RNA preMir23a to overexpress miRNA23a (miR23a) or nonspecific preMir negative control vector (control). n =8 . Median with range presented.

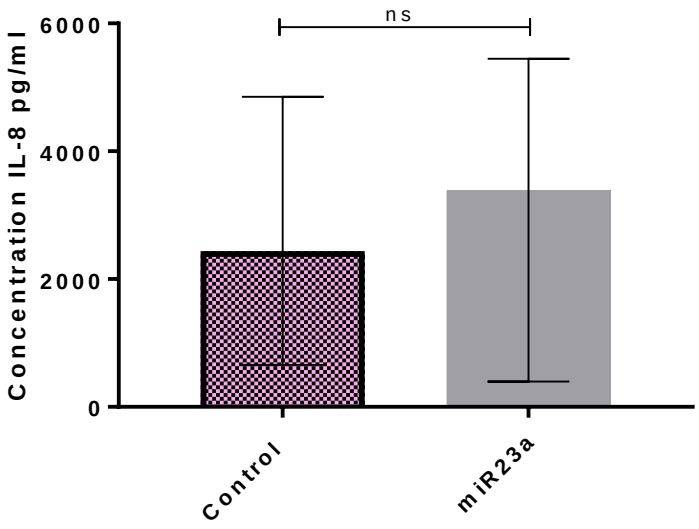


Figure 6.19: Concentration of IL-8 in 24 hour explant tissue culture supernatants. Explant culture of healthy colonic biopsies transfected with RNA preMir23a to overexpress miRNA23a (miR23a) or nonspecific preMir negative control vector (control). n =8 . Median with range presented.

6.8 Discussion

In this chapter I have defined the functional relevance of epithelial TNFAIP3 repression as a result of microRNA23a overexpression. My results show that overexpression of microRNA23a directly reduces TNFAIP3 at the protein level. In the presence of TNFα, reductions in this protein result in increased permeability of the epithelial barrier and enhanced inflammatory cytokine release, both of which are critical features of Crohn's disease.

Epithelial barrier studies have traditionally relied on immortalised cell lines. This approach has several advantages. As cells are cloned the study population can be characterised and the phenotype determined. Cells can be grown for extended periods of time allowing longer term studies to be undertaken. Culture conditions can also be controlled and standardised across duplicate experiments. Growing cells in monolayers allows functional studies to be performed with the measurement of specific outcomes (485). There are several commercially available intestinal cell lines which have been used for such studies. These include T84, caco-2 and HT29 cells. T84 cells were chosen in this study for their close resemblance to colonic epithelial cells. When grown in culture T84 cells are known to form polarised monolayers with the development of tight junctions. This facilitates vectorial solute transport across the monolayer, mimicking in vivo physiology. Typically, these cells also display high transepithelial resistance in cultured monolayers, a surrogate marker of tight junction formation, which can be measured.

Using this T84 model we were first able to shown that overexpression of microRNA23a reduces TNFAIP3 protein. This important finding supports the results of our immunohistochemistry studies in human colonic epithelia which showed a reduction in TNFAIP3 protein in Crohn's disease (chapter 5). We have shown that microRNA23a reduces translation of TNFAIP3 mRNA. Using T84 cells we have now shown that over expression of 23a reduces TNFAIP3 protein, thus proving the link between the microRNA and TNFAIP3 protein changes observed in human colonic epithelium.

An unexpected but intriguing finding from the T84 miRNA23a transfection was the reductions in GAPDH, Beta actin and cytokeratin19 protein. Blots were probed for these proteins as they are traditionally unchanged and can therefore be utilised as a reference for the quantification of the protein of interest. The

initial assumption on finding reductions in these proteins with microRNA23a was that this was due to a toxic effect of the microRNA construct or transfection reagent, resulting in a global protein reduction. However we were able to discredit this by finding that cells transfected without the microRNA or without any transfection reaction and microRNA did not have reductions in these proteins. If this result was due to toxicity of the transfection reagent, reductions would be expected when cells was transfected with this alone. None was seen. This suggests the effect may be a consequence of the microRNA itself. A search of Target Scan and microRNA.org did not reveal any complementarity for this microRNA in the 3'UTR of the mRNA sequences. This suggests either there is complementarity which has not been identified, the microRNA is acting at the 5'UTR (486) or is acting indirectly.

Cytokeratins form part of the cytoskeletal scaffold supporting the epithelial cell. The main role of the cytokeratins is to provide strength to the cell. However they are also important in maintaining cell polarity. Cytokeratin 19 is ubiquitously expressed with intestinal epithelium (487). However, there are currently no firm reports in the literature of diseases associated with alterations in this cytokeratin. Variants in the promotor of this gene have been studied in familial IBD cohorts. A positive association was not observed in this study, although this does not exclude a potential role in sporadic IBD (488). Support for the role of cytokeratins in IBD pathogenesis is provided by studies of cytokeratin 8. Mis-sense mutation in this protein have been found in cohorts of both CD and UC patients (489). In animal knockout studies cytokeratin 8 null mice develop colitis and increased intestinal permeability (490). Cytokeratin's may therefore have a role in CD pathogenesis. Based on their known function supporting the cellular architecture it is plausible that disruptions to one or several cytokeratins would have an adverse effect on cellular integrity and thus epithelial barrier stability. Further studies are needed to confirm this.

Similar to cytokeratin, Beta actin forms an integral part of the cellular cytoskeleton. There are no reports in the literature of alterations in this protein in inflammatory bowel disease. However reductions are seen in epithelial to mesenchymal transition. During this process cell contact are broken, the actin cytoskeleton rearranged, facilitating increased plasticity and movement of the epithelial cells. This is frequently seen in malignancy (491) but more recently has been identified in Crohn's disease associated fibrosis(492). Actin disruption is a fundamental process in this event(493).

