MicroRNA23a overexpression in Crohn’s Disease targets Tumour Necrosis Factor Alpha Inhibitor Protein 3, increasing sensitivity to TNF and modifying the epithelial barrier

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Short Title: MicroRNA23a in Crohn’s Disease

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Abbreviations: IBD, inflammatory bowel disease; TNF, Tumour necrosis factor alpha; NFkB, Nuclear factor kappa B; TNFAIP3, Tumour necrosis factor alpha inhibitory protein 3.
Abstract

Background and Aims: Mucosal healing is important in Crohn’s disease therapies. Epithelial homeostasis becomes dysregulated in Crohn’s with increased permeability, inflammation and diarrhoea. MicroRNAs are small non-coding RNAs, which regulate gene expression and show changes in inflammatory bowel disease. Tumour Necrosis Factor Alpha Inhibitor Protein 3 is raised in Crohn’s and regulates TNFα-mediated activation of NFκB. We investigated TNFα regulation by microRNA in Crohn’s disease and studied effects on epithelial permeability and inflammation.

Methods: Colonic epithelium from CD and healthy donor biopsies was isolated using laser capture microdissection and microRNA quantified. Tumour Necrosis Factor Alpha Inhibitor Protein 3 was characterized immunohistochemically on serial sections. Expression effect of microRNA was confirmed with luciferase reporter assays. Functional barrier permeability studies and innate cytokine release were investigated with cell and explant culture studies.

Results: MicroRNA23a levels significantly increased in colonic Crohn’s epithelium compared to healthy. Luciferase reporter assays in transfected epithelial cells confirmed that microRNA23a repressed expression via the 3’ untranslated region of Tumour Necrosis Factor Alpha Inhibitor Protein 3 mRNA coinciding with increased NFκB-mediated transcription. Immunohistochemical staining of TNFAIP3 protein in colonic biopsies was reduced or absent in adjacent Crohn’s sections, correlating inversely with microRNA23a levels and encompassing some inter-cohort variation. Overexpression of microRNA23a increased epithelial barrier permeability in a colonic epithelial model and increased inflammatory cytokine release in cultured explant biopsies, mimicking Crohn’s disease characteristics.

Conclusion: MicroRNA23a overexpression in colonic Crohn’s epithelium represses Tumour Necrosis Factor Alpha Inhibitor Protein 3, enhancing sensitivity to TNFα with increased intestinal permeability and cytokine release.

Keywords: MicroRNA, Epithelium, TNFAIP3, Inflammatory Bowel Disease, Crohn’s, TNF
**Introduction**

A key outcome for the treatment of Crohn’s disease is the attainment of mucosal healing and deep remission. This is associated with reduced treatment need and possibly an improved long term disease course. Remission of clinical symptoms with anti-TNF is attainable in 45% of patients, but only a small proportion of those achieve mucosal healing. Up to 50% of patients relapse after treatment stops, suggesting persistence of the underlying drivers of inflammation. Strong clinical motivation exists to understand the mechanisms underpinning dysregulation of TNFα responsive pathways in inflammatory bowel disease (IBD), not only to improve mucosal healing rates, but also to maintain remission.

Mucosal healing includes restitution of the intestinal epithelial barrier. In healthy individuals, the intestinal epithelium maintains a selective barrier between the lumen and underlying mucosa, via the coordinated expression and interaction of proteins within circumferential tight and adherens junctions. This is maintained even during the normal shedding of epithelial cells. However in Crohn’s disease the barrier is disrupted with loss of junctional proteins and increased intestinal permeability. Barrier perturbation often coincides with dysregulated activation of the immune system and increased TNFα release. Epithelial tight junctions are dynamic structures, modulated by numerous stimuli of which TNFα is of particular importance in disrupting barrier integrity, with increased intestinal permeability, by a mechanism involving disruption of the actin cytoskeleton via myosin light chain kinase phosphorylation and loss of zonula occludens 1. Induction of NFκB by TNFα caused junction disruption via the down regulation of zonula occludens 1. Additionally TNFα stimulation promoted loss of occludin from the tight junction via endocytosis. Data from cell culture models suggested that TNF antagonists may stabilise epithelial tight junctions and therefore enhance barrier integrity. Disruption of the epithelial barrier is an early feature of disease relapse suggesting an initiating role in the development of mucosal inflammation. Increased epithelial permeability is observed in inactive disease in both animal models and human studies and is strongly predicative of clinical relapse, thus underpinning the importance of the epithelial barrier in disease pathogenesis.

