

ORIGINAL ARTICLE

The guanylate cyclase-C signaling pathway is down-regulated in inflammatory bowel disease

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Abstract

Objective. Activation of membrane receptor guanylate cyclase-C (GC-C) is implicated in gastrointestinal fluid and electrolyte balance, preservation of intestinal barrier integrity, anti-trophic effects and inhibition of pain sensation. To evaluate GC-C signaling, we examined the regulation of GC-C (*GUCY2C/Gucy2c*) and its endogenous ligands guanylin (GN/*GUCA2A/Guca2a*) and uroguanylin (UGN/*GUCA2B/Guca2b*) in colonic Crohn's disease (CD), ulcerative colitis (UC) and in rats with 2,4,6-Trinitrobenzene sulphonic acid (TNBS) colitis. Correlation analyses between expression of *GUCA2A* and *GUCY2C* and expression of inflammatory cytokines (*IL1A*, *IL1B*, *TNFA* and *IFNG*) were conducted. Additionally, expression of transcription factors for *GUCA2A* and *GUCY2C*, and the GC-C signaling pathway, were examined. **Material and methods.** Biopsies from active UC/CD, un-inflamed UC/CD and healthy controls, and inflamed and healthy rat colon were investigated with gene expression microarray, immunohistochemistry (IHC) and *in situ* hybridization (ISH). **Results.** *GUCA2A/Guca2a*, *GUCA2B*, *GUCY2C/Gucy2c*, transcription factors, as well as several cyclic guanosine-3',5'-monophosphate downstream mediators were all significantly down-regulated in both inflamed colonic inflammatory bowel disease (IBD) mucosa and TNBS colitis. Expression of *GUCA2A* and *GUCY2C* negatively correlated to expression of inflammatory cytokines. IHC and ISH confirmed microarray results for *GUCA2A/Guca2a* and *GUCY2C/Gucy2c* in inflamed samples. We identified a highly significant positive correlation between the expression of the transcription factor caudal type homeobox 2 (*CDX2*) and the expression of the downstream target gene *GUCY2C*. **Conclusions.** *GUCA2A*, *GUCA2B* and *GUCY2C* as well as several steps of the GC-C signaling pathway are down-regulated in IBD. This may have implications in IBD pathogenesis.

Key Words: cyclic GMP, guanylate cyclase-C, guanylin, inflammatory bowel disease, uroguanylin

Introduction

The two main types of idiopathic inflammatory bowel disease (IBD), ulcerative colitis (UC) and Crohn's

disease (CD), have a fluctuating disease pattern characterized by remissions and relapses. The prevailing understanding is that IBD has a multifactorial etiology where interaction between environmental and

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microbial triggers in the gut lumen and a dysfunctional intestinal barrier lead to an inappropriate immunological response in genetically susceptible individuals [1,2]. The intestinal epithelium, mucus layer and antimicrobial peptides serve as protection against the luminal environment [3]. Breaches in the intestinal barrier lead to an immunological response with activated T-cells and macrophages and secretion of pro-inflammatory cytokines including interleukin (IL) 1, IL6, IL7, IL17, TNF α and IFN γ [4]. Persistent defects in this protective barrier could sustain inflammation. A large proportion of patients with IBD experience pain, and long-lasting disease is associated with an increased risk for intestinal malignancy [1,2]. Current treatments are only symptomatic and aimed at suppressing the inflammatory response. Consequently, a continuous search for more specific etiological factors and identification of new therapeutic targets is required.

Dysfunctional guanylate cyclase-C (GC-C/*GUCY2C*) signaling has been implicated in diarrhea, constipation, abdominal pain, dysfunctional epithelial barrier function, and polyp and tumor growth [5]. Endogenous ligands for the membrane receptor GC-C are the peptides guanylin (GN/*GUCA2A*) and uroguanylin (UGN/*GUCA2B*). GN and UGN are expressed in several tissues, with high expression in the gastrointestinal (GI) epithelium which is the main source for both GN and UGN [6]. GN and UGN have a similar peptide structure to bacterial derived heat-stable enterotoxin (STa). GC-C signaling is mediated through increased cellular cyclic guanosine-3',5'-monophosphate (cGMP). Downstream mediators of cGMP include intracellular phosphodiesterases (PDE), protein kinases (PK) and membrane-bound proteins and ion channels [7]. In the GI-tract, cGMP activation leads to secretion of chloride and bicarbonate through activation of the cystic fibrosis transmembrane conductance regulator (CFTR). Efflux of cGMP predominantly occurs through basolateral transporters in intestinal cells. Transporters for nucleotide analogues (cGMP) are the multidrug resistance proteins (MRP) 4 and 5 [8], which are expressed throughout the GI-tract [9]. MRP4 is localized predominantly in the apical cellular membrane, whereas MRP5 is localized basolaterally [8,10].

GN and UGN seem to be inversely distributed in the GI tract where UGN is abundant in the upper part with decreasing amounts towards the rectum and vice versa for GN [5]. Several potential transcription factor binding sites have been identified within the *GUCA2A* (GN), *GUCA2B* (UGN) and *GUCY2C* (GC-C) genes. *GUCY2C* transcription is e.g., shown to be regulated by hepatocyte nuclear factor 4-alpha (HNF-4 α /*HNF4A/Hnf4a*) [11] and caudal type homeobox 2 (CDX2/*CDX2*) [12], the latter regulates

intestine-specific *GUCY2C* expression. *GUCA2A* transcription is regulated by hepatocyte nuclear factor 1-alpha (HNF-1 α /*HNF1A/Hnf1a*) [13].

The possible coupling of aberrant GC-C signaling with dysfunctional epithelial barrier function is intriguing in the pathogenesis of IBD. Treatment of a dysfunctional intestinal barrier is an emerging topic and the use of GC-C agonists in UC has been suggested [7]. In a study from 1995, circulating GN was not altered in IBD compared to healthy controls [14], while gene expression data for *GUCA2A*, *GUCA2B* and *GUCY2C* in IBD have only been infrequently investigated. Down-regulation of *GUCA2A* and *GUCA2B* in UC has been suggested to have implications for dysfunctional epithelial electrolyte regulation [15].

We hypothesized that GC-C signaling is dysregulated in IBD. We therefore examined the gene expression of *GUCA2A/Guca2a* (GN), *GUCA2B/Guca2b* (UGN) and *GUCY2C/Gucy2c* (GC-C), proposed transcription factors for GN and GC-C encoding genes, as well as the expression of downstream mediators of cGMP, and cGMP transporters *MRP4/Mrp4* and *MRP5/Mrp5* in IBD and in chemically induced colitis (rats) with 2,4,6-Trinitrobenzene sulphonic acid (TNBS).

