Dig Dis Sci (2008) 53:1013–1019
DOI 10.1007/s10620-007-9973-3

ORIGINAL PAPER

Wnt Pathway-Related Gene Expression in Inflammatory Bowel Disease

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Received: 27 July 2007 / Accepted: 15 August 2007 / Published online: 16 October 2007

Abstract The purpose of this study was to examine the expression of Wnt pathway-related genes in patients with ulcerative colitis (UC). RNA from colonoscopic biopsies from noninflammatory bowel disease (non-IBD) subjects and UC patients were obtained and examined with a Wnt-specific microarray for the expression of Wnt pathway-related genes. Paired samples from uninflamed and inflamed areas of the colon were obtained for the UC patients. *WNT2B*, *WNT3A*, *WNT5B*, *WNT6*, *WNT7A*, *WNT9A*, and *WNT11* exhibited significantly increased expression in UC compared to non-IBD patients. Frizzled 3 (*FZD3*) and *FZD4* exhibited significantly increased expression, and *FZD1* and *FZD5* exhibited significantly decreased expression in UC patients. Genes with increased expression in inflamed mucosa included *DKK4*, *DVL2*, *SOX17*, and *COL1A1*. There was no difference in the expression of a panel of Wnt target genes. The expression of inducible nitric oxide synthase (*INOS*) was variably influenced by inflammation. Significant differences in extracellular and cell-surface components of the Wnt pathway exist in the colonic mucosa of patients with UC compared with non-IBD patients, which may influence the strength or specificity of Wnt signaling. In inflammation, inhibitory components of the Wnt pathway exhibit increased expression, but no changes in Wnt pathway target gene expression are seen. The role and complex regulation of Sox17 and iNOS in IBD warrant further investigation.

Keywords Wnt signaling · Colon cancer · Inflammatory bowel disease · Ulcerative colitis · Microarray analysis

Author contributions: Dr. You and Dr. Nguyen performed microarray experiments and analysis; Dr. Albers participated in tissue acquisition; Dr. Lin provided pathological review of all samples; Dr. Holcombe provided oversight and direction for all aspects of the research.

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Introduction

The Wnt signaling pathway is involved in colon carcinogenesis and in control of proliferation and differentiation in the colonic stem cell compartment [1, 2]. Activating mutations in this pathway are found in more than 80% of sporadic forms of colon cancer [3, 4], as is dysregulation of other components of the Wnt pathway, including Wnt2, Wnt5a, frizzled (Fz) receptors, and the downstream transcriptional regulator LEF1 [5, 6]. Whether this pathway plays an important role in inflammatory-bowel-disease (IBD)-associated colon cancers, or in IBD in general, remains to be specifically defined.

Signaling through the Wnt pathway begins with a Wnt ligand, a secreted growth factor that interacts with a

serpentine cell-surface Fz receptor and LRP 5/6 coreceptor to initiate the signal cascade [7]. Members of the dishevelled (Dsh or dvl in human nomenclature) family interact with Fz [8], APC, and axin, which leads to inhibition of glycogen synthase kinase-3β, preventing phosphorylation and degradation of β-catenin. β-catenin then accumulates and is translocated to the nucleus where it binds to members of the lymphoid-enhancer-factor/T-cell factor (LEF/TCF) family of HMG-box transcription factors [9], inducing the transcription of many target genes including *MYC* [10], cyclooxygenase-2 (*COX-2*) [11], and cyclin D1 (*CCND1*) [12]. β-catenin-mediated Wnt signaling is referred to as the canonical Wnt pathway. Signaling can be blocked by soluble Fz-related proteins (sFRPs, FzBs), which bind to Wnt ligands extracellularly [13] and by proteins of the dickkopf (dkk) family, which bind to the LRP surface molecule [14].

Whereas mutations that result in the stabilization of β-catenin, either in APC or β-catenin itself, are extremely common in sporadic colon cancer, such mutations appear to be rare in tumors from patients with ulcerative colitis (UC) [15–18]. Ulcerative-colitis-associated rat-colon carcinogenesis induced by 1-hydrozyanthraquinone and methylazoxymethanol acetate does not involve APC mutations [19, 20], though β-catenin mutations may be seen [21]. Nucleotide array analysis comparing inflamed intestinal mucosa of patients with ulcerative colitis to inflamed mucosa of patients with Crohn's disease revealed increased expression of three Wnt pathway genes, *SARP1* (*SFRP2*), frizzled (*FZD*), and disheveled (*DVL*), in samples from patients at highest risk for colon cancer—those with longstanding ulcerative colitis [22]. Recent work from our laboratory indicates that the pattern of *DVL* family-member expression is distinct in IBD-associated colon cancers in comparison with sporadic colon cancer [23]. Differential expression of *FZD*, *DVL*, and *SFRP2* suggests that Wnt signaling may contribute to colon carcinogenesis in patients with IBD.

