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# Mucosal Transcriptomics Implicates Under Expression of *BRINP3* in the Pathogenesis of Ulcerative Colitis

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**Background:** Mucosal abnormalities are potentially important in the primary pathogenesis of ulcerative colitis (UC). We investigated the mucosal transcriptomic expression profiles of biopsies from patients with UC and healthy controls, taken from macroscopically noninflamed tissue from the terminal ileum and 3 colonic locations with the objective of identifying abnormal molecules that might be involved in disease development.

**Methods:** Whole-genome transcriptional analysis was performed on intestinal biopsies taken from 24 patients with UC, 26 healthy controls, and 14 patients with Crohn's disease. Differential gene expression analysis was performed at each tissue location separately, and results were then meta-analyzed. Significantly, differentially expressed genes were validated using quantitative polymerase chain reaction. The location of gene expression within the colon was determined using immunohistochemistry, subcellular fractionation, electron and confocal microscopy. DNA methylation was quantified by pyrosequencing.

**Results:** Only 4 probes were abnormally expressed throughout the colon in patients with UC with Bone morphogenetic protein/Retinoic acid Inducible Neural-specific 3 (*BRINP3*) being the most significantly underexpressed. Attenuated expression of *BRINP3* in UC was independent of current inflammation, unrelated to phenotype or treatment, and remained low at rebiopsy an average of 22 months later. *BRINP3* is localized to the brush border of the colonic epithelium and expression is influenced by DNA methylation within its promoter.

**Conclusions:** Genome-wide expression analysis of noninflamed mucosal biopsies from patients with UC identified *BRINP3* as significantly underexpressed throughout the colon in a large subset of patients with UC. Low levels of this gene could predispose or contribute to the maintenance of the characteristic mucosal inflammation seen in this condition.

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**Key Words:** microarray, methylation, colon, inflammatory bowel disease

Ulcerative colitis (UC) is a chronic, relapsing, inflammatory bowel disease (IBD) in which inflammation is largely confined to the colonic mucosa. The disease affects approximately 1 in 1000 of the population.<sup>1</sup> The pathogenesis

of UC is complex and is classically thought to involve an abnormal interaction between the microbial flora, mucosal epithelial barrier, and the host immune system in susceptible individuals.<sup>2</sup>

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UC starts in the rectum and extends proximally in a uniform manner resulting in the 3 major phenotype classifications of proctitis, left-sided, or pancolitis.<sup>3</sup> Twenty percent to 54% of patients develop a more extensive phenotype over the course of their disease.<sup>4,5</sup> Primary defects of the mucosa have been postulated as being important in the etiology of the disease, with reports of abnormalities of mucosal epithelial cell proliferation,<sup>6–8</sup> increased intestinal epithelial permeability,<sup>9</sup> tight junction weakness,<sup>10–12</sup> aberrant mucin production<sup>13</sup> and dysfunctional epithelial cell metabolism.<sup>14</sup>

The study of twins with UC has demonstrated an elevated concordance of 15.4% in monozygotic twins as compared with 3.9% in dizygotic twins, indicative of a present but weak genetic contribution to the etiology of the disease.<sup>15</sup> Of note, this was less pronounced than that seen in Crohn's disease (CD), the other major form of IBD. The discrepancy in concordance between monozygotic and dizygotic twins highlights that environmental factors, potentially mediated by epigenetics, are likely to be important in the etiology of the disease.<sup>16</sup> Genome-wide association studies have identified a large number of loci associated with UC. A large meta-analysis of 15 independent such studies identified 133 loci associated with UC, of which 110 were shared with CD.<sup>17</sup> Several of the 23 loci uniquely associated with UC contain genes involved in the maintenance of mucosal epithelial integrity.<sup>18–20</sup> However, the total disease variance explained by these loci is only 7.5%.<sup>17</sup>

The transcriptomic analysis of the mucosa from patients with UC has the potential to identify abnormally expressed genes that could (1) be due to germline variation, (2) be secondary to environmental factors, or (3) be a consequence of the pathogenic process itself. Gene expression alterations resulting from any of these could be informative for understanding the etiopathogenesis of the disease. In addition, transcriptomic data can be used to develop diagnostic or prognostic signatures.<sup>21,22</sup> Numerous studies have therefore been conducted in UC using both mucosal biopsies and surgical resection specimens.<sup>21–29</sup> In the vast majority of these studies, the samples were obtained from chronically inflamed tissue such that transcriptomic differences observed have been dominated by the consequences of tissue damage and the presence of infiltrating inflammatory cells.<sup>21,24,26,27</sup> A recent meta-analysis of a number of these studies<sup>23</sup> identified the tight junction molecule, claudin 8 (*CLDN8*), to be the most underexpressed gene in the colon in individuals with IBD. *CLDN8* is a “barrier” tight junction molecule located in the apical part of the paracellular junction,<sup>30</sup> and thought to play a role in preventing paracellular back leakage of sodium.<sup>31</sup> In inflamed mucosa, levels of *CLDN8* are greatly reduced within the colon both in UC and CD.<sup>23,32</sup> In contrast “pore forming” claudins such as *CLDN2* and barrier tight junction molecule *CLDN1* have been shown to be upregulated during inflammation,<sup>11,32–34</sup> possibly to compensate for changes in *CLDN8* expression, altering the physical properties of the epithelial barrier. The precise reasons for these changes remain unclear. In this study, we have conducted an analysis of the transcriptome of macroscopically noninflamed

mucosa from patients with UC in clinical remission, and from CD and healthy controls (HC), in an attempt to identify genes which could be causally related to the development of the disease.