Material and methods

Human IBD samples

The human samples were collected from an IBD cohort at Trondheim University Hospital, Trondheim, Norway, approved by the Regional Medical Research Ethics Committee (approval no 5.2007.910) and registered in the Clinical Trials Protocol Registration System (identifier NCT00516776). Written informed consent was obtained from all patients and control individuals.

The previously described [16] sample population consists of 37 active UC, 7 active CD, 44 un-inflamed UC (UC-U), 19 un-inflamed CD (CD-U) and 20 healthy controls (denoted Healthy in tables and figures). Participant data are shown in Table I. Of 44 patients with active inflammation, 15 (34.1%) were not on IBD medication. All participants underwent colonoscopy, and biopsies were collected from endoscopically assessed, maximally inflamed colonic mucosa. Samples from healthy controls and un-inflamed mucosa in IBD patients (when applicable) were taken from the hepatic flexure. Inflamed UC samples were mostly collected from the left colon (descending, sigmoid colon and rectum), as 28 of 37 patients had predominantly left-sided colitis. Un-inflamed IBD (IBD-U) samples were defined as no endoscopic or histological signs of inflammation.

Table I. Number and characteristics of the subjects in the IBD study [16].

	H ^a	UC ^b	CD ^c	UC-U ^d	CD-U ^c
Subjects	20	37	7	44	19
Age, median years (range)	45 (19–71)	38 (19–72)	31 (20–41)	45 (21–71)	39 (20–61)
Female/Male	9/11	22/15	2/5	24/20	6/13
Duration of disease, median years (range)	NA ^f	9 (0–40)	7 (1–12)	13 (0–40)	6 (0–28)
5-ASA ^g /sulphasalazine (%)	0	23 (62)	2 (29)	27 (61)	6 (32)
Systemic corticosteroids (%)	0	9 (26)	2 (29)	4 (9)	8 (42)

^aHealthy, ^bUlcerative colitis, ^cCrohn's disease, ^dUn-inflamed UC, ^eUn-inflamed CD, ^fNot applicable, ^g5-aminosalicylic acid.

Healthy controls were defined from clinical and endoscopic assessment and had no sign of GI disease. Four samples from each area were collected. Three biopsies were snap-frozen in liquid nitrogen, before processing. One biopsy was formalin-fixed and thereafter embedded in paraffin. Hematoxylin-eosin stained samples were evaluated by an experienced pathologist and samples with divergent endoscopic and histological assessment were removed.

Rat TNBS colitis samples

Data were generated from a genome-wide gene expression study comparing TNBS colitis in rats with IBD [17]. Five female Sprague Dawley rats weighing 200–250 g were used (Taconic Farms, Inc., Hallingore, Denmark). The animal studies were approved by the National Animal Research Authority. The general care and use of the animals were in accordance with the European Convention for the protection of Vertebrate Animals used for Experimental and other Scientific purposes.

We followed colitis in individual rats sequentially with colonoscopy and endoscopic biopsies [17]. Briefly, colitis was induced by a rectal enema with TNBS dissolved in 50% ethanol to a concentration of 30 mg/ml in a total volume of 0.6 ml. Colonoscopy was performed and five endoscopic biopsies were collected for both histopathological and gene expression analyses before and at Days 3, 7 and 12 after colitis induction. Colitis was assessed using a modified Murine Endoscopic Index of Colitis severity (MEICS, 0–12).

Genome-wide gene expression analysis

RNA extraction, processing, microarrays from human and rat endoscopic biopsies and corresponding statistical analyses were done as previously described [17,18]; the specific methodology is also available in Supplementary Methods. The data are accessible at ArrayExpress, E-MTAB-184 and ArrayExpress, E-MTAB-1263, respectively.

In the present study, we specifically examined gene expression profiles of *GUCA2A/Guca2a* (GN), *GUCA2B/Guca2b* (UGN) and *GUCY2C/Gucy2c* (GC-C), transcription factors *HNF4A/Hnf4a*, *HNF1A/Hnf1a* and *CDX2/Cdx2*, as well as downstream mediator proteins of cGMP (*PRKG2/Prkg2* [PKGII]; *CNGA1/Cnga1* and *CNGA2/Cnga2* [CNGA]; *PDE3A/Pde3a* and *PDE3B/Pde3b* (PDE3), *PDE5/Pde5*, *CFTR/Cftr*, *SLC9A3/Slc9a3* [NHE3]) and cGMP transporters *MRP4/Mrp4* and *MRP5/Mrp5*. To determine whether there was a correlation between degree of inflammation and expression of *GUCA2A* and *GUCY2C*, we did regression and correlation analyses for the expression of these genes and the expression of inflammatory cytokines *IL1A* (IL1 α), *IL1B* (IL1 β), *TNFA* (TNF α) and *IFNG* (IFN γ). Additionally, we investigated whether there was any differential gene expression of *GUCA2A*, *GUCA2B* and *GUCY2C* in inflamed tissue sampled from the left (descending, sigmoid and rectum) and right (cecum, ascending and transverse) colon in UC and CD (analyzed together).

Immunohistochemistry and in situ hybridization

We used anti-GN (HPA018215, Atlas Antibodies AB, Stockholm, Sweden; dilution 1:100) and anti-GC-C (HPA037655, Atlas Antibodies AB; dilution 1:50) to perform IHC on human endoscopic biopsies from healthy and inflamed colon. A previously described [19] immunoreactivity score (IRS) (0–12) was calculated from the product of the percentage of positive epithelial cells (4: >80%, 3: 51–80%, 2: 10–50%, 1: <10%) and the intensity of the staining (0: non, 1: weak, 2: moderate and 3: strong). IHC against UGN could not be done due to a lack of appropriate antibodies.

In situ hybridization (ISH) was performed using the RNAscope[®] 2.0 assay kit (Advanced Cell Diagnostics, Inc., Hayward, CA) with probes for *GUCA2A/Guca2a*, *GUCA2B/Guca2b* and *GUCY2C/Gucy2c*.

A more detailed description is provided in Supplementary Methods.