In this study, microarray techniques were utilized to evaluate the expression of Wnt pathway-related genes in patients with UC in order to define differences in expression between normal and IBD patients and between uninflamed and inflamed colonic mucosa in patients with UC.

Materials and methods

Patients and sample preparation

Following informed consent, biopsies of normal colonic mucosa were obtained from non-IBD patients undergoing surveillance colonoscopy and from patients with UC undergoing annual colonoscopic screening. From the latter group of patients, biopsies were obtained from both uninflamed and inflamed colonic mucosa. All biopsies were performed in duplicate from an individual area and were uniquely identified. From each matched pair of biopsy specimens, one sample was fixed and submitted for histologic analysis and one sample immediately placed in RNA-later (Qiagen, Inc., Valencia, CA, USA). Samples submitted for histologic analysis were reviewed by an experienced pathologist (FL) who was blinded from the information regarding biopsy inflammation status. Samples from non-IBD patients were utilized for microarray analysis only if deemed histologically normal. Samples from UC patients were utilized for microarray analysis only if paired samples from an uninflamed area and from an inflamed area, based on the histologic review, were both available. In this study, no biopsy specimens were read as having significant dysplasia, and no malignancies or adenomas were identified.

Microarray analysis

Six samples of colonic mucosa from non-IBD patients (normal) and six paired samples from UC patients (six uninflamed IBD and six inflamed IBD) were available for Wnt-specific microarray analysis. Biopsy samples were incubated in RNA-later at 4°C for 24 h, and RNA was then isolated utilizing Trizol reagent. cDNA probes were synthesized using a GeArray AmpoLabeling-LPR kit and hybridized to the GEArray Q Series Human Wnt Signaling Pathway Membrane-based Gene Array (SuperArray Bioscience, Frederick, MD, USA) according to the manufacturers' protocols. These arrays are pathway focused and designed to systematically profile the expression of genes involved in and downstream of Wnt signaling. The array includes the Wnt ligands and their receptors, intracellular signaling molecules, and representative target genes. It can be used to determine the pathway activation profile by chemiluminescence image analysis with a CCD camera system. The GEArray expression analysis suite, an online image data acquisition and analysis software, was utilized to facilitate background normalization, correction for different degrees of exposure, and normalization with multiple housekeeping gene controls on each membrane. Gene expression from individual arrays and collectively from multiple Wnt microarrays were analyzed and compared.

Statistical analysis

Direct comparison between gene expression in tissue between normal and IBD uninflamed and between IBD uninflamed and IBD inflamed was made for the entire population ($n = 6$ in each case). The proportion of patients expressing a specific marker was compared with the

proportion of patients with nonexpression using a two-sided Fisher's exact test. Let:

π_1 = proportion expressing patients

π_2 = proportion non-expressing patients

The null hypothesis is noted as :

$H_N : \pi_1 = \pi_2.$

The two-sided alternative will be tested :

$H_A : \pi_1 \neq \pi_2.$

Significance was set at $\alpha = 0.05$ to test the null hypothesis $\pi_1 = \pi_2$ vs. $\pi_1 \neq \pi_2$. An unpaired *t* test was utilized for comparison of expression in normal mucosa vs. IBD uninflamed mucosa. A paired *t*-test was utilized for matched samples in individual UC patients comparing IBD uninflamed to IBD inflamed mucosa. The level of gene expression was designated in arbitrary units following background correction and normalization, as indicated above, and the mean and standard deviation (SD) calculated for each individual gene marker.