## MATERIALS AND METHODS

### Patients and Controls

Twenty-four patients with histologically confirmed UC, 14 individuals with CD, and 26 non-IBD controls (HC) undergoing routine colonoscopy at University College London Hospital (UCLH) between the ages of 18 and 75 years were included in the study (see Table, Supplemental Digital Content 1, <http://links.lww.com/IBD/A585>). HC were attending for endoscopic investigation of gastrointestinal symptoms; all had macroscopically normal colonoscopies and histologically normal biopsies. No patients were on corticosteroids or biological treatments, and most were on no treatment or 5-aminosalicylates alone. At the time of collection, all patients had clinically quiescent disease with a partial Mayo activity score<sup>35</sup> of 2 or less (UC) or a Harvey–Bradshaw score of less than 3 (CD).<sup>36</sup> Repeated biopsies were taken from 5 patients with UC and 4 new HC at a later time point (median, 22 months; range, 21–23 months) for expression and methylation studies. Rectal biopsies were also obtained from an independent cohort of 5 patients with UC and 16 HC subjects. Written informed consent was obtained from all patients. Ethical approval for this study was obtained from the Joint UCL/UCLH Committees on the Ethics of Human Research (project number 02/0324).

### Tissue Collection

Paired endoscopic pinch biopsies (Radial Jaw 4 2.8 mm Single-Use Biopsy Forceps; Boston Scientific Corporation, Marlborough, MA) were obtained from macroscopically normal mucosa of the terminal ileum, ascending colon, descending colon, and rectum. Where possible samples were taken from all 4 locations in each subject (Fig., Supplemental Digital Content 2, <http://links.lww.com/IBD/A574>). One biopsy was placed in RNAlater stabilization reagent (Qiagen, Hilden, Germany) and stored at  $-80^{\circ}\text{C}$  for messenger RNA (mRNA) preparation, and the other was placed in 4% formaldehyde for histological evaluation.

Blood samples were taken from all study participants at the time of endoscopy for the assessment of systemic inflammatory status<sup>37</sup> and to obtain a sample of genomic DNA from blood leukocytes. Paraffin-embedded colonic and terminal ileum resection tissue from non-IBD controls and stored within the UCLH histological tissue repository was used for immunohistochemistry. Fresh surgical samples from a normal ascending colon were obtained from the Department of Surgery at UCLH and used for the immunogold labeling.

### Histological Grading

All biopsies were graded histologically for microscopic inflammation using a previously described linear histological

grading method by specialist histopathologists who were blinded to the disease status of the participants (see Table, Supplemental Digital Content 3, <http://links.lww.com/IBD/A586>).<sup>38</sup> The combined (acute and chronic) inflammation score, including Paneth cell metaplasia, ranged from 0 to 23.<sup>39</sup> All surgical resection tissue used in this study were graded to be histologically normal.

### RNA Extraction

Biopsies (~25 mg) were lysed in 300- $\mu$ L RNeasy Fibrous Tissue kit (Qiagen) RLT buffer and 0.14 M  $\beta$ -mercaptoethanol ( $\beta$ -ME) (Sigma-Aldrich, St Louis, MO) and then homogenized by centrifugation at 10,000g through a Qiashredder column (Qiagen). Protein was removed by incubation for 10 minutes at 55°C with 10  $\mu$ L Proteinase K (20 mg/mL) (>600 mAU/mL) (Qiagen). Total RNA was extracted on RNeasy Mini spin columns and DNA was removed with RNase free DNase digestion (Qiagen). Samples were taken forward for microarray analysis if they had an optical density ratio of 1.8 to 2.0 OD<sub>260</sub>/OD<sub>280</sub> and >1.8 OD<sub>260</sub>/OD<sub>230</sub> as well as an RNA concentration of greater than 50 ng/ $\mu$ L.

### Microarray Analysis

For each biopsy sample, 500 ng of total RNA was amplified and purified using the Illumina TotalPrep-96 RNA amplification kit (Ambion; Life Technologies, Carlsbad, CA).

Seven hundred fifty nanogram of biotin-labeled complementary RNA (150 ng/ $\mu$ L) was hybridized to Illumina HumanHT-12v4 Expression BeadChips (Illumina, San Diego, CA) for 16 hours at 58°C. After hybridization, BeadChips were washed and stained with streptavidin-Cy3 (GE Healthcare, Little Chalfont, United Kingdom), scanned using the BeadArray reader (Illumina), and processed using Illumina Genome Studio software.

### Quantitative Polymerase Chain Reaction

RNA (1  $\mu$ g) was converted to complementary DNA using oligo d(T) primers and reverse transcription using the Promega reverse transcription kit (Qiagen). Real time quantitative polymerase chain reaction (qPCR) was performed using SensiMix NoRef DNA kit (Bioline Reagents, London, Little Chalfont, United Kingdom), using peptidylprolyl isomerase A (*PPIA*) (Bioline Reagents) as an endogenous control. Primers for qPCR and pyrosequencing are listed in Table, Supplemental Digital Content 4, <http://links.lww.com/IBD/A587>.

All samples and standards were run in triplicate. The relative expression of genes of interest in patients with UC was compared with HC using the comparative Ct ( $\Delta\Delta$  Ct) method.<sup>40</sup>

### Subcellular Localization of BRINP3

Immunohistochemistry, Western blotting, subcellular fractionation, immunogold labeling, and confocal microscopy were used to establish the subcellular location of BRINP3. Details of the full methodology are documented in Methods section, Supplemental Digital Content 5, <http://links.lww.com/IBD/A594>.

### DNA Methylation and Pyrosequencing

Endoscopic pinch biopsies from 5 patients with UC and 4 HC were stored in RNAlater stabilization reagent (Qiagen) at -80°C. DNA was extracted from intestinal biopsies and blood leukocytes using the DNeasy Blood and Tissue kit (Qiagen) with 500 ng DNA bisulfite converted using the EZ-96 DNA Methylation Kit (Deep Well Format) (Zymo Research, Irvine, CA). Sequencing and PCR primers were designed using the PyroMark Assay Design Software 2.0 (Qiagen/Biotage, Uppsala, Sweden) (see Table, Supplemental Digital Content 4, <http://links.lww.com/IBD/A587>). Bisulfite-modified DNA was amplified by PCR using these primers with GoTaq Hot Start Polymerase and reagents (Promega, Madison, WI) and dNTP Mix (Eurogentec, Hampshire, United Kingdom) in a 50- $\mu$ L reaction volume. A 30  $\mu$ L aliquot of the PCR product was transferred to a PSQ HS 96 plate (Qiagen/Biotage), and pyrosequencing was performed following the manufacturer's protocol using a PyroMark Q96 Vacuum Prep Workstation and a PyroMark (Qiagen/Biotage) Q96 MD System. DNA methylation levels were quantified using Pyro Q-CpG Software (Qiagen/Biotage). Percentage methylation was analyzed using GraphPad Prism 4 for Windows (GraphPad Software Inc., La Jolla, CA).

