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IL-10$^{-/-}$ mice, an animal model of Th1-mediated inflammatory bowel disease, were screened for the expression of 600 microRNAs (miRNAs) using colonic tissues and PBLs from animals having either mild inflammation or severe intestinal inflammation. The development of colonic inflammation in IL-10$^{-/-}$ mice was accompanied by upregulation in the expression of 10 miRNAs (miR-19a, miR-21, miR-31, miR-101, miR-223, miR-326, miR-142-3p, miR-142-5p, miR-146a, and miR-155). Notably, the expression of all of these miRNAs plus miR-375 was elevated in PBLs of IL-10$^{-/-}$ mice at a time when colonic inflammation was minimal, suggesting that changes in specific miRNAs in circulating leukocytes may be harbingers of ensuing colonic pathology. In vitro exposure of colonic intraepithelial lymphocytes to IL-10 resulted in downregulation of miR-19a, miR-21, miR-31, miR-101, miR-223, and miR-155. Interestingly, unlike IL-10$^{-/-}$ mice, changes in miRNAs in PBL of dextran sulfate sodium-treated mice were minimal but selectively elevated in the colon after pathology was severe. We further show that miR-223 is a negative regulator of the Roquin ubiquitin ligase, Roquin curtails IL-17A synthesis, and the 3′ untranslated region of Roquin is a target for miR-223, thus defining a molecular pathway by which IL-10 modulates IL-17–mediated inflammation. To identify additional miRNAs that may be involved in the regulation of Roquin, transcriptome analysis was done using cDNAs from HeLa cells transfected with 90 miRNA mimics. Twenty-six miRNAs were identified as potential negative regulators of Roquin, thus demonstrating functional complexity in gene expression regulation by miRNAs. ***The Journal of Immunology*****, 2011, 187: 5834–5841.**

Crohn's disease (CD) and ulcerative colitis (UC) are prominent members of a suite of inflammatory conditions of the small and large intestines grouped under the moniker of inflammatory bowel disease (IBD). Whereas UC is limited to the colon and rectum and typically affects only the mucosa, CD can affect any portion of the gastrointestinal tract and usually involves the entire bowel wall. The precise cause of CD is not fully understood; however, it is known that inappropriate immune responses within the intestine, in particular, IL-17A and other proinflammatory responses, are a hallmark of disease (1). A number of mouse models are available that mimic various aspects of IBD. The IL-10 knockout (IL-10$^{-/-}$) mouse animal of Th1-mediated intestinal inflammation has been particularly useful in that regard (2, 3).

MicroRNAs (miRNAs) are short noncoding RNA species of ~19–24 nt derived from primary mRNA transcripts of intergenic or intronic sources (4). Processing and cleavage of mRNA transcripts by Drosha, DGCR8, and Dicer yields a mature miRNA that incorporates into an active RNA-induced silencing complex (5). Once incorporated into the RNA-induced silencing complex, miRNAs regulate gene expression via two distinct mechanisms based on complementarity between the miRNA and its target, the 3′ untranslated region (UTR) of mRNA transcripts. In the first, complete complementarity between the miRNA and the mRNA results in the target mRNA cleavage and degradation by Argonaute (5). In the second, imperfect or mismatch binding of the 3′ UTR of the target mRNA results in posttranslational repression and mRNA destabilization and degradation resulting from deadenylation and decapping of the target mRNA (6). It is predicted that >50% of the genome may be actively regulated by miRNAs (7). However, aberrant expression of miRNAs has been linked to a growing number of diseases, including cancers (chronic lymphocytic leukemias, gliomas, colorectal cancer, prostate cancer, and uveal melanoma) in which the miRNAs act as tumor suppressors or oncogenes (8–10), and autoimmune-related diseases such as rheumatoid arthritis (11) and systemic lupus erythematosus (12). A role for miRNAs in UC and CD is now also becoming apparent (13, 14).

The rationale for this study was 2-fold. First, we were interested in determining if miRNA expression patterns in colonic tissues in IL-10$^{-/-}$ mice differ depending upon the degree of colonic pathology. Second, we wished to determine if changes in miRNAs that occur in colonic tissues during inflammation are reflective of miRNA changes in PBL. In this study, we demonstrate that changes in specific miRNA expression patterns in circulating

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The sequences presented in this article have been submitted to the the National Center for Biotechnology Information Gene Expression Omnibus under accession number GSE31706 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=vjytpikguqckytg&acc=GSE31706).