Statistical analyses

Gene expression analyses were performed as described in Supplementary Methods. Statistical analyses were performed in Prism[®] Version 6.03 (GraphPad Software, Inc., La Jolla, CA). Statistics for IRS were performed with the nonparametric Kruskal–Wallis test and Dunn’s multiple comparisons test with adjusted *p*-Values. Kruskal–Wallis test and Dunn’s multiple comparisons test were also used for analyses of differential expression of *GUCA2A*, *GUCA2B* and *GUCY2C* between healthy controls, IBD-U, IBD Left colon, and IBD Right colon, as not all data sets of individually expressed genes were normally distributed according to D’Agostino–Pearson omnibus normality test. Correlation and regression analyses of gene expression (*IL1A* and *GUCA2A/GUCY2C*, *IL1B* and *GUCA2A/GUCY2C*, *TNFA* and *GUCA2A/GUCY2C*, *IFNG* and *GUCA2A/GUCY2C*, *HNF1A* and *GUCA2A*, *CDX2* and *GUCY2C*, and *HNF4A* and *GUCY2C*) were done with computation of Pearson correlation coefficient, *r*. *p*-Values of <0.05 were considered significant.

Results

Genes involved in GC-C signaling are down-regulated in IBD and TNBS colitis

Genome-wide gene expression analysis of human IBD samples revealed that mRNAs of the ligands *GUCA2A*, *GUCA2B* and the receptor *GUCY2C* were all significantly down-regulated in IBD with active inflammation compared to healthy controls. Fold change (FC) for *GUCA2A* was -4.91 and -2.86 , for *GUCA2B* -6.06 and -4.64 and for *GUCY2C* -1.61 and -1.36 , in UC and CD, respectively (Table II).

Animal studies have suggested that *Guca2a* expression is decreasing in caudal direction within the colon [20]. To our knowledge, studies regarding potential differential expression of *GUCA2A* between different colonic segments in humans are lacking. We therefore examined whether there was differential gene expression of *GUCA2A*, *GUCA2B* and *GUCY2C* in inflamed mucosa from the left (descending, sigmoid and rectum) compared to right (cecum, ascending and transverse) colon in both UC and CD patients (analyzed together), and compared to IBD-U and healthy controls. Analysis of individual log₂ intensity values showed that gene expression profiles for *GUCA2A*, *GUCA2B* and *GUCY2C* were similar in biopsies sampled from IBD mucosa in predominantly right- and left-sided colitis (Table II and Figure 1A–C).

Expression of inflammatory cytokines (*IL1A*, *IL1B*, *TNFA* and *IFNG*) was significantly increased in IBD compared to healthy controls: *IL1A* with FC 2.74 and 2.01, *IL1B* with FC 8.38 and 5.19, *TNFA* with FC 1.65 and 1.41 and *IFNG* with FC 2.13 and 1.70, in UC and CD, respectively (S1). Individual log₂ expression values of *GUCA2A* and *GUCY2C* negatively correlated with log₂ expression of all inflammatory cytokines, however, not significantly for *IL1A* and *GUCY2C*, in IBD (Figure 1D, E and S2A–F, open circles).

The degree of inflammation in the rats with TNBS colitis was moderate according to the MEICS score. Average MEICS score ($0-12 \pm$ SD) peaked at day 3 (5.6 ± 0.9) and day 7 (5.0 ± 1.2). *Guca2a* and *Gucy2c* were down-regulated in TNBS colitis with FC at Days 3, 7 and 12 of -3.47 , -2.40 and -1.79 for *Guca2a* and -2.88 , -2.36 and -2.02 for *Gucy2c*. *Guca2b*, was dissimilarly regulated in TNBS colitis compared to IBD, and was significantly up-regulated at Days 7 and 12, with FC of 2.22 and 1.78 (Table II). Severity of TNBS colitis assessed endoscopically (as depicted and from MEICS score) and from microarray gene expression of *Il1a* and *Il1b*, demonstrated an inverse relationship with expression of *Guca2a* and *Gucy2c* (Figure 2A, B).

Since mRNAs for GN and GC-C were down-regulated in both IBD and TNBS colitis, gene expression of transcription factors that have been related to control of expression of GN (*HNF1A/Hnf1a*) and GC-C (*HNF4A/Hnf4a* and *CDX2/Cdx2*) encoding genes were identified [11–13]. *HNF1A/Hnf1a* was significantly down-regulated in UC (FC -1.08), CD (FC -1.10) and TNBS colitis (FC -2.24); similarly *CDX2/Cdx2* was significantly down-regulated in UC (FC -1.92), in CD (FC -1.37) and in TNBS colitis (FC -1.91). *HNF4A* was significantly down-regulated in UC (FC -1.24) and CD (FC -1.23), while *Hnf4a* was significantly up-regulated (FC 1.23) in TNBS colitis (Table III). We further examined whether there were significant correlations between the expression levels of these transcription factors and possible target genes in IBD. Although *HNF4A* and *HNF1A* were significantly down-regulated in IBD (CD and UC) compared to healthy controls, we found no correlation neither between expression of *HNF4A* and *GUCY2C* nor between *HNF1A* and *GUCA2A* (S2G and H). However, linear regression analysis showed that there was a significant positive linear relation ($p < 0.0001$) between *CDX2* expression and *GUCY2C* expression in IBD (Figure 1F, open circles). Such significant correlation was not observed in healthy controls (Figure 1F, filled circles), suggesting that *CDX2* is an important transcriptional regulator of intestine-specific membrane receptor GC-C in IBD.

Table II. Gene expression data for *GUCA2A/Guca2a*, *GUCA2B/Guca2b* and *GUCY2C/Gucy2c* in IBD and TNBS colitis.

FC with <i>p</i> -Values										
Human	UC vs. Healthy			CD vs. Healthy						
	FC	<i>p</i>		FC	<i>p</i>					
<i>GUCA2A</i>	-4.91	3.28×10^{-13}		-2.86	0.0037					
<i>GUCA2B</i>	-6.06	1.01×10^{-16}		-4.64	7.12×10^{-6}					
<i>GUCY2C</i>	-1.61	1.27×10^{-11}		-1.36	0.010					
TNBS colitis	Day 3		Day 7		Day 12					
	FC	<i>P</i>	FC	<i>p</i>	FC	<i>p</i>				
<i>Guca2a</i>	-3.47	0.0003	-2.40	0.0006	-1.79	0.0009				
<i>Guca2b</i>	-1.05	0.82	2.22	0.0032	1.78	0.034				
<i>Gucy2c</i>	-2.88	0.0002	-2.36	0.0008	-2.02	0.0024				
Log2 gene expression in Healthy, un-inflamed IBD (IBD-U) and IBD Left colon and IBD Right colon										
Gene	Healthy (<i>n</i> = 20)	IBD-U (<i>n</i> = 61 ^a)		IBD Left colon (<i>n</i> = 33)		IBD Right colon (<i>n</i> = 11)		<i>p</i> ^{b, f}		
	Log2 expression (SD)	Log2 expression (SD)	<i>p</i> ^{b, c}	Log2 expression (SD)	FC vs. Healthy	<i>p</i> ^{b, d}	Log2 expression (SD)		FC vs. Healthy	<i>p</i> ^{b, e}
<i>GUCA2A</i>	13.42 (0.21)	13.32 (0.29)	>0.99	11.29 (1.37)	-4.08	<0.0001	11.11 (1.78)	-4.63	<0.0001	>0.99
<i>GUCA2B</i>	10.85 (0.33)	10.90 (0.56)	>0.99	8.30 (1.21)	-6.06	<0.0001	8.30 (1.44)	-6.06	0.0003	>0.99
<i>GUCY2C</i>	9.92 (0.20)	9.92 (0.25)	>0.99	9.26 (0.42)	-1.59	<0.0001	9.34 (0.38)	-1.51	0.0019	>0.99