### Data Analysis

Expression data for Illumina HumanHT-12v4 BeadChips was normalized after Log<sub>2</sub> transformation within Illumina Genome Studio using cubic spline normalization with background subtraction<sup>41</sup> and analyzed using the LUMI and LIMMA packages in R programming language (Bioconductor).<sup>42,43</sup> Probes that reached a minimum detection *P*-value of <0.01 in at least 2 biopsies in any location were included in the subsequent analyses (*n* = 26,261 probes). Microarray expression data for the verification cohort of rectal biopsies was generated using the Illumina WG6v3 beadarrays and were processed as above (*n* = 14,976 probes). Data were normalized for batch effects using ComBat normalization<sup>44</sup> for each tissue location separately.

Differential expression analysis between groups was performed at each intestinal location using LIMMA software (Bioconductor).<sup>42,43</sup> The *P*-value was adjusted for multiple testing as described by Benjamini and Hochberg<sup>45</sup>; with a corrected *P*-value threshold for significance of 0.05. The differential gene expression results from each location were meta-analyzed using Fisher's method with Brown's extension for nonindependence.<sup>46,47</sup> GraphPad Prism 4 for Windows (GraphPad Software Inc.) was used for the analysis of qPCR and DNA methylation data and for correlation analyses. For group comparisons, an unpaired Student's *t* test was used to assess statistical significance (*P* < 0.05) in each circumstance. The data from Gene Expression Omnibus (GEO)<sup>48</sup> datasets GSE38713,<sup>29</sup> GSE22619 (samples GSM560961-GSM560976),<sup>49</sup> and GSE27899<sup>49</sup> were used as verification and comparison cohorts. Gene-annotation enrichment analysis was performed using DAVID (Database for Annotation, Visualization, and Integrated Discovery) v6.7 Functional Annotation Bioinformatic Software for Microarray Analysis (NIAID; Bethesda, MD).<sup>50</sup> In cases of group sizes of 5 or fewer observations per group, *P*-values should be interpreted with caution.

## RESULTS

### Histological and Biochemical Assessment of Inflammatory Status

Histological assessment revealed that 11 of 20 rectal, 12 of 20 descending colon, 3 of 16 ascending colon, and 2 of 12 terminal ileal biopsies from patients with UC demonstrated microscopic inflammation (a combined inflammation score of 1 or more) with a median inflammation score of 3 across all locations (see Table, Supplemental Digital Content 6, <http://links.lww.com/IBD/A588>). No histological inflammation was observed in the HC cohort. For future analyses, “inflamed UC” biopsies were designated as those with a combined inflammation score of 1 or more and non-inflamed UC biopsies with an inflammation score of 0. Assessment of systemic markers of disease activity confirmed the quiescent status of all patients with UC (Fig., Supplemental Digital Content 7, <http://links.lww.com/IBD/A575>).

### Comparisons of Gene Expression in Noninflamed UC and HC

In an attempt to identify genes that are associated with a primary abnormality within the colonic mucosa in UC, we compared noninflamed UC with HC biopsies from the rectum, descending, and ascending colon. When the results from each location were analyzed independently, no differentially significant expression differences were observed. Meta-analysis of the differential results from each colonic location identified 4 probes (corresponding to 3 genes) that were significantly differentially expressed throughout the colon (Table 1; see Table, Supplemental Digital Content 8, <http://links.lww.com/IBD/A589>). Of these, only 2 probes, both mapping to the gene family with sequence similarity member 5C (*FAM5C*) recently renamed *BRINP3*, were underexpressed. The overexpressed probes were *AKRIC2*, Aldo-Keto Reductase family 1 member C2, and the transcriptional regulator *PITX1*.

The 2 *BRINP3* probes were highly correlated across our entire dataset ( $r^2 = 0.906$ ,  $P < 0.001$ ) (Fig., Supplemental Digital Content 9, <http://links.lww.com/IBD/A576>), and all subsequent analysis was performed with 1 probe (ILMN\_1758943).

### Transcriptomic Analysis of Microscopically Inflamed Mucosa

To identify the genes associated with inflammation in the UC mucosa, we compared gene expression in microscopically inflamed biopsies with HC in the rectum (see Table, Supplemental Digital Content 10, <http://links.lww.com/IBD/A590>). There were 460 probes (416 genes) significantly differentially expressed of which 220 probes (195 genes) were overexpressed, e.g., the defensins genes (*DEFA5* and *DEFA6*, Defensin Alpha 5 and 6) and 240 probes (221 genes) were underexpressed including the tight junction molecule, *CLDN8*. The same analysis was also performed on the descending colonic samples with similar findings (data not shown) and validated using qPCR for both *DEFA5* and *S100A8* (Fig., Supplemental Digital Content 11, <http://links.lww.com/IBD/A577>). Unsurprisingly, gene-annotation enrichment analysis on these differentially expressed genes demonstrated significant overrepresentation of biological processes associated with inflammatory and defense responses to bacteria and wounding. Underexpressed biological processes related to the generation of precursor metabolites, respiration, and electron transport (see Table, Supplemental Digital Content 12, <http://links.lww.com/IBD/A591>).