Given the detrimental effects of TNFα on barrier integrity and NFκB activation, appropriate regulation of TNF signaling is critically important in IBD. One regulator of TNF is Tumor Necrosis
Alpha Inhibitor Protein 3 (TNFAIP3), which negatively regulates the TNF driven NFκB pathway, a fundamental driver of inflammation and epithelial tight junction instability\textsuperscript{25,33,34}. This pathway is active in epithelia in Crohn’s disease\textsuperscript{35}. In addition TNFAIP3 protein may itself directly contribute to the maintenance of junction integrity via the deubiquination and stabilisation of occludin within the junctional complex\textsuperscript{36}. Genome Wide Association Studies have identified the TNFAIP3 gene in a susceptibility loci for CD\textsuperscript{37} suggesting its relevance to inflammatory bowel disease. Reduced TNFAIP3 mRNA expression was observed in mucosal samples from Crohn’s disease patients and is associated with a more severe disease phenotype\textsuperscript{38,39}. It is unclear how this protein is regulated within the epithelium in Crohn’s disease.

MicroRNA have emerged as potential key regulators of cellular responses that are dysregulated in IBD\textsuperscript{40-42}. These small non-coding RNAs regulate gene expression by binding to the 3’ untranslated region (UTR) of mRNA and inhibiting expression\textsuperscript{43,44}. In this way they function to modify cellular protein levels\textsuperscript{45-47}. Limited information exists on the potential role of microRNA in regulating epithelial barrier and innate immune responses in the colonic epithelium. In irritable bowel syndrome (IBS), increased expression of microRNA29a,b correlated with decreased expression of glutamine synthetase, NFκB repressing factor (NKRF) and claudin 1, with these changes contributing to increased intestinal permeability in mice, suggesting an important role for microRNA in regulating barrier stability\textsuperscript{48,49}. In addition, claudin 2, associated with increased barrier permeability\textsuperscript{16} and cingulin were upregulated in IBS, correlating with reductions in the targeting by microRNA16 and 125b, with confirmed functional increases in barrier permeability\textsuperscript{50}. Increased expression of microRNA223 correlated with decreased claudin 8 in whole human biopsies from ulcerative colitis (UC) and Crohn’s disease\textsuperscript{51} and microRNA200b expression in CaCo2 cells attenuated tight junction damage and suppressed IL-8 secretion\textsuperscript{52}.

Existing microRNA studies in Crohn’s disease have mostly defined expression profiles in patient serum or whole mucosal intestinal biopsies\textsuperscript{40,53-55}. In situ hybridization showed increased expression of microRNA21 and microRNA126 in UC lamina propria and endothelial cells respectively\textsuperscript{56}. Here we utilised laser capture microscopy and immunohistochemistry in serial tissue sections. This revealed cell-specific information on microRNA expression in colonic epithelium of mucosal biopsies from patients with Crohn’s disease, relative to expression of TNF-induced TNFAIP3, a negative regulator of NFκB\textsuperscript{33,57-60}. Our results suggest a link between
increased epithelial microRNA23a expression and the dysregulation of TNFα signaling via repression of TNFAIP3. Recapitulating these changes in vitro, significantly increased epithelial barrier permeability and enhanced innate inflammatory cytokine release in ex vivo explant biopsies of human colon, implicating this pathway as a therapeutic target in Crohn’s disease.
Materials and Methods

Additional information is in Supplementary Methods

Human tissue collection

IBD and investigative colonoscopy patients were recruited with informed consent, at Southampton University NHS Trust, under full ethical approval (REC No 10/HO502/69). Pinch biopsies were from sigmoid colon, with active and inactive Crohn’s disease, or healthy mucosa. Diagnosis was confirmed by the presence or absence of active mucosal inflammation by histopathology on an adjacent biopsy to the sample. Features of acute inflammation and presence of ulceration or granulomas defined active Crohn’s, which were absent in inactive disease in the presence or absence of focal or segmental crypt and glandular distortion or ulcer-associated cell lineage changes. Biopsy samples were placed in 10% neutral buffered formalin before being embedded in paraffin blocks.

Laser capture microdissection and RNA extraction

10µm thick biopsy sections were cut from paraffin blocks, dewaxed and stained with 0.1% w/v Cresyl Violet. Epithelial tissue was isolated utilising laser capture microscopy (LCM) (Leica AS LMD microscope). Total RNA was extracted using the Ambion RecoverAll™ Total Nucleic Acid Isolation Kit utilising columns from the Ambion RNAqueous-Micro Kit (Thermofisher Scientific) according to manufacturer’s instructions. RNA integrity was determined using the Nanodrop ND-1000 Spectrophotometer.

microRNA real time PCR

MicroRNA23a is hsa-miR-23a-3p; microRNA29a is hsa-miR-29a-3p; microRNA29b is hsa-miR-29b-3p; microRNA29c is hsa-miR-29c-3p.; microRNA429 is hsa-miR-429. Quantitative RT-PCR was performed using TaqMan® microRNA assays comprising specific RT primers and TaqMan® PCR primers with fast Universal PCR Mastermix, in MicroAmp optical 384 well plates and assayed using Applied Biosystems 7900HT Fast Real Time PCR system (Thermofisher Scientific). All microRNA values were expressed relative to an internal RNU44 housekeeping ribosomal RNA gene value in order to normalise for differences in cell number, RNA isolation and assay consistency. Consistent expression of RNU44 was verified across samples. The ΔΔct method was used to calculate intergroup variation with values compared to one value in the healthy group to show the data spread within and between groups.
RT-qPCR RNA assay

Primers for TNFAIP3, Keratin8 and CD45, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to normalise for RNA integrity and assay consistency, were used to quantify expression using a TaqMan 7900HT machine (Thermofisher Scientific).