If the effects of mirNA23a on beta actin and cytokeratin 19 are accurate this suggests mirNA23a is having a wider effect on epithelial cell stability with far reaching implications. However it should be remembered that this data has been generated using T84 cells. Further work is therefore needed initially using human tissue to confirm that actin and cytokeratin 19 expression change in human Crohn's disease epithelium.

Increased intestinal permeability is an early feature of Crohn's disease. In asymptomatic individuals, defects in permeability have been observed many months before the onset of clinical symptoms or overt mucosal inflammation. This suggests increased permeability is early initiating event in the development of inflammation and is supported by data from the Il-10 $^{-/-}$ mouse model(494). It is known that mice lacking TNFAIP3 within the epithelium develop normally and do not spontaneously develop colitis(324).. However if exposed to TNFα, these animal rapidly develop profuse diarrhoea, breakdown of the epithelial barrier and severe colitis. This inflammatory state is irretrievable, even after withdrawal of the TNFα stimuli and is universally fatal (324).. This suggests TNFAIP3 is playing a crucial role in regulating TNFα responses within the epithelium.

A similar situation is observed in our clinical patient cohort. As described in previous chapters both patients with active and inactive disease have reduced epithelial TNFAIP3 and overexpression of miRNA23a. Patients with inactive disease and therefore lower mucosal TNFα, had no overt mucosal inflammation and no increase in stool frequency. However, in those with active disease and therefore elevated mucosal TNFα, profuse diarrhoea and mucosal inflammation was observed.

The data from our T84 model now crucially provides a link between the mouse data and the observed clinical characteristics of our Crohn's cohorts. Seminal work by Fish at al in the late 1990's provided the first data characterising responses of T84 monolayers to inflammatory cytokines. In this work T84 monolayers cells were observed to be unresponsive to TNFα stimulation with no measurable change in barrier integrity (151). We were able to reproduce these results in our lab, We have now shown that by overexpressing microRNA23 and thus reducing TNFAIP3 in T84 cells , epithelial barrier function is perturbed, but only in the presence of an inflammatory stimulus, in this case TNFα. This suggests that TNFAIP3 supports epithelial barrier function and assists with the regulation of epithelial responses to TNFα in the T84 model.

As discussed in earlier sections there are two measurements that can be made of epithelial permeability enabling a distinction to be made between the ionic and macromolecule paracellular pathways. In our data the increase in epithelial permeability was observed with an increase in FITC dextran flux suggesting that it is the macromolecule permeability pathway that is affected by the reduction of TNFAIP3. This macromolecular pathway is regulated by occludin (178). This important junctional protein has previously been shown to be de ubiquitinated by TNFAIP3 and stabilised at the tight junction (191). Our data is supportive of this

In our study we added 1ng/ml of TNFα to cell cultures. Precise data detailing cytokine concentrations in the colonic mucosa are lacking in Crohn's disease. However measurement of colonic mucosal concentration in UC report a concentration of 1ng/ml (495). This concentration has also been used in the initial T84 studies by Fish etal investigating T84 barrier responses to TNFα. For these reason we too chose to apply 1ng/ml

Dysregulated cytokine production by epithelial cells is implicated in the pathogenesis of Crohn's disease. The transcription factor NFκB is one of the principle drivers of this response. It is known that TNFAIP3 is induced by TNFα driven NFkB expression and functions as a rheostat, modulating canonical NFκB expression (314, 320, 496, 497) . There is also some emerging data to suggest that TNFAIP3 is a positive regulator of the non-canonical NFKB pathway, leading to enhanced expression.(498). NFκB expression needs to be finely balanced. Over or under expression is detrimental to the epithelium and leads to the development of inflammation in experimental models.(300, 301)

In the mouse, epithelial NFκB activation has been shown mediate increased epithelial paracellular permeability with reorganisation of the junctional proteins claudin1 and occludin. Repression of NFKB attenuated these responses, further highlighting the importance of NFκB regulation (300).

Increased NFκB expression and activity is observed in epithelial cells patients with Crohn's disease and correlates with the severity of inflammation (298, 499) . Increased NFκB enhances the ability of the epithelium to produce pro inflammatory cytokines. Cytokines known to be driven in this way include TNF, IL6, IL8 and IL1β (63, 500).

Our data for the first time confirms that via the repression of TNFAIP3, microRNA23a results in increased NFKB activity. Increased transcription was first

demonstrated using a luciferase reporter construct showing that increasing microRNA23a results in increased NFκB transcription. It is known TNFAIP3 represses NFκB and from our previous assays, we know miRNA23 represses TNFAIP3. To then show the functional consequence of this we utilised an explant culture model and were able to show that increasing miRNA23a within colonic epithelium results in increased TNFα secretion. This section of work provides some evidence to suggest that overexpression of miRNA23a not only affects the barrier integrity of the epithelium but also promotes a pro inflammatory response from the epithelial cells.

The explant culture model was used for this work as it provides a more physiologically relevant model of epithelial function. This technique has gained increasing popularity in recent years (501-504). The epithelium functions as an integral part of the mucosal innate immune system. It does not exist in isolation in vivo. Using this explant model our data show that TNFα secretion is increased in explants where the epithelium is overexpressing miRNA23a. Therefore as well as affecting the barrier integrity , this dysregulated microRNA also promotes a pro inflammatory phenotype. TNF is known to have a detrimental effect on tight junction integrity. Increase production may therefore weaken an already fragile barrier, leading to increased antigen presentation and stimulation of lamina propria innate immune responses resulting in further cytokine release. In the experimental model used, it is possible that the epithelium was stimulated by cytokines released by mucosal immune cells as no additional cytokines were added to the biopsy or culture media. It is also possible that factors within the culture media provided the NFkB stimulus.