Results

Wnt ligand and Fz receptor expression in IBD

There were multiple significant differences in the expression of Wnt ligands and Fz receptors in uninflamed IBD colonic mucosa compared with normal, non-IBD mucosa (Table 1). In normal colonic mucosa, *WNT1*, *WNT5A*, and *WNT10A* had the highest levels of expression. In IBD uninflamed colon, *WNT1*, *WNT2B*, *WNT3A*, *WNT5B*, and *WNT9A* had the highest levels of expression. There was significantly higher expression in the IBD samples compared with normal for *WNT2B*, *WNT3A*, *WNT5B*, *WNT6*, *WNT7A*, *WNT9A*, and *WNT11* (Fig. 1A; all *P* values < 0.05; *WNT3A* had the most significant difference, $P < 0.0001$). None of the Wnt ligands showed a decreased level of expression in IBD when compared with normal. The predominantly expressed Fz receptors in the normal colon were *FZD1*, *FZD5*, and *FZD9*, and in the IBD uninflamed mucosa *FZD3*, *FZD4*, and *FZD9*. Fz receptors with significantly decreased expression in IBD compared with normal included *FZD1* and *FZD5* (Fig. 1B; $P = 0.006$, $P = 0.012$ respectively). Fz receptors with significantly increased expression in IBD compared with normal included *FZD3* and *FZD4* (Fig. 1B; $P = 0.002$, $P = 0.029$, respectively).

Wnt pathway-related gene expression in inflamed mucosa in IBD

In individual patients, multiple genes showed differential expression between inflamed and uninflamed mucosa (Table 2). All samples were paired so as to account for differences in clinical characteristics and treatments that may have been present for different patients. When combining microarray data from all six patients, a smaller subset of genes (Fig. 2A) was consistently noted to be differentially expressed, including *DKK4*; $P = 0.008$), *DVL2*; $P = 0.03$), *SOX17* [sex-determining region Y (*SRY*)-box 17; $P = 0.029$], and collagen type I α1 (*COL1A1*; $P = 0.015$). Expression of each of these genes was increased in inflamed mucosa compared with uninflamed mucosa. Four genes were expressed at very high levels in uninflamed mucosa and had slightly decreased levels of expression in inflamed mucosa, which were statistically significant, but expression in the latter remained very high [*SFRP2*, *WISP3*, *WNT3A*, and *CCND3*; *P* values all <0.05]. Interestingly, the presence of inflammation had no effect on the expression of Wnt ligands or Fz receptors in the IBD patients.

The expression of multiple Wnt target genes was examined, including *CNND1*, *CD44*, fibroblast growth factor-4 (*FGF4*), c-*JUN*, *LEF1*, matrix metalloproteinase-26 (*MMP26*), *MMP7* and c-*MYC* (Fig. 2B). None of these genes had differential expression in inflamed IBD mucosa compared with uninflamed IBD mucosa. The expression of *MMP26* was decreased but did not reach statistical significance for this cohort of six paired samples. Specifically, there was no consistent increase in any of the target genes that might indicate augmentation of Wnt pathway signaling.

Inducible nitric oxide synthase (iNOS) has been associated with the initiation and maintenance of inflammation in human IBD, is part of the intestinal antibacterial response [24], and has recently been shown to be a target gene of Wnt/β-catenin signaling [25]. Therefore, the expression of iNOS was carefully examined in each of the uninflamed and inflamed samples from IBD patients. For one patient, iNOS expression was more than three-fold higher in inflamed mucosa. However, for two patients, levels were roughly equivalent and, in three patients, expression levels in inflamed mucosa were markedly reduced (Fig. 3).

Discussion

The Wnt pathway is involved in regulation of homeostasis within the colonic stem cell compartment controlling, along with Notch signaling, the balance between proliferation and differentiation [1, 2]. Colonic mucosal proliferation is increased in UC, especially in the setting of high-grade dysplasia [26]. However, whereas some studies have suggested that Wnt signaling is involved in UC-related colon cancer [23], others suggest that UC-associated colon cancers arise along a different pathway than sporadic, Wnt/APC driven, colon cancers [27]. Characteristics of Wnt signaling in UC mucosa compared with colonic mucosa of non-UC patients has not been previously defined.