Immunohistochemistry

Serial 4µm sections cut from patient biopsies, adjacent to those used for microRNA analysis, underwent antigen retrieval, blocking of endogenous peroxidase and avidin biotin unspecific binding, then incubated overnight at 4°C with rabbit anti-TNFAIP3 (ProSci). Biotinylated Dako swine anti-rabbit (Agilent) was applied and sections were developed with 3,3’-Diaminobenzidine, counterstained with haematoxylin, viewed (Leica DMLB) and photographed (Nikon Coolpix 4500).

Immunohistochemistry Quantification

All slides were coded blind and five random fields per slide were quantified, using an established method.

TNFAIP 3’UTR Construct and Luciferase Reporter Assay

Approximately 600bp of the 3’UTR of TNFAIP3, with putative microRNA23a binding site (seed nucleotides 1823-1830 Target Scan V7.0), was cloned 3 prime to Renilla luciferase in the pRLTK reporter (Promega UK). Genomic DNA was amplified using forward primer GCTAGC-AGACTGGCAATGGCAATGGTCACAGG and reverse primer GCGGCCGC-ATCCAACAAAGAATAGGTGGC. This fragment was cloned Nhe1 into Xba1 (pRLTK base 1971) and Not1 into Not1 (pRLTK base 1978). A paired TNFAIP3 reporter was made with mutant microRNA binding site comprising an ECoR1 restriction site. For overexpression, the genomic region encompassing microRNA23a premiR (NCBI NR_029495.1) was cloned in pcDNA3.1 (Thermofisher Scientific), with empty pcDNA3.1 vector as a negative control. PremiR genomic DNA was amplified and cloned into Xba1 (using forward primer AAGCTTTCTAGA-TGCCAGCCTCTGGCCC) and Kpn-1 (using reverse primer GGTACCACGCGT-CTCCTCAGGCGGCACCAG) restriction sites of the pcDNA3.1 expression vector. TNFAIP3 reporter DNA was transfected into cells, with pGL3 luciferase transfection efficacy control, using Superfect (Qiagen). Lysates were harvested after 16 hours and assayed with Dual Luciferase Reporter Assay System (Promega), according to manufacturer’s instructions. Experiments were performed five times in duplicate.
**NFκB luciferase reporter assay**

The Cignal NFκB reporter (Qiagen) with or without tandem repeats of the NFκB response element cloned upstream of a basal promoter and firefly luciferase coding region was used. All firefly luciferase reporters were normalised to a simultaneously transfected efficacy control *CMV Renilla* Luciferase. Each preMiR construct and NFκB reporter were transfected into HeLa cells using Superfect (Qiagen) and incubated for 48 hours. Two hours prior to cell harvest, 1ng/ml TNFα (R+D Systems) was added to selected wells. Luciferase levels were measured using Dual Luciferase Reporter Assay System (Promega) according to manufacturer’s instructions. Experiments performed five times in duplicate and statistical comparisons were within each NFκB reporter type to control for inherent transcriptional variation between reporter constructs.

**Cell culture**

HeLa cells were cultured in Modified Eagle Media with Earle’s salts, glutamine, penicillin/streptomycin and 10% heat inactivated Fetal Calf Serum. T84 cells were cultured in Advanced Dulbecco Modified Eagle Media: F12 supplemented with L-glutamine, penicillin/streptomycin and 5% heat inactivated Fetal Calf Serum.

**T84 cell model with microRNA transfection and permeability studies**

T84 cells were seeded on 12 well, 0.4um pore Transwell filters (Fisher Scientific UK), transfected with 100nM pre-miR-23a-3p or pre-miR negative control #1, with minimal homology to human microRNA (Ambion, Thermofisher Scientific) using 3μl of HiPerFect reagent (Qiagen), according to manufacturer’s instructions. Amounts of pre-miR RNA and HiPerFect reagent were optimised and transfection efficiency checked prior to experimental assays (Supplementary figure 3C,D). Inhibitory experiments utilised anti-miR hsa-23a-3p inhibitor or anti-miR negative control #1 (Ambion, Thermofisher Scientific) and effect of transfections on miR-23a levels were assayed (Supplementary figure 4). Cells were cultured for 5 days, with media replacement, with or without TNFα 1ng/ml, every 24 hrs to the apical and basolateral compartments. Transepithelial resistance was measured daily, in triplicate at 37°C, a using an EVOM epithelial volt/Ohm meter and STX2 electrode (World Precision Instruments). At day 5 the macromolecule paracellular permeability was measured using 4KDa Fluorescein isothiocyanate dextran (Sigma Aldrich) permeability assay. Three independent experiments were done with 8-10 individual filters per condition.