Abbreviations: CD = Crohn’s disease; FC = Fold change; IBD = Inflammatory bowel disease; TNBS = 2,4,6-Trinitrobenzene sulphonic acid; UC = Ulcerative colitis.

^aDenotes the number of samples from both IBD patients in remission without inflammatory changes, as well as samples from endoscopically and histologically un-inflamed hepatic flexure of IBD patients with inflammatory changes elsewhere in the colon.

^bData analyzed with the Kruskal–Wallis test and Dunn’s multiple comparisons test.

^cComparison between *Healthy* and *IBD-U*.

^dComparison between *IBD Left colon* and *Healthy*.

^eComparison between *IBD Right colon* and *Healthy*.

^fComparison between *IBD Left colon* and *IBD Right colon*.

Since GC-C signaling is mediated through cGMP [6], gene expression of cGMP downstream mediators (*PRKG2/Prkg2* [PKGII]; *CNGA1/Cnga1* and *CNGA2/Cnga2* [CNGA]; *PDE3A/Pde3a* and *PDE3B/Pde3b* (PDE3), *PDE5/Pde5*, *CFTR/Cftr*, *SLC9A3/Slc9a3* [NHE3]) were also examined and all human genes, except *CNGA2* and *PDE3B*, were found significantly down-regulated (Table III).

Expression of cGMP transporter *MRP4* was not significantly changed, while *MRP5* was significantly down-regulated in CD (FC -1.25) and down-regulated in UC (FC -1.23), but not significantly. From the TNBS colitis study, we have no data for *Mrp4* and *Mrp5* was not significantly changed (Table III).

Overall, gene expression of *GUCA2A/Guca2a* (GN), *GUCA2B/Guca2b* (UGN), *GUCY2C/Gucy2c* (GC-C), relevant transcription factors as well as downstream targets for GC-C signaling were mostly down-regulated in IBD and TNBS colitis. Expression of *GUCA2A/Guca2a* and *GUCY2C/Gucy2c* in both IBD and TNBS colitis seems to be inversely related to degree of colitis (MEICS score and/or inflammatory

cytokines). Expressions of *CDX2* and *GUCY2C* are highly correlated in IBD.

Protein (IHC) and mRNA (ISH) in mucosal biopsies support gene expression data

We further used IHC and ISH to investigate localization and expression levels of relevant peptides/proteins and mRNAs in colonic biopsies. IHC demonstrated a markedly less positivity for GN and GC-C in inflamed mucosa compared to healthy controls. A non-significant reduction in IRS compared to control was also observed in biopsies collected from un-inflamed parts of the colon of IBD patients (Figure 3A, B).

GN and GC-C are expressed in the epithelial cells and it is possible that mucosal denudation could have reduced immunoreactivity. However, biopsies were not collected from ulcerated areas and histopathological examination demonstrated that the epithelium was intact with goblet cells and enterocytes. A detailed description of IRS scores, sample location and patient characteristics is included in S3.