Table 1 Expression of Wnt ligands and frizzled (Fz) receptors in normal [noninflammatory bowel disease (IBD)] colonic mucosa and uninflamed IBD colonic mucosa

	Normal (mean ± SD)	IBD, uninflamed (mean ± SD)	*P* value
Wnt ligand			
1	18,991 ± 13,978	21,912 ± 13,444	NS
2	4,210 ± 5,173	6,225 ± 5,070	NS
2b	2,507 ± 1,717	13,199 ± 5,605	0.0028
3	5,118 ± 6,106	4,883 ± 4,475	NS
3a	1,082 ± 1,234	52,541 ± 4,270	<0.0001
4	6,972 ± 9,846	13,187 ± 5,412	NS
5a	8,384 ± 7,158	10,343 ± 8,173	NS
5b	2,257 ± 1,441	25,035 ± 11,143	0.0015
6	2,910 ± 1,865	10,234 ± 6,708	0.0435
7a	1,851 ± 1,473	7,299 ± 3,894	0.0166
7b	2,400 ± 1,528	4,622 ± 3,908	NS
8a	1,046 ± 722	5,447 ± 4,419	NS
9a	5,146 ± 4,349	42,722 ± 13,220	0.0002
9b	7,227 ± 3,909	4,047 ± 4,018	NS
10a	14,147 ± 9,082	7,521 ± 6,427	NS
11	951 ± 364	10,457 ± 7,997	0.0273
Fz receptor			
1	25,159 ± 14,969	3,320 ± 2,820	0.0063
2	12,758 ± 15,067	4,530 ± 4,612	NS
3	2,779 ± 1,795	23,763 ± 10,650	0.0019
4	791 ± 710	10,948 ± 8,621	0.0286
5	20,779 ± 12,330	4,701 ± 2,344	0.0116
6	4,506 ± 3,607	5,582 ± 3,156	NS
7	604 ± 356	2,931 ± 4,507	NS
8	2,003 ± 147	2,044 ± 3,449	NS
9	13,161 ± 15,918	20,296 ± 14,164	NS
10	1,166 ± 734	2,404 ± 4,071	NS

$n = 6$ for normals, $n = 6$ for IBD, uninflamed

NS not significant

We demonstrate here significant differences in the expression of Wnt ligands and Fz receptors in patients with UC compared with non-UC normal controls. Because the function of different Wnt ligands and Fz receptors is poorly understood, it is unclear what functional significance these differences portend or whether the differential expression is a result of, or contributes to, the pathophysiology of IBD. Interestingly, Fz4 has significantly higher expression in the mucosa from the UC patients, and this receptor, in addition to binding Wnt ligands, can bind to the non-Wnt protein norrin to initiate a signaling cascade [28]. The expression of norrin in patients with UC has not been reported and was not tested in this study.

The lack of increase in the expression of numerous Wnt target genes in inflamed UC mucosa suggests that Wnt signaling is not of critical importance in the inflammatory process. Despite a prior report that *CCND1* is increased in active UC [29], no increase was seen in this study. Two Wnt-related genes that did exhibit increased expression in inflammation were *DKK4* and *DVL2*. *DKK4* inhibits Wnt signaling and *DVL2*, because it functions to transduce signals to the β-catenin destruction complex, is also a negative regulator of Wnt throughput. *SOX17*, a HMG box transcription factor, was found to be increased in inflamed mucosa. *Sox17* is known to interact directly with β-catenin and, therefore, also inhibits the Wnt pathway throughput by sequestering β-catenin so that it is unavailable to bind with LEF/TCF transcription factors [30, 31]. *Sox17* affects differentiation [32] and it is expression is required for the development of gut endoderm in the mouse [33]. Overexpression of *Sox17* may be a response to inflammation-induced mucosa damage in active UC.

Inducible nitric oxide synthase (iNOS) is positively regulated by canonical Wnt signaling [25] and has been implicated in the pathogenesis of IBD. Increased expression of iNOS has been reported in inflamed colonic IBD tissue [34–36] which may be beneficial during acute inflammation though potentially detrimental if upregulation is sustained [24]. Lack of iNOS, however, has been implicated in the development of polyps and dysplasia in the IL10(–/–) murine model of IBD, suggesting that iNOS may be protective for the development of colonic