A possible criticism of this approach Is that we cannot be certain that some of the TNFα released was not produced by the epithelium but by the innate immune cells within the biopsy. It is possible that both contributed. However what we can say ,is that we know epithelial over expression of the microRNA was achieved as expression following transfection was checked by RT=qPCR of laser dissected epithelium. This clearly showed a reduction in the cT value for the transfected samples indicating increased expression. In the transfected biopsy TNF secretion was greater so it is therefore likely that the epithelial microRNA expression was a driver of this.

NFκB drives more than just TNF production. Other cytokines released by the epithelium and of relevance in IBD are IL=8, IL-6 and IL-1β (282, 505). In our

study, we did not find a significant difference in these cytokines compared to control. However, there was general trend towards increased expression. It is difficult to draw a firm conclusion from this data. Media was assayed 22 hrs after transfection. This time point was chosen to allow enough time for uptake and processing of the microRNA. However this culture time is significantly longer than has been reported in the literature where culture has typically been up a maximum of 18hrs(504). It is possible that the biopsy was therefore starting to disintegrate with areas of necrosis and cellular apoptosis. This was not apparent in the macroscopic appearance of the biopsy. The half-life of the cytokine and their secretion kinetics also need to be considered. In kinetic modelling studies, mRNA expression of IL6, and IL8 peaked at 2 hrs following stimulation and decreased over the next 8 hrs.(506). It is therefore possible that the sampling at 22 hours missed the peak cytokine release and an earlier time point for supernatant collection would have resulted in higher levels being present. It is also possible that significance would be achieved with a larger sample size, particularly for IL-8 where there was a numerical trend towards greater secretion in the miRNA23a transfected biopsies

In summary, the work in this section has shown that miRNA23a reduces TNFAIP3 protein. As a result of this NFκB transcription is enhanced leading to increased epithelial cytokine production. Epithelial barrier integrity is disrupted, but only in the presence of an inflammatory insult suggesting miRNA23 overexpression is not detrimental if homeostatic control can be maintained and inflammatory signalling restricted. However in the presence of TNF α there is an increase in paracellular permeability, which in turn will lead to increased antigen penetration through the epithelium.

Chapter 7:

Discussion

7.1 Summary

The original hypothesis for this thesis was that microRNA are altered in the colonic epithelium in Crohn's disease and modify the expression of the TNFAIP3 gene.

To test this, colonic biopsies were collected from Crohn's disease patients with active and inactive mucosal inflammation as well as healthy controls. An initial preliminary series of work identified microRNA23a as being differentially expressed with the epithelium. This was predicted to have partial sequence complementarity with the 3'UTR of TNFAIP3, a negative regulator of TNF induced NFκB signalling and was therefore chosen for study.

The epithelium was isolated from a cohort of 16 patients with active Crohn's disease, 7 inactive disease and 10 healthy controls. MicroRNA23a expression was determined using RT-qPCR. Immunohistochemistry staining for the predicted mirNA23A protein target TNFAIP was performed on serial tissue sections from the same biopsies used for the microRNA quantification. Functional studies were then undertaken utilising colonic cell lines and human explant cultures to determine the functional effect of TNFAIP3 repression of epithelial barrier integrity and NFκB regulation.

This led to the following conclusions in support of the hypothesis:

- MicroRNA23 is overexpressed with the colonic epithelium active and inactive colonic Crohn's disease.
- MicroRNA23a represses translation of TNFAIP3 via a complementary sequence within the 3'UTR of TNFAIP3.
- TNFAIP protein is reduced in the colonic epithelium of active and inactive Crohn's disease.
- NFKB transcription is increased by miRNA23a, acting via TNFAIP3 repression
- Increased miRNA23a leads to increased paracellular permeability in the presence of TNFα.
- TNFα secretion by colonic epithelia is enhanced by miRNA 23a expression.

7.2 Discussion

Crohn's disease is an inflammatory disease affecting the gastrointestinal tract. It is characterised by transmural inflammation most commonly in the terminal ileum and colon, although any part of the GI tract can be involved. Typical symptoms include diarrhoea, abdominal pain and a general failure to thrive. Systemic involvement is also observed in some cases. The eyes, skin, joints and oral mucous membranes are areas commonly affected. Uncontrolled inflammation leads to penetrating disease with the development of fistulas and abscesses. Scarring of the intestine subsequently leads to stricture formation.

Globally the incidence of Crohn's disease is increasing (3, 55). This increasing disease burden carries a significant cost to the healthcare economy and has a substantial impact on quality of life for patients. Although the exact aetiology is unknown it is widely accepted that the disease occurs as result of an aberrant immune response within the intestinal mucosa. The conceptual framework for the development of mucosal inflammation centres on impaired epithelial barrier function. This impairment allows an influx of luminal antigens to encounter innate and adaptive immune cells residing in the lamina propria. A dysregulated inflammatory response is initiated leading to the release of pro inflammatory cytokines which further exacerbate the barrier dysfunction. Traditionally Crohn's disease has been described as being driven by a Th-1 response, with TNFα, a fundamental inflammatory cytokine responsible for driving inflammation.