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Abbreviations used in this article: CD, Crohn's disease; cIEL, colonic intraepithelial lymphocyte; DSS, dextran sulfate sodium; IBD, inflammatory bowel disease; IL-10$^{-/-}$, IL-10 knockout; miRNA, microRNA; ML, mononuclear leukocyte; qRT-PCR, quantitative real-time PCR; siRNA, small interfering RNA; snRNA, small nuclear RNA; UC, ulcerative colitis; UTR, untranslated region.

leukocytes occur prior to their expression in the colon, thus providing a potentially important diagnostic approach for predicting the development of colonic inflammation in IBD.

Materials and Methods

Mice, cell/tissue isolation, dextran sulfate sodium treatment, and intestinal pathology scoring

Breeding stocks of homozygous IL-10$^{-/-}$ mice [C.129P2(B6)-*Il10*^{tm1Cg}/J] on a BALB/cJ background were purchased from The Jackson Laboratory (Bar Harbor, ME). Control BALB/c mice were purchased from Harlan Laboratories (Indianapolis, IN). Mice were used in accord with University of Texas Health Science Center institutional animal welfare guidelines.

Male and female IL-10$^{-/-}$ mice 10–45 wk of age were used. Whole blood was collected from the hearts of anesthetized mice using EDTA-treated needles and transferred to tubes containing 50 μl 0.5 M EDTA. Blood was layered onto Histopaque-1077 (Sigma-Aldrich, St. Louis, MO) and centrifuged at 400 × *g* for 30 min. Cells at the blood–Histopaque interface were collected, washed with PBS, and snap-frozen at −80°C until RNA isolation. Colonic tissues were stored in RNA*later* (Ambion, Austin, TX).

Adult 7-wk-old female C57BL/6 mice were given 3% dextran sulfate sodium (DSS; m.w. 36,000–50,000; MP Biomedicals, Solon, OH) in drinking water for 0, 1, 2, or 7 d. At the designated time, animals were euthanized, PBLs were collected from blood, and colonic tissues were taken for histopathological analysis and RNA extraction for miRNA analyses.

Representative H&E-stained tissue sections (3 μm) from midportions of the proximal and distal colons of IL-10$^{-/-}$ mice and control BALB/c mice were used for histopathologic evaluation. Histopathologic scoring was performed in a blinded fashion by an experienced board-certified pathologist. For IL-10$^{-/-}$ mice, the degree of inflammation and associated crypts architectural distortion were scored microscopically on cross-sections of the colon using a five-tier scoring system established based on the published criteria for grading of IBD intestinal pathology (2): score 0, no signs of inflammation or distortion of crypts architecture; score 1, very low level of mononuclear leukocytes (MLs) in the lamina propria; score 2, low level of ML infiltration in the lamina propria; score 3, moderate level of ML infiltrate in the lamina propria with occasional crypt distortion; score 4, high levels of ML infiltrate within the lamina propria with crypt distortion, high vascular density, and thickening of the colon wall; and score 5, transmural ML infiltration, widespread crypt distortion/abscess with loss of goblet cells, high vascular density, and thickening of the colon wall.

For DSS-treated mice, histological scoring of mucosal injury and the degree of inflammation in the colon was performed as previously reported (2) with some modifications, taking into account the histopathologic differences between colitis in DSS-treated mice and IL-10$^{-/-}$ mice. H&E-stained sections were scored for intraepithelial edema, inflammation, erosions, ulceration, and abscesses in the proximal, transverse, and distal colon. Mucosal injury in DSS-induced colitis is more severe and occurs in the early stages than IL-10$^{-/-}$ mice. Hence, the severity of inflammation and mucosal injury were each scored independently from 0–3; the total pathologic grade of a section was obtained by summing the two scores. Severity of mucosal injury and the degree of inflammation increased steadily in a time-dependent manner starting at day 1 and reaching peak at the seventh day, the last time point examined in these experiments.

Cell staining, stimulation, transfection, and ELISA

Colonic intraepithelial lymphocytes (cIELs) used for intracellular expression of IL-17 and IFN-γ were isolated and stained as previously described (2). For in vitro experiments of cIELs, 5 × 10^6 cells/ml were cultured for 24 h in a 37°C incubator with 5% CO_2 in RPMI 1640 containing 10% FBS, penicillin/streptomycin, and L-glutamine (2). In some experiments, IL-10$^{-/-}$ cIEL cells were cultured with 50 ng/ml recombinant mouse IL-10 (BioLegend, San Diego, CA) or with PBS for control stimulation. Cells were assayed for IL-17A using an ELISA Ready-SET-Go! Kit (eBioscience, San Diego, CA), assayed by quantitative real-time PCR (qRT-PCR) for Roquin or IL-17A gene expression, or used to assess the effects of IL-10 on miRNA expression.