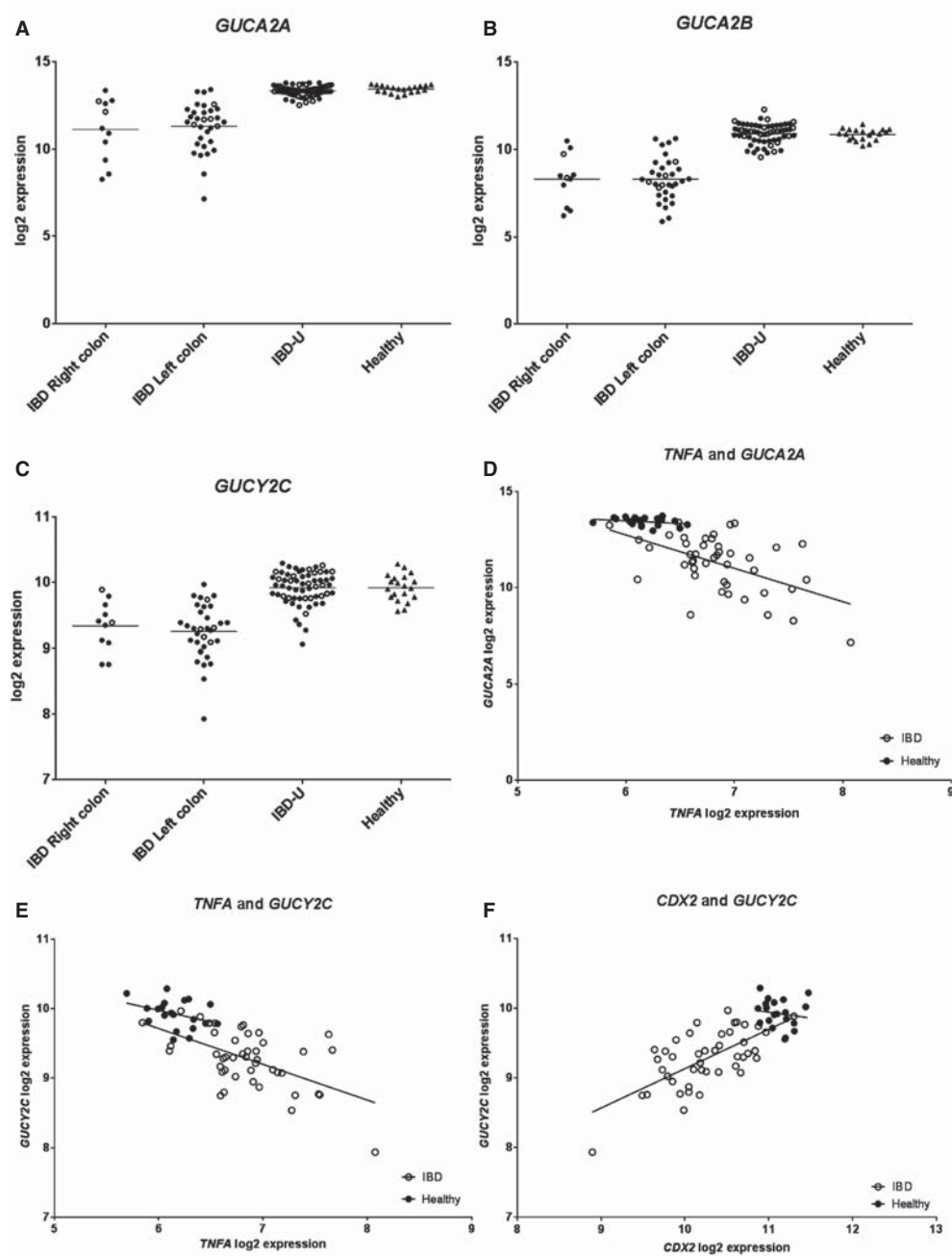


Figure 1. Individual log₂ expression for (A) *GUCA2A*, (B) *GUCA2B* and (C) *GUCY2C* for IBD Right colon, IBD Left colon, IBD-U and Healthy. Log₂ expressions are significantly different for all genes between both *IBD Right colon* and *Healthy* and *IBD Left colon* and *Healthy*. There are no significant differences in log₂ expressions neither between *IBD-U* and *Healthy* nor between *IBD Right colon* and *IBD Left colon*. Filled circles represent UC (*n* = 37) or UC-U (*n* = 42) samples and open circles represent CD (*n* = 7) or CD-U (*n* = 19) samples in A-C. (D) and (E) show individual log₂ expression values for *TNFA* and *GUCA2A* and *TNFA* and *GUCY2C*, respectively. In IBD (both UC and CD) there is negative correlation between both *TNFA* and *GUCA2A* ($r^2 = 0.27$, $p = 0.0003$) and *TNFA* and *GUCY2C* ($r^2 = 0.31$, $p < 0.0001$). (F) In IBD samples (both UC and CD) log₂ expression of *CDX2* positively correlates with log₂ expression of *GUCY2C* ($r^2 = 0.43$, $p < 0.0001$). Figures in D-F are shown with regression lines; open circles represent individual log₂ expression in IBD (both UC and CD) samples, while filled circles represent individual log₂ expression in Healthy.

GUCA2A/Guca2a, *GUCA2B/Guca2b* and *GUCY2C/Gucy2c* were all expressed in the colonic epithelium of healthy human and rat samples assessed by ISH. *GUCA2A/Guca2a* and *GUCY2C/Gucy2c* expression

was localized to both goblet and columnar cells in healthy colonic mucosa. *GUCA2B*, however, was mainly expressed in solitary epithelial cells of the human colon. No expression of *Guca2b* was detectable in rat

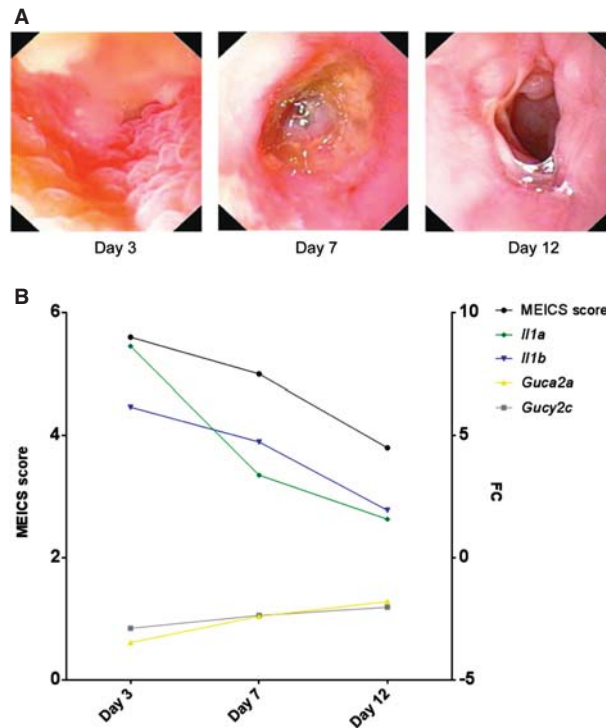


Figure 2. (A) Representative endoscopic pictures from one rat at Days 3, 7 and 12 after induction of colitis with TNBS. (B) Average MEICS score is portrayed with corresponding fold change (FC) of *Guca2a*, *Gucy2c*, *Il1a* and *Il1b* relative to baseline (two days before colitis induction). FC of *Guca2a* and *Gucy2c* is inversely related to both MEICS score and FC of *Il1a* and *Il1b*.

Table III. Gene expression FC and *p*-Values for transcription factors, cGMP mediators and transporters in IBD and TNBS colitis.

Gene/protein	UC vs. Healthy		CD vs. Healthy		TNBS colitis vs. Healthy rat colon	
	FC	<i>p</i>	FC	<i>p</i>	FC	<i>p</i>
Transcription factors						
<i>HNF4A/Hnf4a</i>	-1.24	1.37×10^{-7}	-1.23	0.003	1.23	0.05
<i>CDX2/Cdx2</i>	-1.92	4.55×10^{-17}	-1.37	0.01	-1.91	0.004
<i>HNF1A/Hnf1a</i>	-1.08	0.04	-1.10	0.004	-2.24	0
cGMP downstream mediators						
<i>PRKG2/Prkg2</i>	-2.14	1.2×10^{-15}	-2.19	2.7×10^{-7}	-30.87	0.001
<i>CNGA1/Cnga1</i>	-1.93	2.5×10^{-8}	-1.80	0.004	No data	No data
<i>CNGA2/Cnga2</i>	1.004	0.92	1.04	0.50	No data	No data
<i>PDE3A/Pde3a</i>	-1.17	0.001	-1.13	0.21	-1.08	0.21
<i>PDE3B/Pde3b</i>	-1.05	0.30	1.07	0.56	-1.08	0.39
<i>PDE5/Pde5</i>	-1.34	0.007	-1.15	0.59	1.33	0.05
<i>CFTR/Cftr</i>	-1.84	2.2×10^{-10}	-1.59	0.004	-2.64	0.0002
<i>SLC9A3/Slc9a3</i>	-1.21	2.6×10^{-5}	-1.17	0.06	-4.59	0.002
cGMP transporters						
<i>MRP4/Mrp4</i>	1.06	0.94	1.02	0.52	No data	No data
<i>MRP5/Mrp5</i>	-1.23	0.12	-1.25	0.007	-1.15	0.29