### Relationship Between BRINP3 Expression and Bowel Location

Given that *BRINP3* was the most significantly abnormally expressed gene, subsequent analyses focused on this gene. *BRINP3* expression was the highest in the terminal ileum and decreased proximally (Fig. 1A). When compared with the HC cohort and patients with CD, expression levels in all UC samples, irrespective of inflammation status, were normal in the terminal ileum but significantly lower in all 3 locations in the colon (Fig. 1A). qPCR verified the reduced *BRINP3* levels in the descending colon of patients with UC (Fig. 1B). Patients with UC with reduced *BRINP3* mRNA expression also demonstrated approximately 70% lower levels of protein within the descending colonic biopsies compared with HC (Fig. 1C, D). *BRINP3* protein levels were determined in the descending colon due to the relatively high level of expression at this location and

**TABLE 1.** Comparisons of Gene Expression in Noninflamed UC and HC

Gene	Probe ID	Fisher's Test with Brown's Adjustment			Rectum		Descending Colon		Ascending Colon	
		Mean Log <sub>2</sub> FC	P	Adjusted P	Log <sub>2</sub> FC	P	Log <sub>2</sub> FC	P	Log <sub>2</sub> FC	P
<i>FAM5C/BRINP3</i>	ILMN_1758943	-0.55	8.7E-09	2.3E-04	-0.76	3.0E-06	-0.56	1.4E-04	-0.33	3.0E-02
<i>FAM5C/BRINP3</i>	ILMN_2092589	-0.40	1.4E-07	1.8E-03	-0.54	2.2E-05	-0.48	1.4E-04	-0.19	9.1E-02
<i>AKRIC2</i>	ILMN_2412336	0.76	4.5E-07	3.9E-03	0.44	2.7E-02	0.74	6.8E-03	1.09	6.0E-06
<i>PITX1</i>	ILMN_1674386	0.72	2.4E-06	1.5E-02	0.89	4.5E-05	0.82	3.3E-03	0.46	5.0E-02

Differential comparison analyses of noninflamed UC (inflammation score of 0) biopsies against HCs at each location were meta-analyzed. Log<sub>2</sub> expression fold changes (FC) for each probe at each location are shown as well as the mean Log<sub>2</sub> FC across all 3 colonic locations. P values and adjusted P values ( $P < 0.05$ ) postcorrection for multiple testing are shown for the meta-analysis.

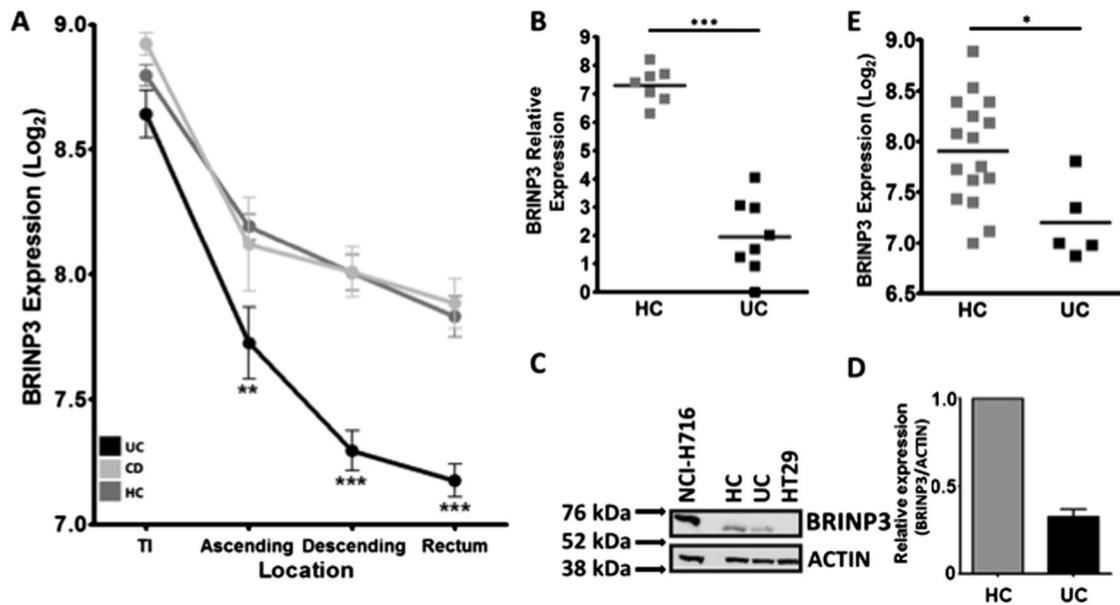


FIGURE 1. Relationship between *BRINP3* expression, disease, and bowel location. A, Log<sub>2</sub> mean expression ( $\pm$ SEM) of *BRINP3* in all locations in UC, HC, and CD in the terminal ileum (TI) (UC = 12, CD = 5, HC = 13), ascending colon (UC = 16, CD = 7, HC = 13), descending colon (UC = 20, CD = 9, HC = 17), and rectum (UC = 20, CD = 13, HC = 26). B, qPCR verification of low *BRINP3* in the descending colon in patients with UC (n = 8) identified as having low expression in the initial dataset and HCs (n = 7). C, Protein level of *BRINP3* in biopsies taken from the descending colon from patients with UC with low mRNA expression was determined by Western blotting and compared against HC. *BRINP3*-positive (NCI-H716) and *BRINP3*-negative (HT29) colonic epithelial cell lines were used as controls. D, Relative *BRINP3* protein levels (using relative luminescence BRINP3/ACTIN) within the descending colon of the patients with UC (n = 3) was compared with HC (n = 2). E, Verification of low *BRINP3* in patients with UC in the rectum ( $P = 0.013$ ) of the second cohort of patients with UC (n = 5) and HCs (n = 16). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

the availability of biopsies from individuals who were shown to express low *BRINP3* mRNA on the microarrays. Independent replication of the reduced expression of *BRINP3* in UC was provided by the second smaller cohort of UC patients (n = 5) and HC (n = 16) (see Table, Supplemental Digital Content 13, <http://links.lww.com/IBD/A592>) ( $P = 0.0131$ ) (Fig. 1E). Additional evidence of a reduced expression of *BRINP3* associated with UC was provided by 2 recent microarray studies on the intestinal mucosal biopsies obtained from patients with UC and unaffected controls (Fig., Supplemental Digital Content 14, <http://links.lww.com/IBD/A578>).<sup>29,49</sup> Both studies support our finding of reduced *BRINP3* expression in the colons of individuals with UC.