EL4 cells were transfected using siPORT NeoFX (Applied Biosystems, Austin, TX) or Oligofectamine (Invitrogen, Carlsbad, CA) with 240 nM Roquin-specific small interfering RNA (siRNA) or control siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) or were mock transfected. Forty-eight hours posttransfection, RNA was isolated and assayed for Roquin and IL-17A gene expression. For determination of the effects of miR-223 on Roquin expression, cIELs were transfected with 30 nM anti–miR-223, miR-223 mimic, or matching Cy3-labeled negative controls (Applied Biosystems).

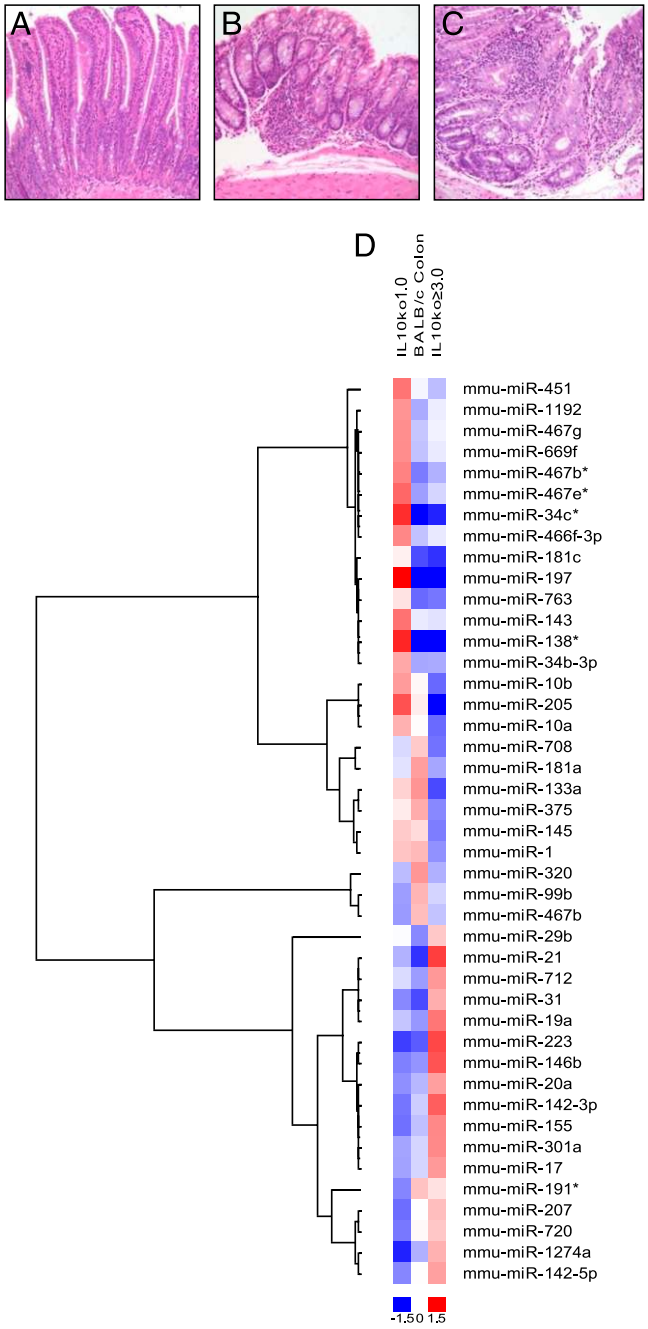

FIGURE 1. Colonic tissues from normal BALB/c mice without pathology (*A*), grade 1.0 pathology of colon tissue from IL-10$^{-/-}$ mouse (*B*), and grade 4 pathology of colon tissue from IL-10$^{-/-}$ mouse (*C*). Original magnification ×200 (*A–C*). *D*, Heat map and unsupervised hierarchical clustering of miRNAs in IL-10$^{-/-}$ and BALB/c mouse colon samples indicate dysregulation of miRNA expression. The miRCURY LNA microarray miRNA profiling service was used to examine miRNA expression in pooled total RNA samples (three mice each) from colonic tissue sections from normal BALB/c mice, IL-10$^{-/-}$ mice with intestinal pathology score of 1 (IL10ko 1.0), and IL-10$^{-/-}$ mice with intestinal pathology scores ≥3 (IL10ko ≥ 3.0). Red color represents an expression level above mean, and blue color represents expression lower than the mean. A Δ LogMedian ratio of ±1.0 is equal to a fold change of ±2.0.