Abbreviations: CD = Crohn's disease; CFTR = Cystic fibrosis transmembrane conductance regulator; cGMP = Cyclic guanosine-3',5'-monophosphate; FC = Fold change; IBD = Inflammatory bowel disease; MRP = Multidrug resistance protein; PDE = Phosphodiesterase; TNBS = 2,4,6-Trinitrobenzene sulphonic acid; UC = Ulcerative colitis.

colon. In the inflamed rat colon, expression of *Guca2a* and *Gucy2c* was decreased and was only detectable in a few cells (Figure 3C).

Discussion

This descriptive gene expression study of GN, UGN and GC-C encoding genes and the GC-C signaling pathway in IBD illuminates the molecular premise for an evolving research topic in IBD. A reduced transcription of *GUCA2A* (GN) and *GUCA2B* (UGN) in IBD has previously been demonstrated [15] and is confirmed in the current study. In addition, the receptor for GN, UGN and STa, *GUCY2C* (GC-C), is down-regulated in IBD, which to our knowledge, has not previously been reported. Consequently, the combined reduction of both the agonists and their corresponding receptor should lead to a pronounced downstream reduction in colonic GC-C signaling. Semi-quantitative determination of GN and GC-C by IHC and ISH confirmed the microarray findings for *GUCA2A/Guca2a* and *GUCY2C/Gucy2c*.

GC-C signaling has received increasing interest in GI disease in general and in the study of IBD in particular. It was recently demonstrated that the GC-C agonist Linaclotide, used to treat constipation, also has an analgesic effect through increased production and release of cGMP. cGMP is transported through the basolateral cellular membrane and acts on and inhibits colonic afferent nociceptors [21]. Interestingly *MRP5*, one of the proposed transporters for cGMP localized in the basolateral membrane [10], is among the down-regulated genes in our IBD cohort. It is thus possible that this in conjunction with reduced expression of *GUCA2A*, *GUCA2B* and *GUCY2C* and diminished cGMP release to the submucosa may affect pain sensation also in IBD.

We demonstrated that *Guca2a* and *Gucy2c* were down-regulated in rats with moderate TNBS colitis. The expression profiles of *Guca2a* and *Gucy2c* were inversely related to the MEICS score and the expression of inflammatory cytokines *Il1a* and *Il1b*. Similarly, *Guca2a* down-regulation has been demonstrated in inflamed colon of *Il10*^{-/-} mice and in cell culture after TNF α stimulation [22]. The human data also showed a negative correlation between increased expression of inflammatory cytokines (*IL1A*, *IL1B*, *TNFA* and *IFNG*) and expression of both *GUCA2A* and *GUCY2C*. Thus, it seems that down-regulation of *GUCA2A/Guca2a* and *GUCY2C/Gucy2c* is a common feature during colonic mucosal inflammation. This could be a phylogenetically conserved mechanism to prevent fluid and electrolyte loss – as described in loss-of-function mutations for *GUCY2C* [23].

However, other mechanisms of diarrhea in IBD seem to override this [24]. The results are, nevertheless, in accordance with previous results showing that net absorption of chloride, sodium and fluid is decreased in IBD (without increased chloride secretion) through, for example, cytokine-mediated (TNF α , IFN γ and IL1 β) down-regulation of NHE and epithelial sodium channels [24]. Additionally, reduced GC-C signaling through down-regulation of *GUCY2C* in intestinal inflammation might also facilitate tissue regeneration and healing by reducing the anti-proliferative effect of GN and UGN [5].

The mechanism for the regulation of transcription of *GUCA2A/Guca2a*, *GUCA2B/Guca2b* and *GUCY2C/Gucy2c* probably involves the role of several transcription factors. We studied the expression of the transcription factors *CDX2*, *HNF4A* and *HNF1A*. Our gene expression data demonstrated that the expression of *CDX2/Cdx2* was significantly down-regulated in both UC, CD and TNBS colitis. *CDX2* together with *HNF4A* could explain the reduced transcription of *GUCY2C* in IBD [11,12], while down-regulation of *HNF1A* is in accordance with reduced expression of *GUCA2A* [13]. In addition to down-regulation of *GUCY2C*, we found a positive correlation between *CDX2* and *GUCY2C* expression in IBD. This corresponds with previous studies investigating *CDX2* in intestinal inflammation and as an intestine-specific transcription factor for *GUCY2C/Gucy2c* [12,25]. Both *HNF4A* and *CDX2* have been found decreased in IBD [26,27] and *Hnf4a* deficient mice are susceptible to dextran sulfate sodium (DSS) colitis [26]. Both HNF-4 α and CDX2 have been postulated to function as tumor suppressors in the colon [28,29], which in conjunction with their role as transcription factors for *GUCY2C* corresponds with the anti-proliferative effect of increased GC-C signaling. In accordance with this, an increased number of intestinal crypts and proliferation of epithelial cells as well as increased tumorigenesis and attenuated apoptosis has been observed in *Gucy2c*^{-/-} mice and cell lines [30–32], supporting the concept that reduced GC-C signaling may be favorable for growth and regeneration of a damaged intestinal epithelium. Moreover, *GUCY2C* is expressed in colonic adenoma and cancer [33]. The GC-C ligand GN, abundantly expressed in the colonic epithelium, however, is lost in colon cancer, leading to reduced GC-C stimulation and oncogenesis [34]. Due to the chronic nature of UC and CD, these mechanisms may be both a necessity for tissue regeneration but may also be a disadvantage if similar mechanisms drive dysplasia and cancer development.