### ***BRINP3* Expression and Relationship to Ongoing Inflammation**

*BRINP3* expression in UC was unrelated to the presence or absence of microscopic inflammation (Fig. 2A–D). Rectal biopsies from the 3 lowest expressers of *BRINP3* were shown to be devoid of any signs of inflammation (Fig. 2E (1, 2, 3)). Similar results were also obtained from the other tissue locations (data not shown). *BRINP3* expression in the descending colon was not correlated with previously identified markers of colonic inflammation (upregulated genes *IL-8*, *S100A8*, *DEFA5* and downregulated *CLDN8*) (Fig., Supplemental Digital Content 15, <http://links.lww.com/IBD/A579>).

A lack of correlation between *BRINP3* and inflammatory markers was also evident in the rectum and ascending colonic biopsies (data not shown). In agreement with previous reports, the loss in *CLDN8* expression seen in our cohort was strongly associated with the presence of microscopic inflammation (Fig., Supplemental Digital Content 16, <http://links.lww.com/IBD/A580>).

### ***BRINP3* Association with Disease Phenotype and Treatment**

To assess whether low *BRINP3* expression was associated with a particular disease phenotype, biopsies obtained from the ascending colon of patients with UC with either pancolitis or left-sided colitis were compared (Fig. 3). Individuals with left-sided disease were defined as those having no historical evidence of inflammation in the ascending colon. Therefore, alterations in *BRINP3* expression at this location in these individuals would suggest that reduced expression is not associated with previous inflammation and support the hypothesis that low *BRINP3* may predispose to disease onset. Although the overall expression levels compared with HC were lower in individuals with a history of pancolitis ( $P < 0.0008$ ) as opposed to patients with left-sided disease ( $P = 0.183$ ) (Fig. 3), *BRINP3* levels were not consistent with phenotype as normal expression was observed in 30% of patients with pancolitis and low in 2 out of 7 individuals with left-sided colitis. To address the possible effects of treatment on

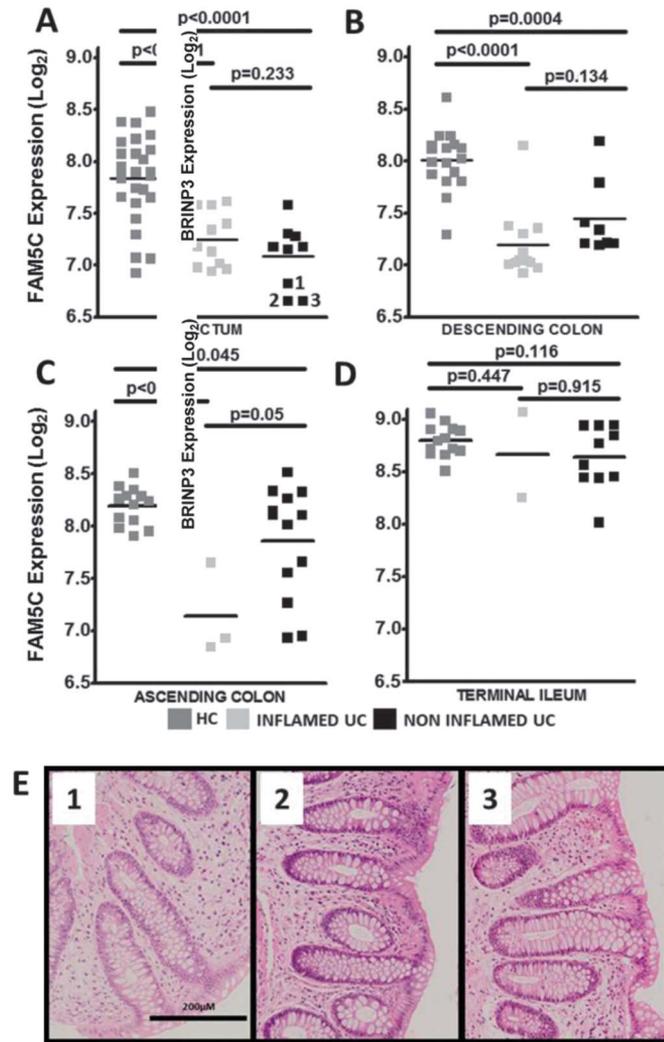


FIGURE 2. *BRINP3* expression and relationship to ongoing inflammation. Dot plots of *BRINP3* Log<sub>2</sub> expression in HCs, noninflamed UC (NIUC, inflammation score = 0), inflamed UC (IUC, inflammation score ≥ 1) in: (A) rectum (NIUC = 9, IUC = 11, HC = 26), (B) descending colon (NIUC = 8, IUC = 12, HC = 17), (C) ascending colon (NIUC = 3, IUC = 13, HC = 13), and (D) terminal ileum (NIUC = 10, IUC = 2, HC = 13), separated according to their histological status. E, In the rectum, the UC biopsies with the lowest expression of *BRINP3* in the colon, graded as being noninflamed, are highlighted (1–3), with their respective histological images.

*BRINP3* expression, patients were separated into subgroups according to their medication at the time biopsies were taken. There was no difference between expression of *BRINP3* with treatment (Fig., Supplemental Digital Content 8, <http://links.lww.com/IBD/A581>) in the rectum or at any other location (data not shown).