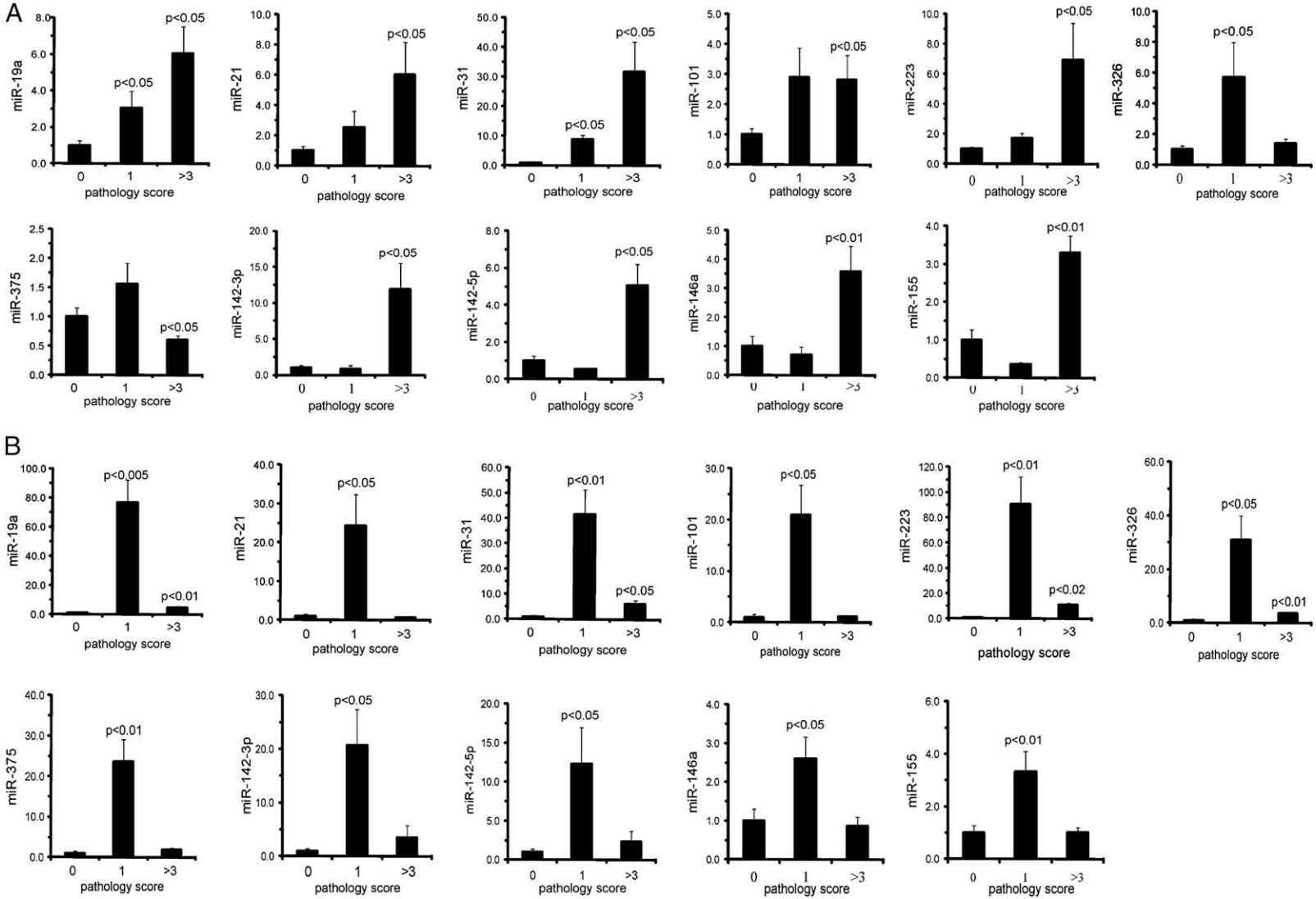

FIGURE 2. Total RNA from IL-$10^{-/-}$ and BALB/c colon was used for TaqMan qRT-PCR analysis of 11 miRNAs in colonic tissues (*A*) and PBLs (*B*) from normal mice (score 0) and IL-$10^{-/-}$ mice with low pathology (score 1) or high pathology (score ≥3.0). Expression values were calculated by normalizing to U6 snRNA levels in a sample and recorded as values relative to the expression of normal mice, which was arbitrarily designated as 1.0. Note that miRNAs were elevated in PBLs of IL-$10^{-/-}$ mice with mild intestinal pathology, but not in IL-$10^{-/-}$ mice with severe intestinal pathology. Determination of statistical significance was calculated using Student *t* test relative to BALB/c control values.

miRNA profiling, transcriptome analysis, and qRT-PCR

Total RNA was isolated using the miRNeasy Minikit (Qiagen, Valencia, CA) according to the manufacturer's instructions. cDNA was synthesized using either the High-Capacity cDNA Reverse Transcription Kit or the Taqman miRNA Reverse Transcription Kit (Applied Biosystems).

miRNA profiling of colonic tissue RNAs from normal BALB/c mice, IL-$10^{-/-}$ mice with a pathology score of 1.0, and IL-$10^{-/-}$ mice with a pathology score ≥3.0 were done by Exiqon (Woburn, MA) using the miRCURY LNA array version 11.0, with dChip software that contained capture probes targeting all miRNAs for human, mouse, or rat registered in the miRBASE version 14.0 at the Sanger Institute. Data sets have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus with accession number GSE31706 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=vjytpikguqckytg&acc=GSE31706).