Sustained loss of GC-C signaling in IBD might lead to a vicious cycle, demonstrated by *Gucy2c*^{-/-} mice that have disrupted tight junction assembly, increased

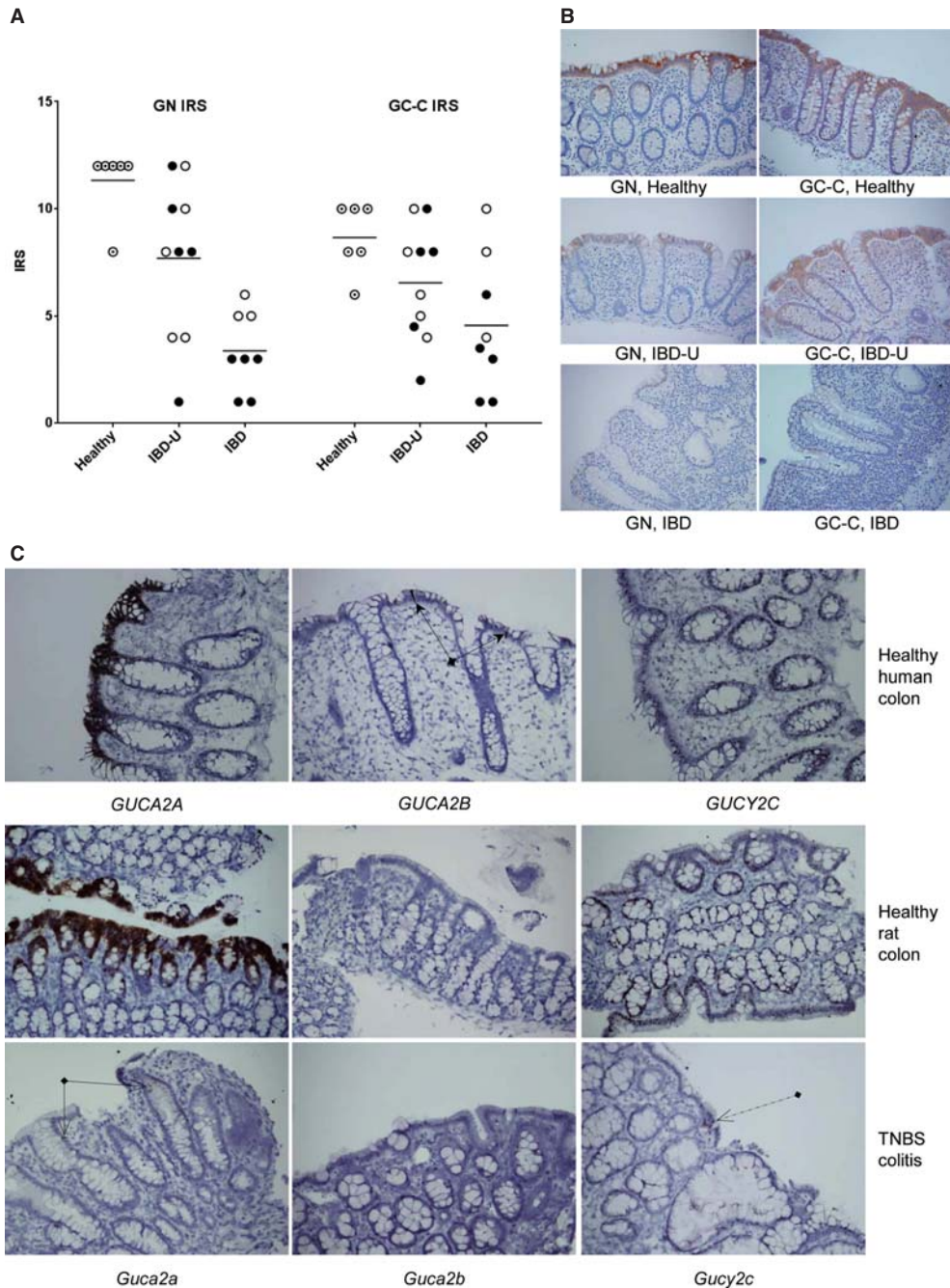


Figure 3. Detection of peptides/proteins (IHC) and mRNA (ISH). (A) Immunoreactivity score (IRS) for GN and GC-C in healthy mucosa, un-inflamed IBD patients (IBD-U) and inflamed IBD. Healthy ($n = 6$) is indicated with target circle, UC ($n = 5$) and UC-U ($n = 5$) with filled circles and CD ($n = 3$) and CD-U ($n = 5$) with open circles. Mean IRS for GN was 11.33, 7.70 and 3.38 for Healthy, IBD-U and IBD. Mean rank difference for GN was 6.50 for Healthy vs. IBD-U ($p = 0.14$) and 13.63 for Healthy vs. IBD ($p = 0.0006$). Mean IRS for GC-C was 8.67, 6.55 and 4.56 for Healthy, IBD-U and IBD. Mean rank difference for GC-C was 5.05 for Healthy vs. IBD-U ($p = 0.32$) and 9.44 for Healthy vs. IBD ($p = 0.02$). (B) Representative GN and GC-C stained tissue specimens from Healthy, IBD-U and IBD (Magnification, 200 \times). (C) Mucosal expression of *GUCA2A*/*Guca2a*, *GUCA2B*/*Guca2b* and *GUCY2C*/*Gucy2c* in endoscopic biopsies from healthy human colon and from endoscopic biopsies from healthy and inflamed rat colon after induction of TNBS colitis. There is marked expression of *GUCA2A*/*Guca2a* and quite significant expression of *GUCY2C*/*Gucy2c* in healthy colonic mucosa. After induction of TNBS colitis only a few cells are positive for *Guca2a* (arrows with open arrowheads) and *Gucy2c* (dashed arrow). The pattern of expression is similar in healthy human and rat colon. *GUCA2A*/*Guca2a* and *GUCY2C*/*Gucy2c* are expressed in both goblet and columnar cells. *Guca2b* has no noticeable expression in rat samples neither before nor after colitis induction, whereas in human colonic mucosa *GUCA2B* is strongly expressed in solitary epithelial cells (arrows with solid arrowheads) and weaker in other epithelial cells (Magnification, 200 \times).