TNFα is known to disrupt the epithelial barrier leading to increases in intestinal permeability in active inflammation. It is also known that increased permeability is present in inactive disease and if present, is predicative of clinical relapse. Restitution of the epithelial barrier and healing of the mucosa has been shown to lead to improved long term outcomes for patients and lasting remission. There is therefore a clinical motivation to further understand the causes of epithelial barrier dysfunction and investigate ways in which these may be addressed

Antagonism of TNFα forms a cornerstone of modern IBD treatment. In vitro studies using immortalised colonic cell lines have suggested that such an approach is able to prevent internalisation of tight junction proteins in response to TNF α and thus preserve barrier integrity (507). This suggests anti TNF treatments may in part exert their function by modification of epithelial tight junctions, leading to preservation of epithelial barrier integrity. Despite this, results from clinical data sets are somewhat disappointing. In patients mucosal

healing occurs in only 45% when treated with a TNFα antagonist (124, 129. Adding an immunomodulator makes little difference {Colombel, 2010 #571). Discontinuation of treatment leads to relapse in up to 50% of patients (121). This suggests that although TNFα antagonism is important, additional regulation of TNFα responses may be needed to provide lasting remission.

In this study we have focussed on the role of TNFAP3 and its modification by microRNA. TNFAIP3 was chosen for several reasons. Genome Wide Association Studies have identified the TNFAIP3 gene in a susceptibility loci for CD (328) suggesting its relevance to inflammatory bowel disease. Reduced TNFAIP3 mRNA expression is observed in mucosal samples from Crohn's disease patients and has been shown to be associated with a more severe disease phenotype (331, 332). TNFAIP3 protein is known to negatively regulate the TNF driven NFκB pathway, a fundamental driver of inflammation and tight junction instability (256, 314, 508). This pathway is known to be active in the epithelium in Crohn's disease (293). In addition TNFAIP3 proteins may itself directly contribute to the maintenance of junction integrity via the deubiquitination and stabilisation of occludin within the junctional complex (191). MicroRNA have emerged as key regulators of cellular responses and are dysregulated in IBD. Little is known about their role in the regulation of TNFAIP3. This work therefore addressed this issue.

A fundamental and novel concept of our work has been to study TNFAIP3 regulation by microRNA in a defined epithelial cell population from human colonic biopsies. As discussed in the introduction the use of heterogamous samples in previous microRNA studies has hampered the interpretation of this data, making it difficult to determine the cellular location or function of particular microRNA. Recently data was presented from a small cohort of 6 ulcerative colitis patients in which RNA sequencing was undertaken on laser dissected epithelium(509). This represents a significant advance from previous studies and highlights the wealth of data which is accessible from defined cell populations. However, studies of microRNA expression and function in defined populations are still lacking. Our study has addressed this. We have now shown for the first time that microRNA can be successfully quantitated from defined cell populations in human samples. This is technically feasible from both inflamed and healthy tissue.

As a proof of concept we initially performed a series of laser dissections and microRNA screening analysis in a small number of UC and CD patients. As

discussed in chapter 3, several microRNA showed increased expression in this samples set although they not achieve statistical significance. This is most likely a reflection of this small initial sample size. As microRNA23a was the only one to achieve significance in and only in Crohn's disease, this was chosen for further study.

Our patient cohort for these studies were carefully characterised and was representative of the general IBD population (510). It should be noted the mean age in our healthy cohort was generally older than those with Crohn's disease. Age matched controls were not possible in this study as all recruited patients were undergoing colonoscopy as part of their routine clinical care. By definition this means that all patients had symptoms warranting investigation. To exclude bias, patients with a diagnosis of irritable bowel syndrome were excluded as this is known to causes diarrhoea and has associated mucosal microRNA changes (404) . The normal cohort patients were therefore selected from those undergoing investigation for either rectal bleeding or cancer surveillance, which typically occurs in the older population.

MicroRNA expression differences have been observed depending on the anatomical location of the sample Wu, 2010 #24}. To control for this all samples were in our study were taken from the sigmoid colon. This is broadly in keeping with the majority of microRNA studies in IBD, the majority of which have utilised colonic samples (419, 420, 434).

In our initial preliminary work expression of miRNA23, although greater than healthy control, did not achieve statistical significance in UC and was therefore not chosen. It is interesting to consider at his point that in whole biopsies from UC, miRNA 23a has been reported to be significantly over expressed (420). There are several potential reasons for this discrepancy. Firstly the sample size in our initial data set was small – just 5 patients. It is plausible that a larger sample size would be more representative and achieve significance. Secondly the normalisation between studies differs. Wu et al has used U6 as the internal control (420), compared to RNU44 in our present study. The relative expression levels of these could differ accounting for the observed differences in results. Finally it is possible that in UC the majority of miRNA23a expression is produced in inflammatory cells of the lamina propria and not the epithelium. Macrophages for example, have been shown to express high levels of miRNA23a (511). This may mask lower expression levels in the epithelium, with results from whole biopsies reflecting the cell type with the dominant expression.

Very little is known about the role of miRNA23a in inflammatory bowel disease. Both 23a and 23b have previously been shown to be overexpressed within the whole colonic biopsies of CD and UC (420, 434) with increased expression in the serum of CD patients (432) . However our work is the first time that epithelial specific expression has been reported.

Using a variety of methods we have shown that microRNA 23a reduces TNFAIP3 protein TNFAIP3 mRNA is known to be reduced in the colonic mucosa in Crohn's disease (331). Our study is the first to demonstrate reduced expression at the protein level in a defined cell population. Our work has identified dysregulation of TNFAIP3 specifically within the epithelium in Crohn's disease as a cause of increased epithelial permeability. TNFAIP3 stabilises the epithelial barrier by deubiquitinating occludin, preventing its internalisation from the junction (191). In support of this. we have shown that loss of TNFAIP3 causes an increase in epithelial permeability with an increase in macromolecular flux, a pathway which is regulated by occludin(178). This is the same mechanism by which anti TNF antibodies prevent TNF induced barrier dysfunction (507).