**Ex vivo Culture and MicroRNA Transfection of Intestinal Biopsies**
Approximately four sigmoid colon pinch biopsies, with macroscopic and histologically normal colonic mucosa, were collected from each patient and rinsed and plated in 1ml ice cold RPMI 1640 medium, containing L glutamine, penicillin/streptomycin and 10% heat-inactivated fetal calf serum. Biopsies were transfected with 100nM of pre-miR23a-3p RNA precursor mimic or pre-miR negative control #1 (Ambion UK) using Interferin (PolyPlus Transfection) according to manufacturer’s instructions. At 24 hours, culture supernatants were stored at -20°C. Biopsies were fixed in neutral buffered formalin and embedded in paraffin blocks. Epithelium from transfected and negative control biopsies was isolated with LCM and microRNA23a overexpression was confirmed with RT-qPCR.

**Total protein extracts T84 cells**

Cells and supernatants were lysed for SDS_PAGE in 0.5M Tris pH6.8, 10% sodium dododecyl sulphate, 1mM dithiothreitol (DTT), glycerol and protease inhibitor cocktail (Sigma Aldrich). Protein concentration was determined using a bicinchoninic acid (BCA) assay (Thermo Fisher Scientific) and adjusted to 1M DTT prior to loading on gels.

**Western Blot**

Samples were run on 10% Mini Protean TGX gels and transferred to PVDF membranes using a Trans-Blot®Turbo™ system (BioRad). TNFAIP3 rabbit antibody (Cell signalling Technology) was used with horseradish peroxidase linked donkey anti-rabbit IgG (GE Healthcare), with 1:5000 mouse anti-beta actin HRP linked (Abcam) loading control antibody applied for 1 hour at room temperature. Detection was with ECL Select™ (BioRad) and blots viewed using the Biorad ChemiDoc Imaging system.

**Cytokine assay**

Inflammatory cytokines were assayed simultaneously in biopsy culture supernatant using a high sensitivity MesoScale Discovery V-PLEX assay (MSD) according to manufactures instructions.

**Statistical Analysis**

Statistical analysis for RT-qPCR data was analysed using unpaired 2 tailed student’s t test. Immunohistochemistry quantification, barrier permeability measurements, luciferase assays and cytokine measurements were analysed with non-parametric tests as appropriate, using Graph Pad Prism 6. A p value of <0.05 was considered significant. R correlations were tested with Spearman Rank Coefficient and Pearson correlation coefficient as outlined in figure legends.
**Results**

MicroRNA23a expression was higher in colonic epithelium from Crohn’s disease relative to healthy donor epithelium

Previous microRNA studies have analysed whole mucosal biopsies thus hampering interpretation of results due to cell type heterogeneity. Data detailing cell specific microRNA expression and function is lacking in IBD studies. We therefore isolated epithelial cells from colonic biopsies using Laser Capture Microscopy (Supplementary Figure 1A,B) and isolated total RNA to include small RNAs and microRNA. The epithelial enrichment of samples was confirmed using RT-qPCR for epithelial-specific keratin 8 and immune cell marker CD45. Samples showed high levels of mRNA for keratin 8 and reduced levels of CD45 compared with THP-1 control mRNA indicating epithelial differentiation (Supplementary Figure 1C,D). In preliminary work, we performed bioinformatic sequence analysis for microRNA seed complementarity within the 3’ UTR of TNFAIP3. This analysis showed potential binding of several microRNAs to the 3’UTR of TNFAIP3 mRNA, therefore the expression of microRNA29a, 29b, 29c, 429, 23a (Supplementary Figure 2) was assayed in a subset of 5 healthy controls and 5 active Crohn’s disease patients. These data demonstrated that microRNA quantitation from LCM isolated epithelium is feasible. Only microRNA23a showed significant over expression compared to the healthy controls and was studied further in a larger patient cohort.

Sigmoid colonic biopsies from 16 patients showing active Crohn’s disease, 7 with inactive disease without mucosal inflammation and 10 healthy donors were analysed for comparison. Clinical characteristics of each patient group are in Table 1.

**Table 1:** Clinical characteristics of tissue donors
MicroRNA23a was increased in isolated colonic epithelium in both active (p=0.04) and inactive Crohn’s disease (p=0.02) compared to healthy controls in these groups (Figure 1A). Review of the Crohn’s disease data showed the presence of individuals with low/normal 23a expression and those with expression greater than the mean of the healthy group. We interrogated the associated clinical data of these patients to identify any characteristics that might correlate with this observation. Across all Crohn’s Disease and healthy donors, a positive correlation of $r=0.347$, $p=0.04$ (Spearman) was found between relative microRNA23a expression and loose stool frequency (Figure 1B). Importantly, active disease alone showed a correlation of $r=0.5269$, $p=0.036$ (Figure 1C).