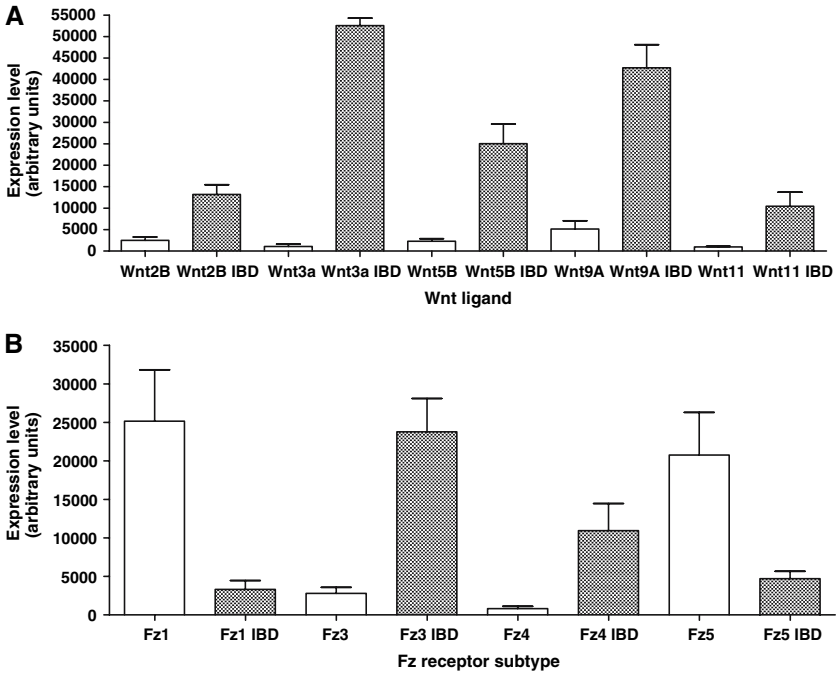

Fig. 1 Panel **A**: expression of Wnt ligands with statistically significant differential expression between normal [noninflammatory bowel disease (IBD)] colonic mucosa and uninflamed IBD colonic mucosa (mean ± SEM). Panel **B**: expression of frizzled (Fz) receptors with statistically significant differential expression between normal (non-IBD) colonic mucosa and uninflamed IBD colonic mucosa (mean ± SEM)

Table 2 Expression of genes in uninflamed inflammatory bowel disease (IBD) colonic mucosa and inflamed IBD colonic mucosa (genes with >2.5-fold difference between uninflamed and inflamed in at least one patient)

Gene	IBD, uninflamed (mean ± SD)	IBD, inflamed (mean ± SD)	*P* value
APC	11,625 ± 10,382	8,538 ± 2,925	NS
DVL2	1,374 ± 555	4,245 ± 2,979	0.03
SFRP2	52,613 ± 4,304	47,909 ± 6,011	0.046
MYC	10,580 ± 7,489	9,364 ± 1,909	NS
INOS	23,466 ± 18,140	9,518 ± 3,507	NS
SOX17	11,902 ± 9,086	30,926 ± 10,343	0.029
TCF7L1	40,383 ± 11,690	25,970 ± 16,799	NS
AXIN1	32,631 ± 13,999	31,569 ± 11,938	NS
COL1A1	25,293 ± 11,313	35,910 ± 7,248	0.015
DKK2	32,868 ± 13,167	35,135 ± 12,597	NS
DKK4	12,139 ± 6,532	20,446 ± 8,232	0.0083
ERG1	23,998 ± 15,496	26,790 ± 13,790	NS
FZB	5,789 ± 3,474	11,130 ± 5,812	NS
FST	942 ± 1,117	5,424 ± 4,912	NS
FZD4	10,948 ± 8,621	10,819 ± 6,501	NS
JUN	12,094 ± 9,655	11,226 ± 6,525	NS
IRP5	6,181 ± 2,838	14,738 ± 12,173	NS
MSX1	18,524 ± 10,499	20,707 ± 8,849	NS
WISP3	52,646 ± 4,262	47,920 ± 6,111	0.046
WNT3A	52,541 ± 4,270	46,257 ± 7,055	0.0098
CNND3	52,695 ± 4,202	47,915 ± 5,997	0.042
MMP26	43,109 ± 17,101	24,739 ± 17,909	NS