In addition to detrimental effects on the barrier, TNFα induces transcription of NFkB, which is also elevated in the mucosa of IBD patients (293, 499). Enhanced NFκB transcription causes further inflammatory cytokine release and disruption to the epithelial barrier directly via the loss of occludin (82, 256, 512). TNFAIP is known to repress NFkB. Our data demonstrates the functional consequence of the loss of this repression due to a microRNA. We have shown utilising an ex-vivo colonic culture model that epithelium with repressed TNFAIP3 due to miRNA23a produces increased TNFα, indicative of a pro inflammatory phenotype and aberrant NFκB transcription. This cytokine is overexpressed in inflammatory bowel disease and as discussed plays pivotal roles in disruption of barrier integrity and recruitment of innate and adaptive immune cells (513) (71). Our current treatments , which antagonise TNFα, have recently been shown to reduce epithelial NFκB activity (507). Thus it appears our current treatments are also attempting to replicating some of the NFκB regulatory functions exerted by TNFAIP3.

MicroRNA23a overexpression and TNFAIP3 repression were present in both active and inactive disease, suggesting this dysregulation is an intrinsic feature of the epithelium in Crohn's disease. However, barrier dysfunction only occurred in the presence of TNFα. This suggests that in the absence of an inflammatory stimulus,

epithelial integrity can be still supported despite the reduction of TNFAIP3. However this is fragile and when TNFα is encountered the epithelium is unable to adequately regulate its response, leading to barrier disruption, increased permeability and inflammation. This supports data from mouse studies in which animals lacking TNFAIP3 specifically within the epithelium were healthy in the absence of any inflammatory stimulus. However all animals were hypersensitive to sub therapeutic doses of TNFα with profuse diarrhoea, inflammatory cytokine release and death occurring in all subjects (324).

Taken together these data may help to explain why patients relapse when discontinuing an anti TNFα, particularly if the intrinsic TNF regulatory mechanisms remain dysregulated with the epithelium because of aberrant microRNA expression. Our data supports this and demonstrates a direct link between aberrant microRNA expression, reduced target protein and TNF dependent barrier dysfunction. Our data suggests loss of TNFAIP3 due to microRNA is detrimental to the stability of the barrier. This leads to the additional hypothesis that augmentation of TNFAIP3 levels could be beneficial and improve the efficacy of our current anti TNF treatments, thereby improving mucosal healing. It follows that if endogenous TNFAIP3 can be restored, the epithelium may be able to maintain control of TNF responses once treatment is discontinued, thereby improving lasting remission rates and clinical outcome.

Restoration of TNFAIP has already been shown to be beneficial as described in the murine epithelial TNFAIP3 expression model (314). Murphy and colleagues have also reported reduced inflammation in a murine model with epithelial overexpression of TNFAIP. This mechanism was shown to involve alteration of antimicrobial peptide expression, enhance microbial colonisation and induction of IL-10 responses (514). In a respiratory cell model TNFAIP3 responses could be augmented by glucocorticoids(515) suggesting it is possible to modify TNFAIP3.

The results from our study suggest that antagonism of microRNA23 is potential therapeutic option which should be explored. This concept is not new and the development of antiMir therapy is an evolving field(516). In recent years this approach has entered the clinical area with a successful phase 2 study of MirVirsen an anti-microRNA against hepatitis C (517). However as yet this approach has not been applied to IBD research.

The reason for this takes us back to where we started. MicroRNA studies in IBD until now, have simply not addressed cell specific expression and function. By utilising laser capture dissection we have defined epithelial cell specific

expression of microRNA23a as cause of TNFAIP3 repression. This exciting discovery paves the way for further study and potentially the development a new therapeutic treatment. Ultimately this may lead to treatment which will restore epithelial integrity, thus improving the efficacy of our current treatment and important provide sustained clinical remission and mucosal healing for patients with Crohn's disease.

7.3 Limitations

There are several limitations to this work that merit discussion. Although novel, this work has been limited to the study of sigmoid colonic epithelium. Crohn's disease also commonly affects the terminal ileum. Our study would therefore benefit from including small bowel disease samples. As such our conclusions can only be applied to colonic disease. It is unclear if isolated small bowel or ileocolonic disease would exhibit a different phenotype.

Control samples were taken from healthy individuals. As discussed these were not age matched with the disease group. An alternative approach would have been to include internal controls form each Crohn's patient, with samples taken from healthy mucosa as well as disease.

It should be remembered that although the determination of microRNA and TNFIP3 expression was undertaken in human tissue, the mechanistic studies utilised immortalised colonic cell lines. These have been widely used in studies and have many features of the epithelium. However by their very nature they may have genetic drift over time resulting in altered phenotypes and responses. To control for this the lowest passage cells available were used and repeated passage of thawed cells was avoided. However there are other cell lines available and some of the work could have been repeated in HT 29 cells or Caco 2 cells. If similar results were obtained with these cells it would increase confidence that the responses observed the T84 cells was not unique to this cell line. Of course the gold standard would have been to utilise the organoid culture models which are gaining in popularity.

Finally it should be noted that although slides for immunohistochemistry were independently blinded before being viewed for TNFAIP3 immunohistochemistry quantification, it was not possible to avoid bias. The differences in cellular morphology between inactive diseases and healthy were subtle and may not be appreciated. However there was a significant morphological difference between active Crohn's disease and healthy tissue. Therefore even though the codes for each slide were blinded it may still have been possible to distinguish that there was more than one group of slides being viewed.