### Longitudinal Studies into *BRINP3* Expression

To ascertain whether low expression levels of *BRINP3* in UC were stable over time, patients with attenuated expression were rebiopsied after a period of 21 to 23 months (see Table, Supplemental Digital Content 17, <http://links.lww.com/IBD/A593>). The

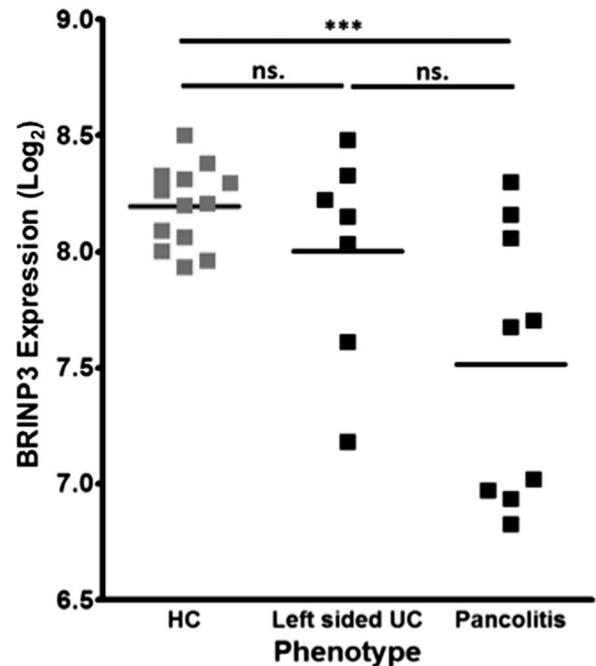


FIGURE 3. *BRINP3* expression relationship to UC phenotype. Dot plot of *BRINP3* Log<sub>2</sub> expression in the ascending colon in HCs (n = 13) and in patients with UC (n = 16) divided according to phenotype using the Montreal classification (E3, pancolitis = 9; E2, left-sided colitis = 7).<sup>3</sup> \*\*\**P* < 0.001; ns, nonsignificant.

expression of *BRINP3* in these individuals remained low in comparison with control individuals in both the rectum (*P* = 0.0001) and the descending colon (*P* = 0.0072) in all patients rebiopsied (Fig. 4A, B). These patients had all remained in clinical remission over the intervening time period (see Table, Supplemental Digital Content 17, <http://links.lww.com/IBD/A593>).

### Cellular Localization of *BRINP3* in the Lower Gastrointestinal Tract

Immunohistochemistry was performed on resected ileal and colonic tissue from subjects without IBD and with normal histology. *BRINP3* was observed to be located in the brush border region of ileal and colonic epithelial cells (Fig. 5A). Electron microscopy immunogold staining localized *BRINP3* to the region of the brush border microvilli just under the luminal plasma membrane of human colonic epithelial cells (Fig. 5B). Minimal staining was seen elsewhere.

The NCI-H716 cell line, a human colorectal adenocarcinoma found to have the highest level of expression of *BRINP3* in the Cancer Cell Line Encyclopaedia (GEO dataset GSE36133),<sup>51</sup> was used for cell biology experiments. After fractionation of the cells on continuous sucrose gradients, the protein was identified in the fractions by Western blotting. It was mainly found in the region of the gradient normally occupied by the light membrane fraction<sup>52</sup> (Fig. 5C). The mitochondrial marker GRP75 showed minimal overlap with *BRINP3* (Fig. 5C). Confocal microscopy confirmed the lack

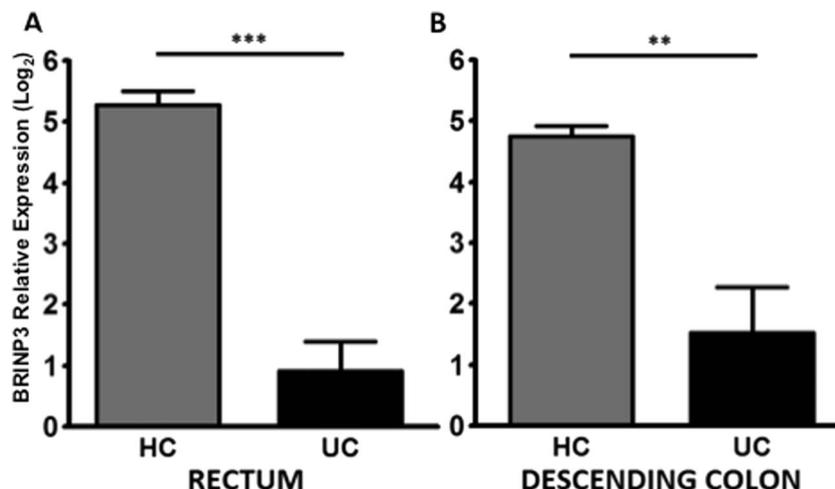


FIGURE 4. Longitudinal studies into *BRINP3* expression. Low expression of *BRINP3* in patients with previously documented low *BRINP3* expression was verified by qPCR using repeat biopsies taken from the: (A) rectum, UC (n = 5) and HCs (n = 4); (B) descending colon, UC (n = 5) and HCs (n = 4). Results expressed as mean  $\pm$  SEM. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

of association between *BRINP3* staining and mitochondria identified with an antibody to GRP75 (Fig., Supplemental Digital Content 18, <http://links.lww.com/IBD/A582>). It was possible that *BRINP3* migrated from the cytosol into the denser fractions as part of a macromolecular complex, and evidence for this was its colocalization with vinculin, a component of the actin cytoskeleton. To distinguish this from a membrane location, we subjected a *BRINP3*-positive fraction to an additional centrifugation step in which membranes would not sediment at their buoyant density and would remain in suspension, whereas macromolecular protein complexes would sediment into the pellet. *BRINP3* and vinculin were found almost exclusively in the pellet, indicative of their association with large protein complexes (Fig. 5D). Confocal microscopy of NCI-H716 cells demonstrated colocalization of *BRINP3* and vinculin just under the plasma membrane, providing additional evidence of a direct association with the cytoskeleton (Fig. 5E).