Paired samples from IBD uninflamed and IBD inflamed; $n = 6$

NS not significant

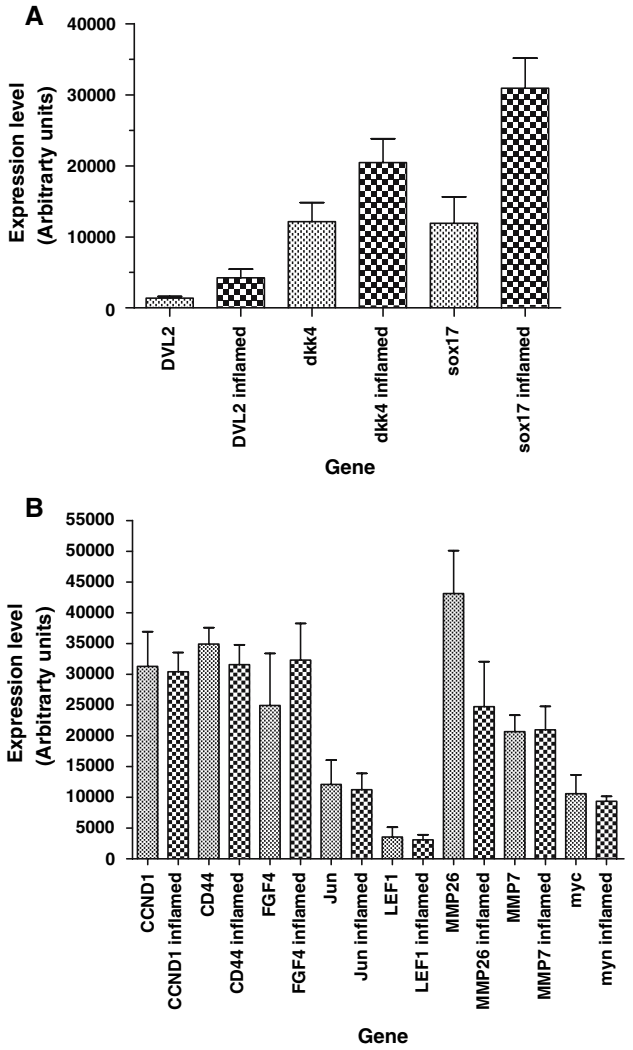

Fig. 2 Panel **A**: Wnt pathway-related genes with statistically significant differential expression between uninflamed inflammatory bowel disease (IBD) colonic mucosa and inflamed IBD colonic mucosa (mean ± SEM). Panel **B**: Wnt pathway target genes in uninflamed IBD colonic mucosa and inflamed IBD colonic mucosa (mean ± SEM). None of the differences are statistically significant

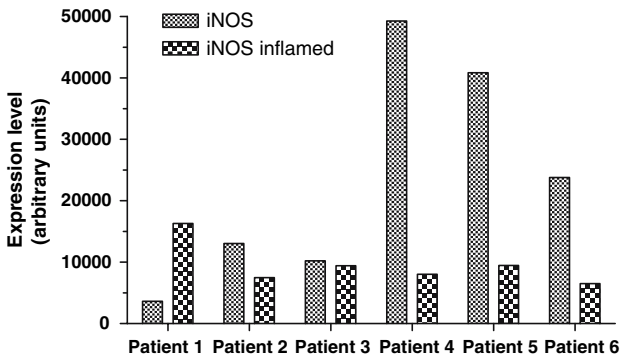

Fig. 3 Expression of inducible nitric oxide synthase (iNOS) in uninflamed and inflamed colonic mucosa from each of six patients with inflammatory bowel disease (IBD)

malignancy [37]. We demonstrate significant but not consistent changes in iNOS expression in inflamed mucosa compared with uninflamed mucosa in the six patients studied. Three patients demonstrated a marked decrease in iNOS expression, one a five-fold increase and two others minimally changed. As there was no evidence of activation of Wnt signaling in inflamed mucosa in our study, regulation of iNOS in this setting appears to be Wnt independent and may vary dependent upon specific patient characteristics.

In summary, significant differences in extracellular and cell-surface components of the Wnt pathway exist in the colonic mucosa of patients with UC compared with non-IBD patients. These may influence the strength or specificity of Wnt signaling in the colon. In inflammation, inhibitory components of the Wnt pathway exhibit increased expression and no changes in Wnt pathway throughput, as measured by the expression of a panel of target genes, is seen. Finally, the role and complex regulation of Sox17 and iNOS in IBD warrant further investigation.

Acknowledgements The authors thank Dr. Kestus Planutis and Dr. Chris Hope for their advice on experimental methodologies. This work supported by NIH grant DK-65642 (RFH) and a grant from the Broad Medical Research Foundation (IBD-0065R, RFH).

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