7.4 Future Work

This thesis has addressed an important question regarding the control of TNFAIP3 within the epithelium by microRNA and has identified some important results as discussed. However there remain several important lines of further investigation.

Our study was limited to sigmoid colonic samples. It would benefit from being extended to include samples from the terminal ileum. An attempt could then be made to explore if there are any differences between epithelial microRNA expression in small bowel and colonic Crohn's. This would be important to know if antagonism of microRNA23a is to be considered as a therapeutic option. Principally this information would potentially influence the method of delivery of a anti microRNA therapy. Left sided colonic disease could be reached using rectal preparations. However these would not be appropriate for more proximal or small bowel disease.

Our results have shown that TNFAIP3 repression by microRNA23a impairs barrier function and that antagonism of miRNA23a restores TNFAIP3 levels. The logical extension of this is to expand work this further and investigate if restoration of TNFAIP by antagonising miRNA23a restores barrier integrity in the presence of TNF. This could initially be done in the T84 cell model used in this thesis. However a growing field is the use of human intestinal organoids. These have the significant advantage of

Finally consideration should be given to the unexpected finding of beta actin and cytokeratin 19 repression by microRNA23a. This work was not pursued as it was not the focus of this thesis. However if the results are correct this has wider reaching implications and would suggest microRNA23a has a fundamental role not only on TNF and tight junction regulation but also on cytoskeletal stability. In the first instance human epithelial samples from Crohn's disease and healthy subjects should be stained for these proteins. If this replicates the findings then further work should attempt to define a potential mechanism for this repression.

Appendices

Appendix A : Reagents

Advanced DMEM/F12 media (1x)
Life Technologies (1263010)

Advanced DMEM media
Life Technologies (12491015)

Agaraose
ThermoFischer (BPE 1356-100)

Ampicillin 200mg
Life Technologies (11593027)

A20/TNFAIP3 antibody (Western Blot)
Cell Signalling Technology (4625)

Amersham™ ECL™ Anti Rabbit IgG Horseradish Peroxidase linked Whole Antibody (from donkey)
GE Healthcare (NA934-100µl)

Amersham™ ECL™ Anti Mouse IgG Horseradish Peroxidase linked Whole Antibody (from sheep)
GE Healthcare (NA931-100µl)

Amersham™ ECL™ Select Western Blot Detection Reagent
GE Healthcare (RPN2235)

Amersham™ ECL™ Prime Blocking Agent
GE Healthcare (RPN418)

Anti-miR™ miRNA Inhibitor 23a-3p (MH10644)
Life Technologies (4464084)

Anti-miR™ miRNA Inhibitors Negative Control #1
Life Technologies (AM10644)

Arcturus®PEN membrane glass slide
Applied Biosystems (LCM0522)

Avidin Biotin Blocking Kit
Vector Labs (SP-2001)

Avidin Biotin complexes (peroxidase)
Vectastain ABC
Vector Labs (ZA0204)

Bovine Collagen I
Life Technologies (A10644-01)

Bovine Serum Albumin (used in cytokine reconstitution)
Sigma Life Sciences (A8806-5G)

Bromophenol Blue

Cignal NFkB Pathway Reporter Assay Kit (LUC) (Product number 336841)
Qiagen (CCS-013L)

Chloroform
Sigma Aldrich (02432 -23ml)

Cresyl Violet Acetate
Sigma Aldrich (05042-10G)

Cytokeratin 19 Antibody (4E8)
Thermo Fischer MA5-15884

DAB buffer (3'3 diaminobenzidine) 100ml
Biogenax (HK520-YAK)

Diethyl pyrocarbonate (DEPC)
Aldrich Chemistry (40718-25ml)

Dimethyl Sulphoxide (DMSO)
Sigma (D2650)

Dual Luciferase®Reporter Assay System
Promega (E1910)

Fluorescein Isothiocyanate Dextran (MW 3000-5000)
Sigma Life Sciences (101441911)
Fetal Bovine Serum (Heat Inactivated)
Life Technologies (10108-165)

GAPDH PCR primer (Hs02758991-g1)
Life Technologies (4331182)

Glycine
Fisher Scientific (BP381-1)

HighRanger Plus 100bp DNA Ladder
Norgen (12000)

Hiperfect Transfection Reagent
Qiagen (301704)

Interferin® SiRNA Transfection Reagent
Polyplus (409-01)

LB Agar
Sigma (L3027-1kg)

LB Broth
Sigma (L3022)

L Glutamine
Thermo Fisher (25030081)

Meso Scale Discovery Human Proinflammatory II (4 Plex) Kit
Meso Scale Discovery (K15053D-1)

MiRNEASY mini Kit (for cell lysate RNA extraction)
Qiagen (217004)

MicroRNA primer 23a (ID000399)
Life Technologies (4427975)

MicroRNA primer 27a (ID000408)
Life Technologies (4427975)

MicroRNA primer 24 (ID000402)
Life Technologies (4427975)

MicroRNA primer RNU44 (ID001094)
Life Technologies (4427975)

MiniProtean® TGX Stain-free™ Gels
BioRad (456-8033)

Modified Eagle Media (with Earls salts and stable glutamine)
GE Healthcare (E15-888)

Mouse monoclonal antibody to Beta Actin HRP linked
Abcam (ab20272)

One shot Mach1 T1 phage resistant chemically competent E.coli
Life Technologies (C862003)

Penicillin/Streptomycin (500U/ml)
Life Technologies (15070-063)