### Methylation of the *BRINP3* Promoter in the Distal Colon

Previous genome-wide association studies have revealed no significant association between the *BRINP3* locus and UC (Fig., Supplemental Digital Content 19, <http://links.lww.com/IBD/A583>).<sup>53,54</sup> A recent microarray study on the intestinal mucosa of monozygotic twins discordant for UC demonstrated that *BRINP3* expression was significantly reduced in the affected as compared with the unaffected twin (Fig., Supplemental Digital Content 14, <http://links.lww.com/IBD/A578>).<sup>49</sup> These findings suggest that alterations in *BRINP3* expression may not be attributable to germline variation and could result from epigenetic modifications. DNA methylation is a major regulator of gene expression and has been previously shown to be abnormal in patients with UC.<sup>20,49,55</sup> *BRINP3* contains a CpG rich sequence (“CpG island”

(chr1: 190446552–190447574) in the promoter region (Fig., Supplemental Digital Content 19, <http://links.lww.com/IBD/A583>). The methylation status of the *BRINP3* promoter region was determined in the rectal mucosa of *BRINP3* low UC patients (n=5) and HC (n = 4) as well as in circulating leukocytes from the same individuals (Fig. 6). *BRINP3* expression was also quantitated by qPCR in the same rectal biopsy specimens. Patients with UC expressing *BRINP3* at low levels in the rectum (Fig. 6A) had significantly increased methylation in the promoter region ( $P < 0.01$ ) (Fig. 6B). There was an inverse relationship between *BRINP3* expression and methylation ( $r^2 = 0.6991$ ,  $P = 0.005$ ) (Fig., Supplemental Digital Content 20, <http://links.lww.com/IBD/A584>). There was no significant increase in the extent of *BRINP3* promoter methylation in the circulating leukocyte population in patients with UC indicating a possible tissue-specific modification (Fig. 5B).

### DISCUSSION

The pathology of UC, such as its superficial nature, is in keeping with a primary abnormality of the mucosa. Numerous studies have reported defects in mucosal integrity, epithelial turnover, and mucin production.<sup>7,9,12,13</sup> To identify molecular abnormalities that might be responsible for these mucosal defects, we compared macroscopically normal colonic mucosal tissue from patients with UC in remission with HC individuals. Although the mucosa appeared macroscopically normal in all cases, approximately 30% of the biopsies had evidence of microscopic inflammation; a previously recognized phenomenon.<sup>56,57</sup> Through a transcriptome-wide approach using biopsies with no evidence of microscopic inflammation, we identified 3 genes which were differentially expressed throughout the entire colon. Of these, the only one that was underexpressed was *BRINP3*, which was also the most significantly different gene from HCs.