**MicroRNA23a repressed expression via a predicted seed binding sequence on the 3’UTR of TNFAIP3**

A search of bioinformatic data (TargetScan V7.0 and MicroRNA.org (August 2010 release) indicated a microRNA-23a predicted binding site in the 3’UTR of TNFAIP3 (Figure 2A). To test this we generated an expression vector construct for microRNA23a, plus *Renilla* luciferase reporter constructs harboring approximately 600bps of the 3’UTR of TNFAIP3, cloned three prime to the luciferase coding region, with either wild type or mutant microRNA23a predicted seed binding sequences (Figure 2B). Transfecting Hela cells with the wild type 3’UTR and expression vector for microRNA23a resulted in a 31% reduction in luciferase activity $p=0.02$ (Figure 2C). This was not...
seen when the mutated 3'UTR or empty microRNA vectors were transfected. This confirms that microRNA23a is able to repress expression via complementary nucleotide sequences in the 3'UTR of TNFAIP3. Reduction in TNFAIP3 encoded A20 protein in T84 colonic epithelial cells, transfected with pre-miR-23a to overexpress microRNA23a (miRNA23a) or pre-miR negative control (Negative Control), was confirmed with semi-quantitative western blotting (Figure 2D). Conversely, transfection with anti-miR-23a in T84 cells was observed to maintain TNFAIP3 encoded A20 protein at levels similar to anti-miR control (Figure 2E). The effect of transfection on miR-23a levels in T84 cells was confirmed with RT-qPCR (Supplementary Figure 3C,D and Supplementary Figure 4).

TNFAIP3/A20 protein staining was reduced in colonic epithelia in Crohn's disease

After observing that microRNA23a was overexpressed within the epithelium in Crohn's disease, with potential effects on TNFAIP3, we next sought to determine expression of TNFAIP3 mRNA and its encoded protein TNFAIP3/A20, within the colonic epithelium. Using RT-qPCR analysis, total RNA extracted from LCM dissected epithelium, was assayed for expression of TNFAIP3 mRNA in Crohn’s donors compared to the healthy cohort. Expression in active disease was not significantly changed compared to healthy controls (p=0.333). The expression of mRNA in inactive disease was also not significantly different from healthy controls with a 0.37 fold change (p=0.294) (Supplementary Figure 5).

Changes were observed in TNFAIP3/A20 protein staining in tissue sections adjacent to LCM sections assayed for RNA. In biopsies from healthy controls strong immunostaining was seen at apical tight junction and subapical membrane regions of epithelial cells (Figure 3A). In biopsy sections from active and inactive Crohn’s disease, there was an observed loss of staining from these cellular regions, with full loss, partial loss with reduced intensity, or patchy staining, (Figure 3A-D). Consistent with these observations, quantification of the immunohistochemical staining by an independent blinded observer confirmed there was a significant reduction in TNFAIP3/A20 protein at apical and subapical membrane locations in both active and inactive Crohn’s disease (Figure 3E). Further analysis of data supporting Figure 1A and Figure 3E, correlated specific individual donor values of microRNA 23a in epithelial biopsy sections and TNFAIP3 histochemistry score from adjacent sections (Supplementary Figure 6A,B). This shows there is variation in the
level of microRNA23a relative to the immunohistochemistry score in individual snapshots of these dynamic processes.

**MicroRNA23a increases NFκB transcriptional activity**

TNFAIP3/A20 is known to function as a negative regulator of NFκB signalling. We observed that increased levels of microRNA23a in the epithelium in Crohn’s Disease coincided with decreased expression of TNFAIP3/A20 protein in adjacent cell sections. Therefore, we investigated whether microRNA23a expression promotes an increase in NFκB transcriptional activity, using a luciferase reporter activated via NFκB response elements upstream of the promoter. There was no difference in basal NFκB transcription when microRNA23a was transfected into cells with the NFκB or non-reporter. The relative luciferase activity of NFκB/23a transfected cells compared to NFκB reporter alone was 1.08 (p=0.1602) (Figure 3F). However when NFκB mediated transcription was stimulated by the addition of 1ng/ml TNFα, a significantly greater increase in luciferase activity was observed, confirming the efficacy of the assay. Relative luciferase expression after TNFα stimulation increased 12.9 fold in the absence of microRNA23a, whereas in microRNA23a transfected cells, a 17.43 fold increase was achieved with TNFα (p=0.0098) (Figure 3F). This data confirms that microRNA23a expression increased the transcriptional activity of NFκB in the presence of TNFα.