PreMir 23a-3p (MC10644)
Life Technologies (4464066)

PreMir precursor negative control #1
Life Technologies (AM17110)

Phosphate buffered saline 0.0067 M (PO4)
Lonza (BE17-516F)

Pierce™ BCA protein assay kit
ThermoScientific (23227)

Protease Inhibitor Cocktail
Sigma Aldrich (P8340-1ml)

Polyclonal swine anti rabbit immunoglobulins / Biotinylated swine F(ab')2
Dako (E0431)

QIAzole lysis reagent 50ml
Qiagen (79306)

Random Hexamers 50uM (100uL)
Life Technologies (N8080127)
RecoverAll™ Total Nucleic Acid Isolation kit
Ambion (AM1975)

RNAqueous- Micro Kit (Use the tubes only)
Ambion (AM 1931)

Recombinant Human IFNγ (100µg)
R&D Systems (285-IF-100)

Recombinant Human TNFα (5µg)
R&D Systems (201-TA-005)

RPMI 1640 media
Thermo Fischer (11875-093)

SOC medium
Invitrogen (15544034)

Sodium Dodecyl Sulphate
Thermo Fischer (5200/53)

Superfect Transfection Reagent
Qiagen (301305)

TaqMan MicroRNA Reverse Transcription Kit (1000 reaction)
Applied Biosystems (4366597)

TaqMan®Fast Universal PCR Master Mix (2x)
Applied Biosystems (4352042)

TNFAIP3 antibody (Polyclonal rabbit)
ProSci (5145)

TNFAIP3 PCR primer (Hs00234713-m1)
Life Technologies (4331182)

Tween®20
Sigma Life Sciences (P1379-500ml)

Trans-Blot® Turbo™ Transfer Pack Mini Format
BioRad (170-4156)

Thermoscientific Spectra Multicolour High Range Protein Ladder
ThermoScientific (26625)

TRIS Base
Fisher Scientific (BP152-1)

Trypsin-EDTA solution (1x)
Sigma (T3924)

2-Mercaptoethanol
Sigma Life Sciences (M3148-25m)

Appendix B: Equipment

Arcturis Pen Membrane Glass Slides for Laser dissection (Thermo Fischer)

Beckman Avanti J25 High speed centrifuge

Beckman Coulter Allegra™ 6R Centrifuge

ChemiDioc Imaging System (BioRad)

DNA Engine TETRAD™ 2 Peltier Thermal Cycler (Esco Technologies)

Dynatech MR7000 Plate Reader

Eppendorph 5415D benchtop centrifuge

EVOM2 Epithelial Voltohmmeter (World Precision Instruments)

Hematocytometer (Fischer Scientific)

Leica LMD Laser Dissection Microscope

Leica DMLB light microscope

MesoScale Discovery Sector 2400 Imager

Nanodrop (Fischer Scientific)

Nikon Coolpix 4500 4MP digital camera

STX2 Electrode (World Precision Instruments)

TD20/20 Luminometer (Turner Biosystems)

Transwell-clear 12 inserts in 12 well plates 0.4um pore size, (Fischer Scientific)

7900HT Fast Real-Time PCR System (Applied Biosystems)

Appendix C

Immunohistochemistry solutions

Tris Buffered Saline

Sodium Chloride	80g	Merck
Tris	6.05g	Merck
1M Hydrochloric acid	38mls	Merck
Distilled water	10L	

Mix buffer salts in 1L of distilled water, adjust pH to 7.65 and add to remaining 9L of water to give a final pH of 7.6. Aliquot in 200ml water bottle for use.

0.5% Hydrogen Peroxidase Solution

Methanol	5.9ml
Hydrogen peroxide	100µL

Blocking medium

Dulbecco's modified Eagles medium	80mls	Sigma
Fetal calf serum	20mls	PAA
Bovine serum albumin	1g	Sigma

Dissolve BSA in DMEM and FCS. Aliquot and store at -20°C until required.

Citrate buffer 0.01M pH6.0

Citric acid crystals	2.1g
Distilled water	1000ml

Mix and adjust to pH6.0 with 1M sodium hydroxide (approx. 25mls)