The SureFIND Immunopathology miRNA Transcriptome PCR Array (Qiagen) was used according to the manufacturer's instructions. Briefly, a multiplex quantitative PCR assay was set up using the TaqMan Universal Master Mix II, No UNG reagent, and TaqMan gene expression assays for human Roquin (VIC-labeled probe) and GAPDH (FAM-labeled probe) (Applied Biosystems) to screen HeLa cell cDNAs transfected with a panel of 90 miRNA mimics. Samples were analyzed using the StepOnePlus real-time thermal cycler and the Excel-based data analysis software provided with the SureFIND Immunopathology miRNA Transcriptome PCR Array. Roquin gene expression was compared after normalization of samples to GAPDH.

To measure Roquin and IL-17A transcript levels, the Power SYBR Green PCR Master Mix (Applied Biosystems) was used according to the manufacturer's instructions. miRNA expression was quantified using miRNA-specific primers and probes (TaqMan microRNA assays; Applied Biosystems) with the TaqMan Universal Master Mix II, No UNG (Applied Biosystems). Samples were analyzed using the StepOnePlus real-time thermal cycler (Applied Biosystems) and software. Relative gene expression was normalized to either GAPDH or U6 small nuclear RNA (snRNA). Roquin, IL-17A, and GAPDH gene-specific primers were designed and purchased from Integrated DNA Technologies (Coralville, IA). Primers used were: mouse Roquin forward, 5′-GGCTGCTCGATCTTTAGGTG-3′; mouse Roquin reverse 5′-TGTTCTCTCCTCAGAGCTTCG-3′; mouse IL-17a forward, 5′-CTCCAGAAGGCCCTCAGACTAC-3′; mouse IL-17a reverse, 5′-GGGTCTTCATTGCGGTGG-3′; mouse GAPDH forward, 5′-AGAACATCATCCCTGCATCC-3′; mouse GAPDH reverse, 5′-AGCCGTATTCATTGTCATACC-3′; human Roquin forward, 5′-ACCAACCTTGCCTCCTACCT-3′; human Roquin reverse, 5′-TAATCGCTGGTCCCTCATTC-3′; human GAPDH forward, 5′-TGCACCACCAACTGCTTAGC-3′; and human GAPDH reverse, 5′-GGCATGGACTGTGGTCATGAG-3′.

Luciferase assay

The 599-bp section (P6496–7095) of the *Rc3h1* 3′ UTR incorporating the predicted miR-223 site at position 6638 was PCR-cloned into the HindIII site of the pMIR-REPORT Luciferase plasmid (Applied Biosystems) to yield the Rc3h1 P6496 3′ UTR pMIR plasmid. 293T cells were plated in 24-well plates and cotransfected with 400 ng Rc3h1 P6496 3′ UTR pMIR, 400 ng pMIR-REPORT β-galactosidase vector, and 30 nM miR-223 or Cy3-labeled Negative Control PremiR miRNA Precursors (Applied Biosystems) using 5 μl/well Endofectin Lenti (Genecopoeia, Rockville, MD). Forty-eight hours later, the cells were lysed using a 5× Cell Culture Lysis Reagent (Promega, Madison, WI), and the luciferase activities were measured on a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA) using an Enhanced Luciferase Assay kit (BD Biosciences, San Diego, CA). Luciferase activities were normalized to β-galactosidase activity. Luciferase activity with the Rc3h1 P6496 3′ UTR pMIR vector alone was regarded as 1.0.

Statistical analysis

The statistical significance of data was determined using an unpaired two-sided Student's *t* test. Data are presented as mean values ± SEM.

Results

Regulated expression of miRNAs in the colon and PBLs of IL-$10^{-/-}$ mice

Previous studies have demonstrated extensive cytokine disruption in the colon of IL-$10^{-/-}$ mice (2, 15). This led us to hypothesize that miRNAs may also be dysregulated in a disease-specific pattern. To address this, we examined global expression patterns of miRNAs in colon samples from IL-$10^{-/-}$ and parental BALB/c mice by miRNA microarray analysis. To compare miRNA expression in IL-$10^{-/-}$ mice with mild intestinal pathology to mice with severe intestinal pathology, PBLs were collected and frozen, and colonic tissues were stored in RNA*later* until scoring for pathology had been completed for colonic tissues. Analysis of miRNA expression was then done using tissues from normal BALB/c mice (Fig. 1*A*), IL-$10^{-/-}$ mice that had intestinal pathology scores of 1.0 (Fig. 1*B*), and IL-$10^{-/-}$ mice that had intestinal pathology scores ≥3.0 (Fig. 1*C*). Each group consisted of RNA pooled from three mice. Out of >600 miRNAs analyzed, 43 miRNAs were differentially expressed at least 2-fold between the comparison groups as represented in the heat map (Fig. 1*D*). This identified several miRNAs that were elevated in IL-$10^{-/-}$ mice having high colonic pathology compared with IL-$10^{-/-}$ mice with minimal pathology and to normal BALB/c mice.