**Overexpression of microRNA23a impairs epithelial barrier function.**

Dysregulated epithelial barrier function is linked to inflammatory bowel disease pathogenesis. A separate study suggested that TNFAIP3/A20 stabilised occludin at the tight junction, contributing to maintenance of epithelial barrier integrity. The observed increase in microRNA23a levels and correlation with reduced TNFAIP3/A20 staining in Crohn’s, led us to investigate the functional effect of overexpressing microRNA23a on epithelial barrier function. We used the T84 colonic epithelial model as these cells show limited effects on barrier loss with TNFα alone, requiring synergy with Interferon γ. This facilitated barrier analysis of increased microRNA23a, in the presence of TNFα, as often found in the disease milieu. There was no significant difference in transepithelial ionic permeability over days 1-5 with or without TNFα or increased microRNA23a (Supplementary Figure 3; Figure 4A). Macromolecular barrier function between transfected cells overexpressing microRNA23a or negative control precursor, assessed at day 5 by FITC dextran permeability
measurements, was not significantly different (Figure 4B). However, in the presence of TNFα, FITC-dextran permeability in negative precursor transfected cells was increased (Figure 4B, lanes NC and NC TNF3), and microRNA23a transfection caused a greater increase in permeability with TNFα in the medium (Figure 4B, lanes NC TNF and 23a TNF). Median FITC permeability was 0.24mcg/ml in negative precursor cells and 0.68 mcg/ml with TNFα, compared to 1.11mcg/ml in TNFα with microRNA23a transfection ($p=0.0009$) (Figure 4B). These data show that increased expression of microRNA23a, in the presence of TNFα, leads to significant increase in the permeability of the epithelial macromolecular barrier.

**Overexpression of microRNA23a enhances inflammatory cytokine release in an ex vivo colonic culture system.**

In our study, the NFκB luciferase reporter assay confirmed that in the presence of microRNA23a there was an increase in NFκB-mediated transcription (Figure 3F). NFκB shows enhanced activity in inflammatory bowel disease leading to increased inflammatory cytokine release $^{35,67}$. We therefore investigated the effect of microRNA23a overexpression on proinflammatory cytokine release in an ex vivo model of human colon. We utilised an explant biopsy culture model $^{68}$, transfected with pre-miR-23a-3p RNA precursor mimic to overexpress microRNA23a, or pre-MiR negative control. LCM with RT-qPCR of mature microRNA23a confirmed transfection of biopsy epithelium (Supplementary Figure 7). Overexpression of microRNA23a resulted in significant release of TNFα at 24hours into explant culture medium (Figure 5A). Median baseline TNFα concentration from explants transfected with empty vector was 14.37pg/ml, with 42.26pg/ml ($p=0.0391$) in microRNA23a overexpressing explants. A trend towards enhanced IL-1β, IL6 and IL8 release was not statistically significant (Figure 5 B-D). However, the marked increase in TNFα release suggested a significant proinflammatory effect of microRNA23a in human colonic explant tissue. Increased detection levels of TNF mRNA, in LCM isolated epithelia from active Crohn’s disease patient biopsies, correlated positively with increased microRNA23a expression in these samples ($r=0.8025$ $p=0.0165$) (Figure 6) supporting this interpretation.
Discussion

In this study, we have demonstrated for the first time that microRNA23a is overexpressed specifically within the colonic epithelium in Crohn’s disease compared to healthy controls. Importantly, we have shown that increased microRNA23a was present in both active and inactive disease epithelium, suggesting it is an intrinsic feature of pathogenesis, predisposing the mucosa to increased sensitivity to effects of proinflammatory mediators, including TNFα, by reducing the negative feedback on inflammatory effects of NFκB. Differential expression of microRNA has previously been reported in several Crohn’s disease cohorts. Interestingly, increased microRNA23a levels were reported in the serum of Crohn’s disease patients. However, mucosal expression profiles remain poorly characterised. Previous mucosal studies have utilised whole biopsies. Due to the heterogeneous cellular nature of these samples, it is difficult to define the cell populations responsible for the observed microRNA changes. Our innovative approach, using laser capture dissection to isolate epithelial cells from colonic biopsies, has allowed us to define microRNA23a expression within a defined colonic cell population and importantly to investigate a functional consequence of this change in epithelial cells.

Interestingly, TNFAIP3 was defined here as a putative target, due to a microRNA23a seed binding sequence in the 3’ untranslated mRNA. Importantly TNFAIP3 is a negative regulator of the TNFα/NFκB pathway that was identified in a susceptibility locus for Crohn’s with reduced TNFAIP3 mRNA expression in Crohn’s disease mucosa associated with a more severe disease phenotype. Our study showed that overexpression of microRNA23a by transfection, in reporter assays with the 3’ UTR of TNFAIP3, caused reduced luciferase activity via the intact microRNA23a binding site. MicroRNA23a also reduced TNFAIP3 protein on blots of colonic epithelial cells, although we cannot exclude alternative target mRNA effects. Increased microRNA23a in serial sections of Crohn’s epithelium correlated with reduced staining for TNFAIP3/A20 protein. Assays of mRNA for TNFAIP3/A20 were not significantly different between healthy and disease donor biopsies. Therefore taken together these data suggest that microRNA23a overexpression effects on translation are an important factor in the repression of TNFAIP3/A20 protein in Crohn’s colonic epithelia. Effects of microRNA on translation has previously been reported.
Our work has identified dysregulation of TNFAIP3, specifically within the epithelium in Crohn’s disease, as a contributing factor for increased epithelial permeability. Modulation of TNFα responses is critical for epithelial barrier stability. In this study, increased expression of microRNA23a per se, did not affect ionic or macromolecular permeability. However, dextran permeability was sensitive to low doses of TNFα in the presence of increased microRNA23a where TNFAIP3 protein expression was reduced. TNFAIP3 was reported to stabilise the epithelial barrier by altering ubiquitination of occludin, preventing its internalisation from the junction. In support of this, we have shown that increased microRNA23a, targeting TNFAIP3 mRNA 3’UTR, caused an increase in epithelial macromolecular flux in the presence of TNFα, a pathway which is mechanistically influenced by occludin.