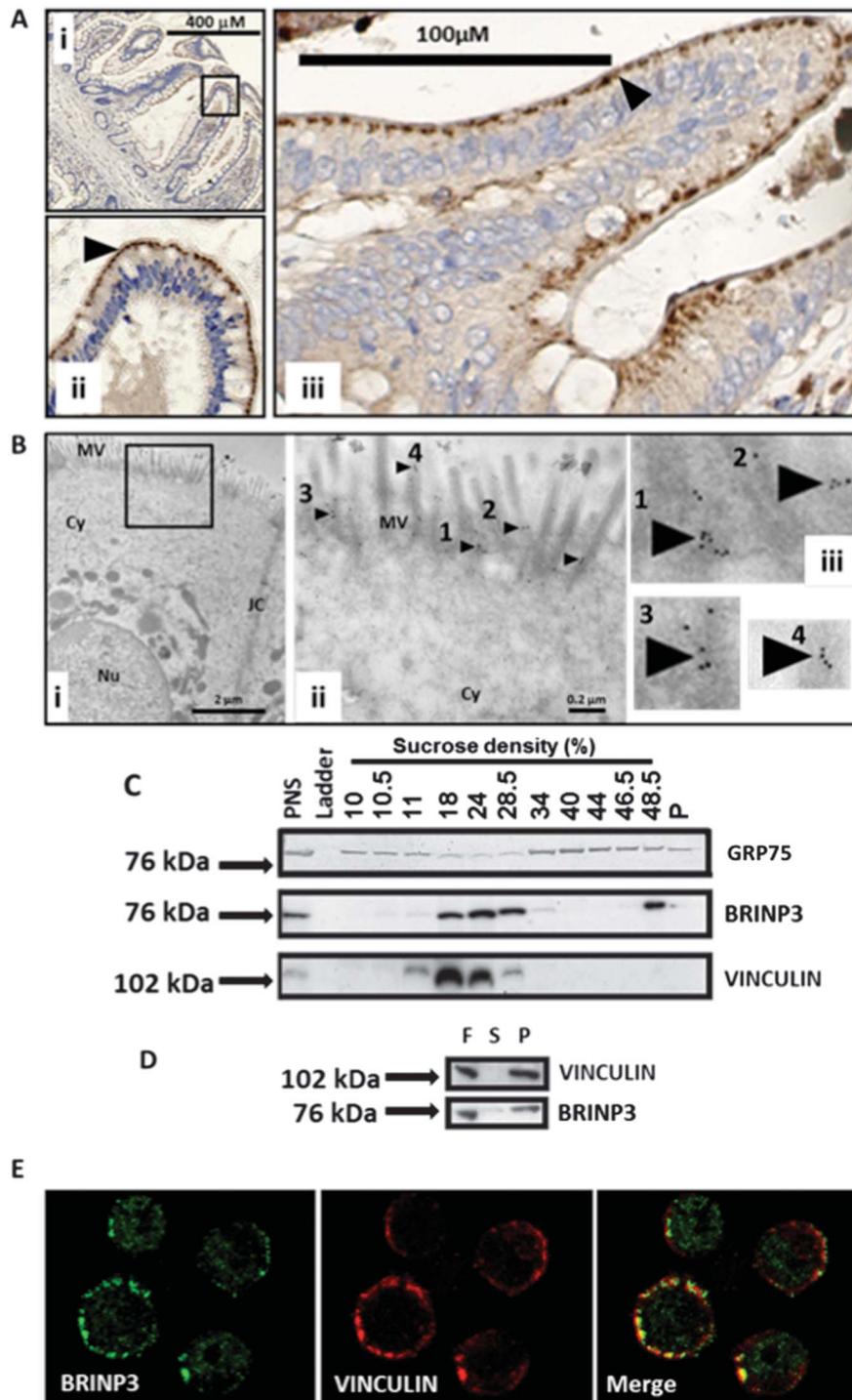


FIGURE 5. Cellular localization of BRINP3 in the lower gastrointestinal tract. A, Immunohistochemistry of BRINP3 performed on resection specimens from HC in the terminal ileum (i and ii) and colonic mucosa (iii) (black arrows denote BRINP3 expression). B, Electron micrographs of the brush border epithelium in a human colonic epithelial cell using anti-BRINP3 and immunogold secondary antibodies (i and iii). B (ii and iii), immunogold staining of the microvilli shown in (i) (black box) at higher magnification (black arrows and numbered 1–4). MV, microvilli; Cy, cytoplasm; JC, epithelial cell junctional complex; Nu, nucleus. C, Subcellular fractionation of the NCI-H716 adenocarcinoma epithelial cell line followed by Western blotting with anti-BRINP3, anti-GRP75 (a mitochondrial protein), and anti-vinculin (an epithelial cytoskeletal protein). PNS, postnuclear supernatant. D, Separation of the soluble (S, supernatant) and insoluble (P, pellet) material in the 24% sucrose fraction (F) by ultracentrifugation, followed by Western blotting of BRINP3 and vinculin. E, confocal microscopy of the NCI-H716 epithelial cell line stained with BRINP3 and vinculin.

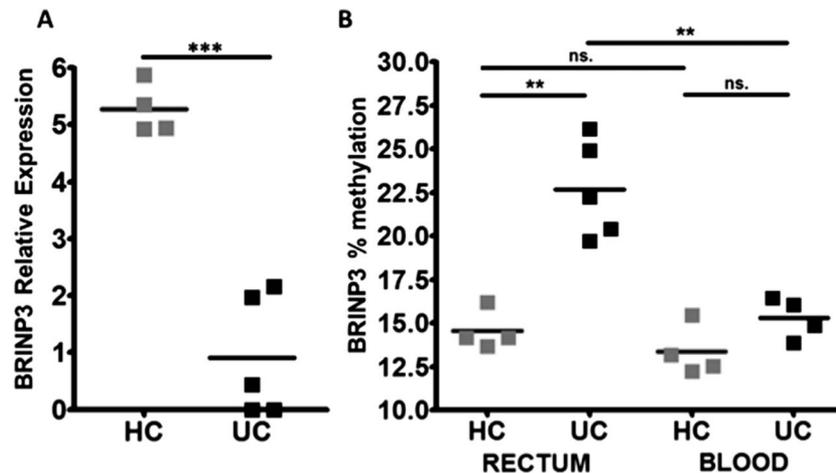


FIGURE 6. Methylation of the *BRINP3* promoter in the distal colon. A, quantification of *BRINP3* Log<sub>2</sub> relative expression in the rectum in rebiopsied patients with UC (n = 5) and additional HC (n = 4) by qPCR. B, Percentage (%) methylation status of the *BRINP3* promoter in UC (n = 5) and HC (n = 4) rectal biopsies and in circulating leukocytes in the same patients with UC (n = 4) and HC (n = 4). \*\*\**P* < 0.001; \*\**P* < 0.01; ns, nonsignificant.

Analysis of the microscopically inflamed samples identified 460 probes which strongly overlapped with the findings of from previous microarray studies into UC.<sup>21,23,26,27</sup> The genes which demonstrated the largest alteration in expression were associated with epithelial integrity such as *CLDN8* and Paneth cell metaplasia (*DEFA5*, *DEFA6*, and *REG1*). The majority of genes reported to be associated with UC in previous studies are attributable to chronic inflammation and leukocyte infiltration into the mucosal tissue such as the *SAAI* (serum amyloid A1), chemokine (C-X-C motif) ligands (*CXCL6*, *CXCL11*, *CXCL13*), and the matrix metalloproteinases (*MMP1*, *MMP3*, *MMP12*).<sup>23,26</sup> These genes were not found to be significantly abnormal in the noninflamed samples in our study. These results demonstrate a strong correlation between the histological appearance of microscopic inflammation and inflammatory markers, indicating that a suitable collection of appropriate markers on molecular arrays could provide a rapid and cost-effective alternative to histological examination.

Low levels of *BRINP3* expression were replicated in a previous microarray study of biopsies taken from quiescent patients with UC.<sup>29</sup> This suggests that low *BRINP3* may identify a subgroup of patients within the general UC cohort and supports the previously reported heterogeneous genetic character of this disease.<sup>53</sup>

Abnormal *BRINP3* expression is specific for colonic mucosal tissue in UC and not a generic response to inflammation or therapy. Tissue-specific hypermethylation of the *BRINP3* promoter may be responsible for the reduced expression. Previous studies have demonstrated a role for epigenetic modification and hypermethylation in UC and UC-associated colorectal carcinoma, but none of these studies have reported results for *BRINP3*.<sup>16,20,58</sup> Hypermethylation of the *BRINP3* promoter in individuals with gastric cancer has been proposed as a prewarning marker for its development.<sup>59</sup> Recent epigenome-wide association studies have identified genes in which DNA methylation is associated with

gene expression changes in patients with UC.<sup>49,55</sup> Abnormalities in *BRINP3* were not reported in these studies, although the presence of ongoing inflammation may have influenced the results. The discrepancies between the current findings and previous investigations are most likely due to the heterogeneous nature of UC; our methylation studies were restricted to patients with confirmed low *BRINP3* expression.

Very little is currently known about the function of the *BRINP3* protein. The *BRINP3* gene has been correlated with myocardial infarction<sup>60</sup> and aggressive periodontitis<sup>61</sup> in genetic studies. It has been postulated that it could play a role in osteoblast differentiation.<sup>62</sup> Other investigations conducted in pituitary adenoma cells, identified *BRINP3* as a mitochondrial associated protein, and linked it to tumor proliferation, migration, and invasion.<sup>63</sup> In contrast, in colonic tissue and a colonic epithelial cell line, *BRINP3* appears to be associated with the cytoskeleton in the brush border membrane.

*BRINP3* expression appears to be restricted to the microvilli and cytoskeletal region of the brush border in the epithelium. Impaired integrity of a cytoskeletal complex just under the plasma membrane interface with the bowel might be expected to predispose to an increased chance of membrane damage. It is therefore possible that reduced *BRINP3* levels due to promoter hypermethylation predates the onset of disease and may be a susceptibility factor in its development.

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