qRT-PCR was done to validate expression levels of 11 miRNAs that were specifically elevated or lowered in the heat map (miR-19a, miR-21, miR-31, miR-142-3p, miR-142-5p, miR-155, miR-223, and miR-375) or have been shown to be linked to chronic inflammatory conditions (miR-101, miR-146a, and miR-326) (16–18). In colonic tissues of IL-$10^{-/-}$ mice with pathology score ≥3.0, miR-19a, mir-21, miR-31, miR-101, miR-223, miR-142-3p, miR-142-5p, miR-146a, and miR-155 were significantly elevated compared with colonic tissues from normal mice. miR-326 expression was significantly elevated in mice with pathology score of 1.0, but not in mice with scores ≥3.0. miR-375 expression was significantly lower in mice with pathology scores ≥3.0 compared with normal mice (Fig. 2*A*).

To determine if changes in miRNA expression in colonic tissues also were present in PBLs, miRNA expression levels for the 11 miRNAs used in Fig. 2*A* were measured in PBLs from normal mice, IL-$10^{-/-}$ mice with pathology score of 1.0, and IL-$10^{-/-}$ mice with pathology score ≥3.0. Importantly, miRNA expression levels were highest in PBLs of IL-$10^{-/-}$ mice with low intestinal pathology (score 1.0) compared with mice with severe intestinal pathology (score ≥3.0) (Fig. 2*B*), suggesting that the elevation of specific miRNAs in circulating leukocytes may be an early indicator of developing intestinal pathology.

To test whether exposure to IL-10 would alter miRNA expression, cIELs from IL-$10^{-/-}$ mice were cultured overnight with 50 ng/ml rIL-10 or PBS. miRNA expression was quantified relative to that of PBS control cells. Expression levels for 6 of 11 miRNAs (miR-19a, miR-21, miR-31, miR-101, miR-155, and miR-223) were significantly suppressed in cIELs from IL-$10^{-/-}$ mice following exposure to IL-10 compared with cIELs cultured with PBS (Fig. 3*A*). IL-10 exposure had no significant effect on miR-142-3p, miR-142-5p, miR-146, miR-326, and miR-375 expression. To test for the specificity of IL-10, cIELs were cultured with IL-2, which did not alter miRNA expression levels relative to PBS-supplemented cultures (data not shown).

Roquin modulates IL-17 expression via IL-10 and is negatively regulated by miR-223

We used Web-based algorithms (19) to identify potential targets for miRNAs that were elevated in this study. From that, miR-223 was predicted to target Roquin (Fig. 3*B*). Dysregulation of Roquin expression has been linked to various autoimmune diseases (20), although an association between Roquin and IL-17 expression has not been established. IL-17 production by cIELs is a common feature of IL-$10^{-/-}$ mice (2) (Fig. 3*C*). The ameliorating effects of IL-10 on IL-17 synthesis can be seen by ex vivo treatment of cIELs with IL-10 (Fig. 3*D*, 3*E*). Of interest, Roquin expression was suppressed in cIELs of IL-$10^{-/-}$ mice (Fig. 3*F*) and was