EDTA buffer 1mM pH8.0

EDTA	0.37g
Distilled water	1000ml

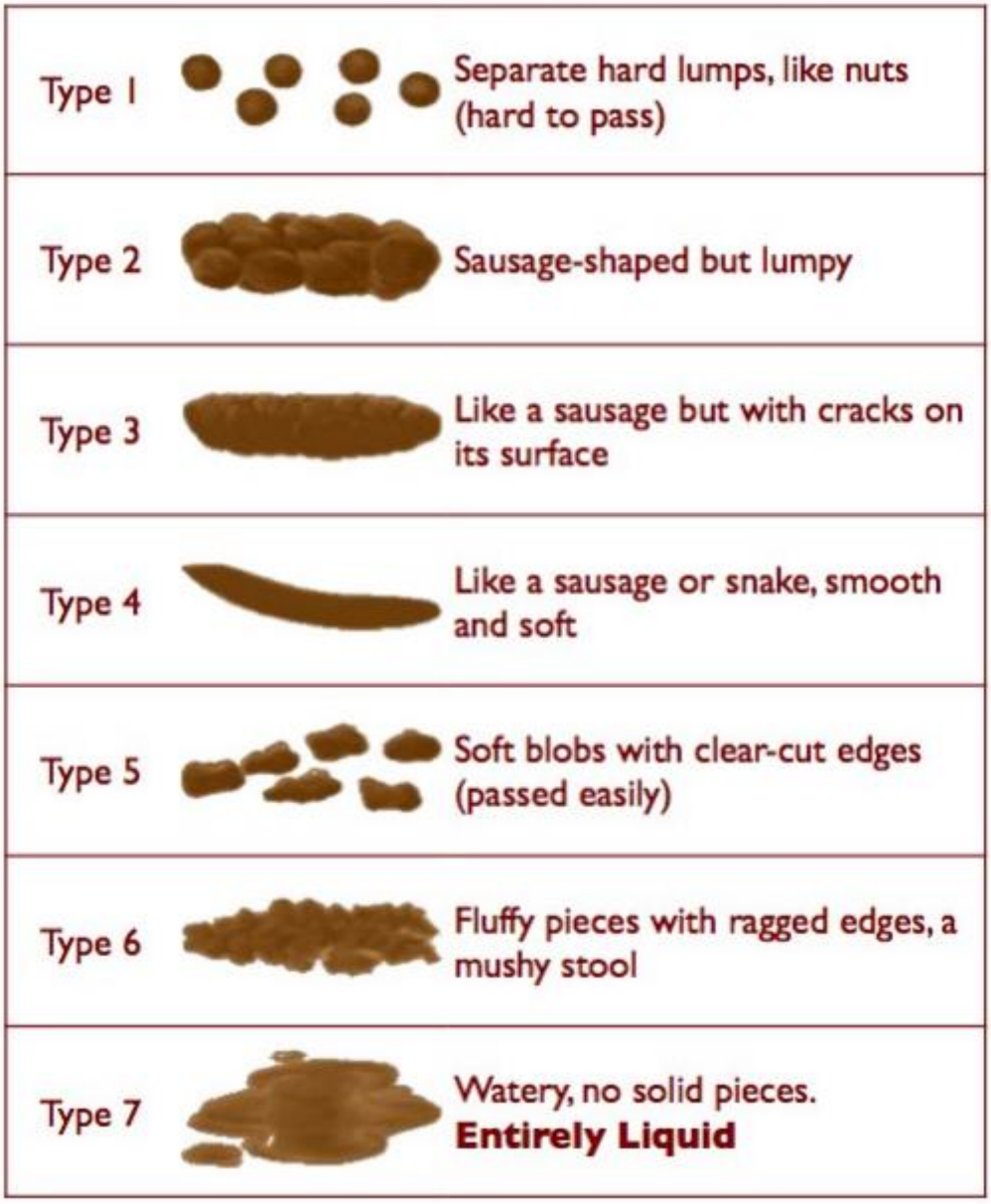
Mix and adjust to pH8.0 with 0.1M sodium hydroxide (approx. 8ML)

DAB

Buffer	1ml
Chromagen	32µl
15% Azide	50µl

Scale up as needed. Allow 200µl per slide

Appendix D: Bristol Stool Chart



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Appendix E: Harvey-Bradshaw Index

A clinical score of Crohn's disease severity. The scores of each question are added with higher scores indicating more severe disease. A score of <5 is considered clinical remission; Mild disease 5-7; Moderate disease 8-16; severe disease >16 (466).

General well-being	Very well = 0 Slightly below par = 1 Poor =2 Very poor = 3 Terrible = 4
Abdominal pain	None = 0 Mild = 1 Moderate = 2 Severe = 3
Number of liquid or soft stools per day *(score 1 for each)*	
Abdominal mass	None = 0 Dubious = 1 Definite = 2 Definite and tender =3
Complications *(Score 1 for each)*	None Arthralgia Uveitis Erythema nodosum Apthous ulcers Pyoderma gangrenosum Anal fissure New fistula Abscess

Appendix F: Patient Consent Form

University of Southampton

Southampton NHS
University Hospitals NHS Trust

CONSENT FORM

Thank you for reading the information about our research project. If you would like to take part, please place your initials in the boxes on the right side beside each statement and sign the form at the bottom.

Title of project: MicroRNA coordination of inflammation-mediated changes in human gut epithelial tight junctions and innate immune cytokine expression.

Centre: **SOUTHAMPTON**
R&D Reference: RHM MED0942 **REC No.: 10/H0502/69**

Name of researchers: **Dr Jane Collins, Dr Tilman Sanchez-Elsner Dr Sylvia Pender and Dr Fraser Cummings**

1. I confirm that I have read understood and have had time to consider the information sheet dated 21/07/10 version 1.0 and have been given a copy to keep. I have had the opportunity to ask questions about this project. ☐
2. I agree to give samples of blood for research in this project as detailed in the Patient Information Sheet ☐
3. I agree to give samples of tissue for research in this project as detailed in the Patient Information Sheet ☐
4. I understand how the sample(s) will be collected, that giving sample(s) for this research is voluntary and that I am free to withdraw my approval for use of the sample(s) at any time without giving a reason and without my medical treatment or legal rights being affected. ☐
5. I understand and agree that parts of my medical information may be passed to other organisations involved in the research on the understanding that my personal patient confidentiality will be maintained. ☐
6. I understand that I will not benefit financially if this research leads to the development of a new treatment or medical test. ☐
7. I agree that the sample I have given and the information gathered about me can be stored for possible use in future projects, and that anonymised samples may be used in other UK centres as described in the attached information sheet. ☐
8. I agree that the samples I give may be used for research aimed at understanding what causes inflammatory bowel disease and how to prevent it. The material may also be used for genetic testing. I understand that the results of these investigations are unlikely to change my future treatment in any way. ☐

Patient's name (PRINT) Date

Signature

Name of person taking consent Date

Signature

Name of Researcher Date

Signature

miRNAs and IBD Consent form 21/07/2010 version 1.0

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