TNFα induces transcription of NFκB, which is elevated in the mucosa of IBD patients. Our data suggests that one effect of microRNA23a is to increase NFκB activation in response to TNFα in cell culture. Enhanced NFκB transcription was previously reported to cause inflammatory cytokine release and disruption to the epithelial barrier directly via the loss of occludin. TNFAIP/A20 represses NFκB activation. Our data in an ex vivo colonic model demonstrate a functional consequence of microRNA23a overexpression. The gut mucosal explants released baseline levels of TNFα. We cannot exclude that only explant epithelial cells were transfected with microRNA23a, however, the functional consequence was release of increased amounts of TNFα, indicative of a proinflammatory phenotype and increased NFκB transcription, consistent with effects seen in IBD.

Antagonism of TNF is the cornerstone of current IBD treatment and has been shown to improve epithelial barrier permeability. Recent work has focussed on understanding the mechanisms underpinning the efficacy of anti-TNF antibodies in the restitution of the barrier. It has been shown that anti-TNFα antibodies prevent internalisation of occludin from epithelial tight junctions, suggesting that they prevent structural impairment of the barrier. In patients where NFκB is endogenously activated via additional mechanisms to TNFα stimulation, including regulation by microRNA, it would be anticipated that increased TNFAIP3 and TNFα synthesis would occur. Antibody neutralisation of TNFα would allow the TNFAIP3 protein to play a significant role in stabilisation of tight junctions and augmentation of its own synthesis in response to glucocorticoids.
Therefore, the observation from our work that increased microRNA23a may inhibit expression of TNFAIP3/A20 is relevant to enhancing disease. In the cytokine milieu associated with active inflammation, the increase in microRNA23a could significantly impair epithelial cells to mitigate the effect of TNFα negatively by TNFAIP3 protein. The effect of glucocorticoids, on expression of TNFAIP3/A20 to mitigate inflammation, would also be reduced leading to increased NFκB activation and further increasing cytokine release. Thus, treatments that target microRNA23a may restore the negative feedback provided by TNFAIP3/A20 and help to enable longer lasting remission within the mucosa after anti-TNFα therapies are clinically discontinued.

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. A further study of a larger active and inactive Crohn’s patient cohort would be of value, as we observed donor variation in the level of microRNA23a relative to the immunohistochemistry histology score. In the present study, barrier dysfunction only occurred in the presence of TNFα. This suggests that in the absence of an inflammatory stimulus, epithelial integrity is maintained despite the reduction of TNFAIP3. However, this is fragile and when TNFα is encountered, the epithelium maybe less able to adequately regulate its response, leading to barrier disruption, increased permeability and inflammation with enhanced TNFα expression. This is supported by 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.

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 within an epithelium that is sensitised to the effects of TNF because of aberrant microRNA23a expression.

This study’s principal strength has been the characterisation of a dysregulated TNF regulatory pathway in a defined colonic epithelial cell population. The use of laser capture microscopy to isolate defined cell populations from colonic biopsies has been poorly utilised in IBD research. We have shown that this approach is technically feasible and can enable functionally relevant microRNA protein studies. Current treatments for Crohn’s disease antagonise the action of TNFα. Within the epithelium, anti-TNF antibodies exert their effect by stabilising occludin in tight junctions and...
supporting the gut barrier, with similarity to the function of TNFAIP3 suggesting that augmentation of epithelial TNFAI3 may be beneficial. We have identified microRNA23a as a potential therapeutic target to restore epithelial TNFAIP3 levels. This could augment and improve the efficacy of our current treatments ultimately leading to improved clinical outcomes, greater rates of mucosal healing and lasting remission.

Strategies for the development of anti-microRNA therapy is an evolving field. The approach has entered the clinical area with a successful phase 2 study of MiraVirsen, a chemically-modified anti-microRNA against hepatitis C in human subjects. Targeting of epithelia in inflammatory bowel disease requires delivery of high affinity anti-microRNA oligonucleotides with nuclease resistance and efficient cellular uptake, without immune activation. Administration of miRNA antagonists in several murine colitis models, including via intracolonic injection, reduced colitis severity scores suggesting this could be a viable therapeutic option. Chemical modifications of anti-miR oligonucleotides and improved delivery mechanisms are in development, including the use of nanoparticle-miR oligonucleotide complexes and engineered bacteria which may offer promise in IBD. Taken together these results have identified a novel epithelial-specific mechanism contributing to the disruption of the epithelial barrier and impairment of mucosal healing in Crohn's disease. Further research is required to translate microRNA therapeutics to the field of IBD.