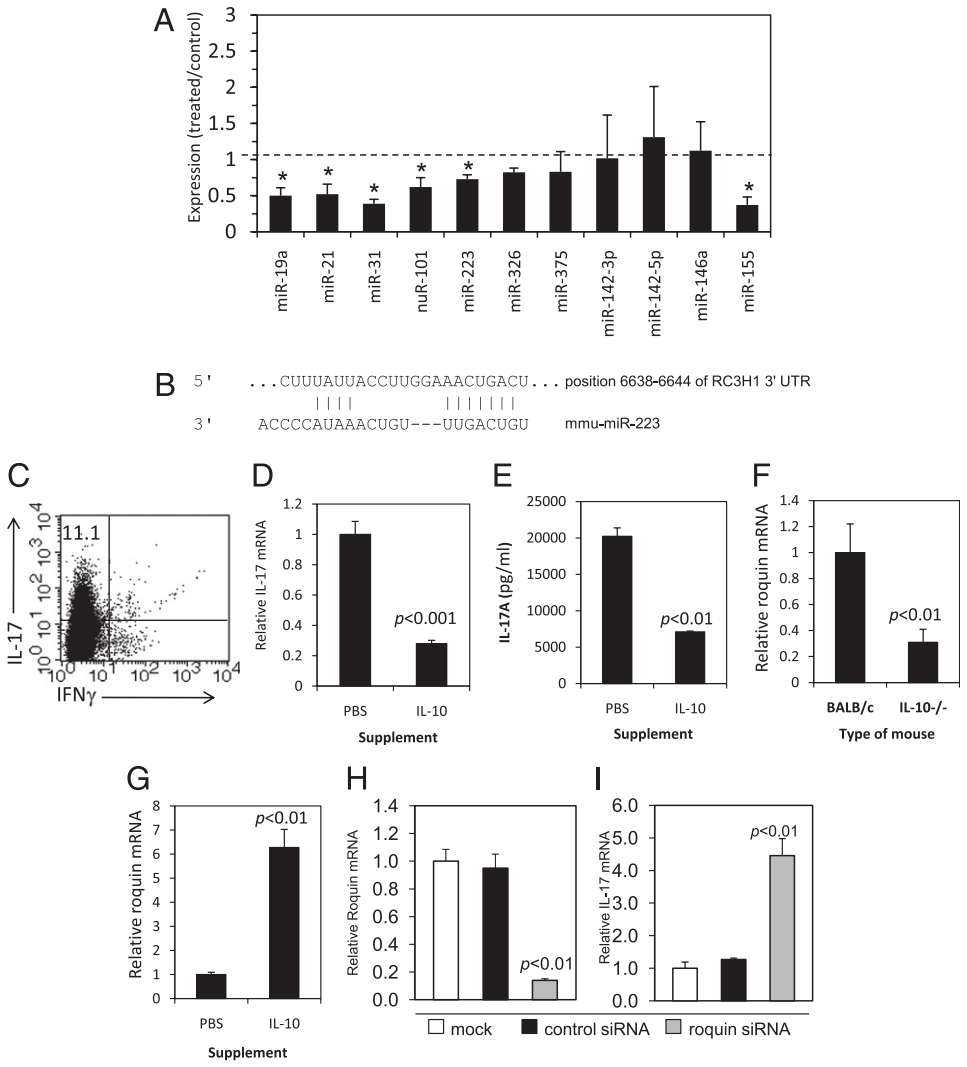

FIGURE 3. *A*, In vitro treatment of cIELs from IL-10$^{-/-}$ mice with severe pathology overnight with 50 ng/ml rIL-10 resulted in the suppression of 6 out of 11 miRNAs relative to cells cultured with PBS (*$p \leq 0.05$). *B*, TargetScan (Release 5.1) analysis of the mouse Roquin (*Rc3h1*) 3′ UTR revealed a potential target site for mmu-miR-223. Alignment of mmu-miR-223 to the conserved site is shown. *C*, Intracellular expression of IL-17 and IFN-γ in IL-10$^{-/-}$ cIELs. In vitro IL-10 treatment of cIELs from IL-10$^{-/-}$ mice resulted in significant reduction in IL-17 mRNA expression (*D*) and IL-17A secretion (*E*) as determined by ELISA. *F*, cIELs from IL-10$^{-/-}$ mice have lower Roquin gene expression. *G*, Culture of cIELs from IL-10$^{-/-}$ mice for 24 h with 50 ng rIL-10 results in an increase in Roquin gene expression. *H* and *I*, Transient transfection of EL4 cells with Roquin-specific siRNA oligonucleotides resulted in suppression of Roquin gene expression and enhanced IL-17 gene expression. Determination of statistical significance was calculated using Student *t* test.

restored following exposure to IL-10 (Fig. 3*G*), thus indicating that IL-10 influences Roquin expression. Further documentation of this was evident in experiments in which EL4 cells transfected with Roquin siRNA had significantly lower levels of Roquin gene expression (Fig. 3*H*) and significantly higher levels of IL-17A gene expression (Fig. 3*I*).

Transfection of cIELs with anti–miR-223 resulted in an increase in Roquin gene expression and a decrease in IL-17A gene expression; miR-223 suppressed Roquin expression and enhanced IL-17A expression (Fig. 4*A*), suggesting a role for miR-223 in Roquin regulation. This was confirmed by luciferase reporter assays, which demonstrated that the 3′ UTR of the Roquin gene was a target for miR-223, as seen by the negative regulatory effects (Fig. 4*B*).