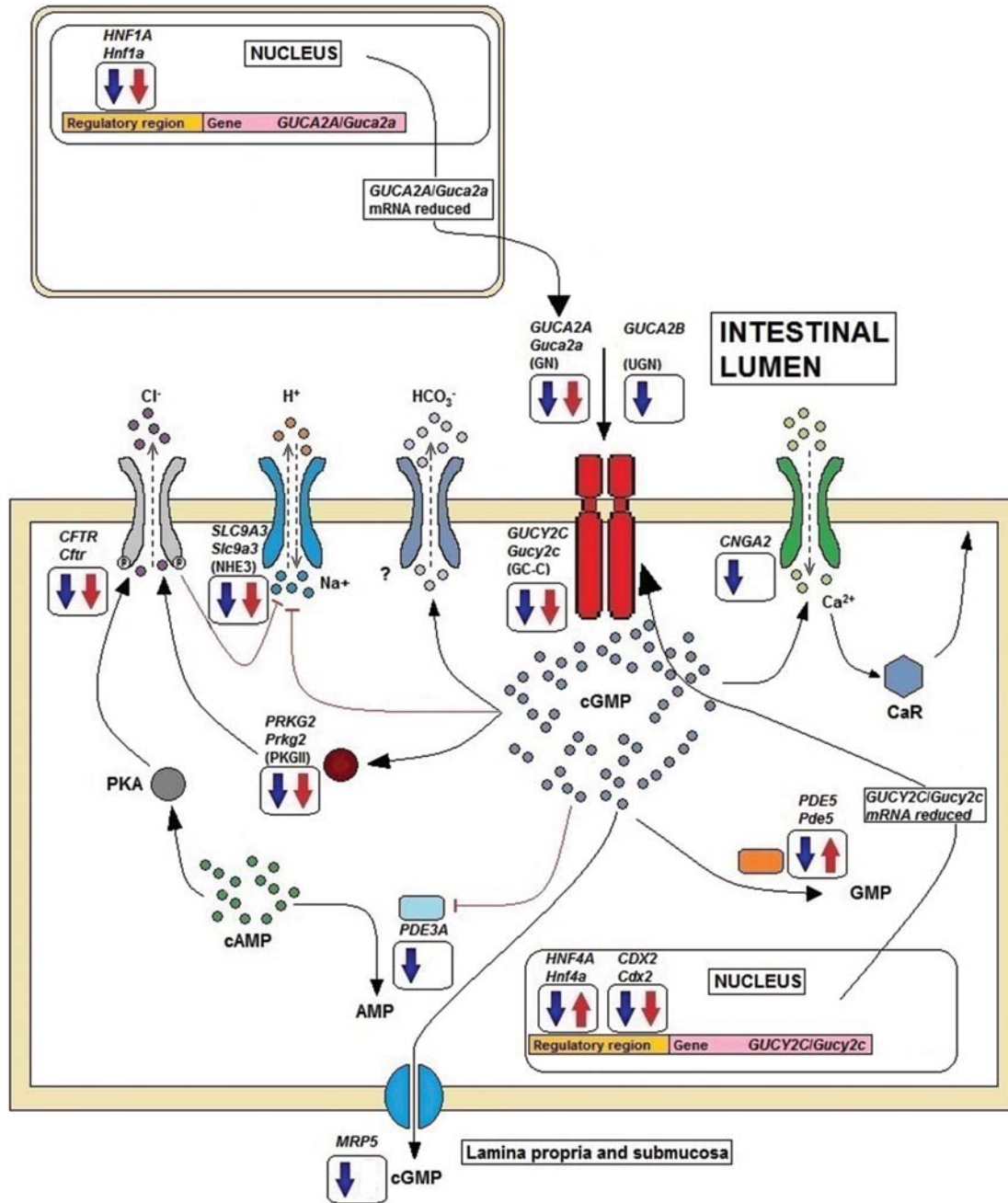


Figure 4. Proposed GC-C signaling in colitis. Direction of filled arrows in frames indicates significantly down- or up-regulated expression of genes encoding transcription factors, GC-C, GC-C ligands (GN and UGN) or cGMP downstream mediators in IBD (blue) and TNBS colitis (red). Down-regulation of transcription factors leads to reduced synthesis of GN (and UGN), which both act in a paracrine, luminoendocrine and autocrine manner, and reduced synthesis of GC-C, with subsequent decreased intracellular cGMP generation. Reduced cGMP and down-regulation of cGMP-dependent protein kinase II (PKGII) reduce phosphorylation of CFTR and diminish chloride efflux. In addition bicarbonate efflux through an unknown channel decreases. The ion channels CFTR and sodium-hydrogen exchanger (NHE) are also down-regulated. These processes have implications for fluid and ion homeostasis. Both lower cGMP levels and down-regulation of cyclic nucleotide-gated channels (CNG) lead to diminished calcium influx and calcium-sensing receptor (CaR) signaling, with reduced anti-proliferative effects as a consequence [38]. cGMP-dependent phosphodiesterase (PDE5) which facilitates conversion of cGMP to GMP is down-regulated in IBD and up-regulated in TNBS colitis. cGMP is transported through the basolateral cell membrane, possibly by multidrug-resistance transporter (MRP) 5. Reduced availability of cGMP in the lamina propria and submucosa could hamper inhibition of nociceptors. The figure was adapted and modified from Basu et al. and Fiskerstrand et al. [37,39].

intestinal permeability and susceptibility to colitis [35]. Some conflicting data exist, as DSS colitis has been found to be less severe in *Gucy2c*^{-/-} and *Guca2a*^{-/-} mice [36], and in a described gain of function mutation of *GUCY2C* with increased activation of GC-C, susceptibility for intestinal obstruction and ileal inflammation were observed [37]. Such discrepancies are difficult to explain. It seems likely that GC-C signaling is important in intestinal homeostasis, with delicate regulation of growth, epithelial renewal and barrier integrity, required for a healthy epithelium.

We also found down-regulation of several genes encoding cGMP downstream mediators, which are involved in processes including the regulation of electrolyte balance and water secretion [7]. Altogether, genes encoding for GN, UGN and GC-C, together with effector proteins involved in downstream signaling of cGMP, as well as transcription factors associated with transcription of GN and GC-C are down-regulated in IBD. Figure 4 displays proposed GC-C signaling pathway aberrations in colitis. The dysregulatory mechanisms include reduced levels of transcription factors HNF-1 α for *GUCA2A*, and HNF-4 α and CDX2 for *GUCY2C*, with reduced levels of ligand (GN) and reduced receptor (GC-C) availability. Lower cGMP levels diminish activation of PKGII and reduce activation of CFTR. Likewise, reduced availability of cGMP reduces activation of CNG calcium channels. Finally, the expression of several downstream mediators, ion channels and also the cGMP transporter MRP5 are down-regulated.

A possible limitation of the current study is related to the IBD cohort as the number of CD patients is low, making comparison to UC difficult. The data would also have been strengthened if a larger proportion of patients were treatment naïve and if clinical activity index and endoscopic activity were recorded.

In conclusion, we have demonstrated reduced expression of the GC-C activating peptides GN and UGN in IBD. Furthermore, the GC-C signaling pathway and corresponding down-stream signaling is reduced. This indicates possible pathogenetic significance given cGMP's multiple cellular downstream mediators and effects.

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Supplementary material available online

Supplementary material and methods.