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Author Contributions

JC, RF and FC designed the study. RF and FC recruited patients. RF undertook most experimental work, with GD and RMN doing cloning work, with TSE giving advice on microRNA assays and transfection experiments. JC and RF analysed the data and wrote the paper with FC, with important contributions from all authors in completing and reviewing the manuscript.

The manuscript, including related data, figures and tables has not been previously published and that the manuscript is not under consideration elsewhere.
Conflict of interest - There is no conflict for Richard Felwick, Geraint Dingley, Tilman Sanchez-Elsner, Rocio Martinez-Nunez, Fraser Cummings or Jane Collins of any financial interests or connections, direct or indirect, or other situations that might raise the question of bias in the work reported or the conclusions, implications, or opinions stated. This includes pertinent commercial or other sources of funding for the individual author(s) or for the associated department(s) or organization(s), personal relationships, or direct academic competition.

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References:


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Figure 1: MicroRNA23a expression in Crohn’s Disease epithelium and correlation with stool frequency. MicroRNA23a expression in active and inactive Crohn’s disease relative to healthy controls with median and interquartile range (A), *$P < .05$. Correlation between stool frequency and microRNA23a in Crohn’s disease for all donors $R=0.347$ $P=.05$ (B) and correlation for active disease only $R=0.5269$ $P=.05$ (C), both assessed with Spearman rank coefficient. Each point represents an individual value, healthy (circles), active Crohn’s (squares), inactive disease (triangles).
Figure 2: MicroRNA23a represses luciferase via TNFAIP3 3’UTR. Bioinformatic data (MicroRNA.org) showing predicted microRNA23a binding site in 3’UTR TNFAIP3 (A). Sequence of 3’UTR of TNFAIP3 with microRNA23a binding site underlined above, with mutated microRNA23a binding site in bold underlined below (B). Relative Renilla luciferase activity (normalised to CMV Firefly luciferase) showing a reduction in Hela cells when microRNA23a is transfected with the 3’UTR of TNFAIP3, which recovers when the 3’UTR is mutated (C) *P<0.05. Quantification of western blots (D) showing reduction in TNFAIP3 protein in T84 cells transfected with pre-miR-23a to overexpress microRNA23a (miRNA23a) or pre-miR negative control (Negative Control) relative to beta actin staining (n=3) *P<0.05. Representative blot (E) of T84 cells transfected with anti-miR-23a (anti23a) compared to control anti-miR inhibitor (Negative Control) showing TNFAIP3 protein levels relative to beta actin P=0.29.
Figure 3: Immunohistochemical staining of TNFAIP3 (brown) in colonic biopsies from healthy (A) active Crohn’s disease (B,C) and inactive Crohn’s (D). Strong apical staining is seen in healthy tissue (arrows in A). Apical staining is present but reduced in intensity in both active and inactive disease (arrows B-D) with discreet areas of staining loss (arrow heads). Immunohistochemistry quantification (E), each value is one donor with data shown as median and interquartile range.
**P<0.01, ***P<0.001. Effect of microRNA23a on NFκB reporter activity in Hela cells (F). Cells were transfected with NFκB luciferase reporter with (nfkb) or without (-) NFκB response elements, in the presence of pcDNA3.1-23a (23a) or empty vector (pcDNA3.1) with or without TNFα at 1ng/ml (tnf).

Data presented from n=5 duplicates, **P<0.01.

**Figure 4**

**Figure 4**: Effect of microRNA23a transfection on ionic permeability (TER) of T84 cells transfected with pre-miR-23a (23a) or pre-miR negative control (NC) plus or minus addition of TNFα at 1ng/ml (23a TNF) and (NC TNF) on day 5, showing no significant differences (A). FITC dextran permeability on day 5 in same cultures, showing effect of microRNA23a (23a) or pcDNA3.1 empty (NC), minus or plus TNFα (TNF) (B). All data show as median with interquartile range from n=3 duplicates, ***P<0.001 ****P<0.0001.
Figure 5: Cytokine assays of supernatants from 24 hour explant cultures of healthy human colonic biopsies transfected with pre-miR-23a to overexpress microRNA23a (microRNA23a) or nonspecific pre-miR negative control vector (Control). Data shown as median with interquartile range (n=8). (A) TNFα *P<0.05. (B) IL-1β P<1.0. (C) 24 hour supernatant IL-6 concentration P<1.0. (D) IL-8 P<1.0.
Figure 6: MicroRNA23a expression and corresponding TNFα mRNA expression in active Crohn’s disease LCM isolated epithelium from individual donors. Correlation strength was tested with Pearson correlation coefficient $r=0.8025$ *$P<.05$