To identify additional miRNAs that may be involved in the regulation of Roquin, transcriptome analysis was done using

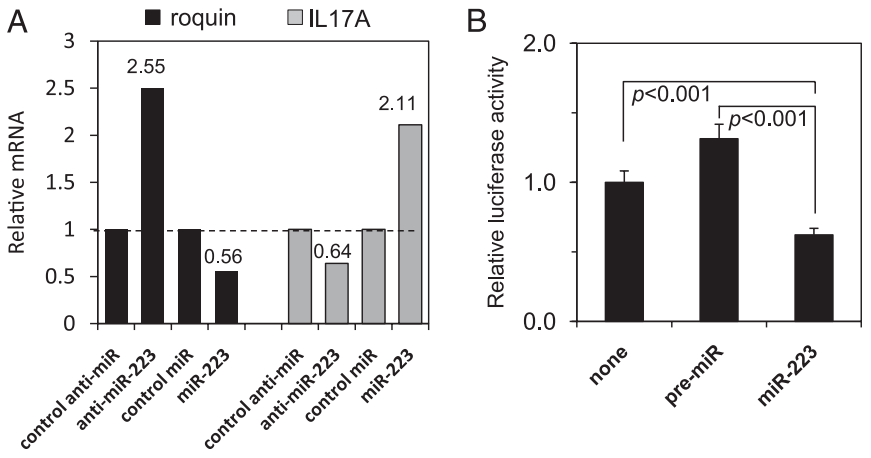

FIGURE 4. *A*, Transient transfection of cIELs with anti–miR-223 resulted in enhanced Roquin expression and suppressed IL-17A expression, whereas transfection with miR-223 suppressed Roquin expression and enhanced IL-17A expression. *B*, Results of luciferase assay using the 3′ UTR of Roquin cloned into the pMIR-REPORT luciferase plasmid and transfected into 239T cells for 48 h with either 30 nM of miR-223 or pre-miR control. miR-223 suppressed luciferase activity relative to nontransfected and pre-miR control levels.

Table I. Negative regulators of Rc3h1

Symbol	Fold Change
miR-191	−2.02
miR-302b	−2.02
miR-105	−2.03
miR-181a	−2.04
miR-29c	−2.04
miR-142-3p	−2.04
miR-20a	−2.05
miR-182	−2.08
miR-223	−2.09
miR-200a	−2.12
miR-194	−2.13
miR-205	−2.15
miR-302c	−2.24
miR-125b	−2.27
miR-99b	−2.37
miR-451	−2.38
miR-135b	−2.39
miR-149	−2.44
miR-155	−2.46
miR-27a	−2.70
miR-183	−2.78
miR-184	−2.78
miR-147	−3.14
miR-31	−3.16
miR-185	−3.82
miR-146a	−3.95

cDNAs from HeLa cells transfected with 90 miRNA mimics (Supplemental Fig. 1). Twenty-six miRNAs were identified as potential negative regulators of Roquin (Table I). This confirmed the involvement of miR-223, as described above, and it identified 25 other functionally important miRNAs, 2 of which (miR-146a and miR-155) are associated with IL-17 regulation (11, 21).

Colonic inflammation in DSS-treated mice is accompanied by selective changes in miRNA expression in the colon but not in PBLs

To determine if the patterns of miRNA expression observed in IL-$10^{-/-}$ mice also occurred in other animal models of colonic inflammation, miRNA expression was examined in PBLs and colonic tissues of DSS-treated mice at early (days 1 and 2) and late (day 7) times of exposure to DSS. The miRNAs studied were the same as those used for IL-$10^{-/-}$ mice (Fig. 2). The severity of mucosal injury and the degree of inflammation increased in a time-dependent manner during days 1–7 of DSS treatment. Untreated mice had no inflammation (Fig. 5*A*). At day 1, the colonic mucosa had surface erosion, intraepithelial edema, and increased infiltration of polymorphonuclear and mononuclear leukocytes within the lamina propria (Fig. 5*B*). By day 2, there was diffuse mucosal ulceration, crypt and submucosal abscess, and diffuse infiltrate of leukocytes extending into the submucosa (Fig. 5*C*). Maximal colitis resulting in ulceration with extensive submucosal and transmural inflammation, abscess formation, and necrosis occurred at day 7 (Fig. 5*D*). Similar to other studies of experimental colitis (22), numerous eosinophils were present among the inflammatory cell infiltrate starting at day 2 of DSS exposure. Pathology scores for all mice per group are shown in Fig. 5*E*.

Using PBL and colonic tissues from DSS-treated animals on days 0, 1, 2, and 7 of DSS treatment, qRT-PCR analysis was done for the same 11 miRNAs studied for IL-$10^{-/-}$ mice. Although some miRNAs were elevated in PBLs early during the treatment period (day 1), these were not statistically significant differences compared with nontreated mice (Fig. 6*A*). However, five miRNAs

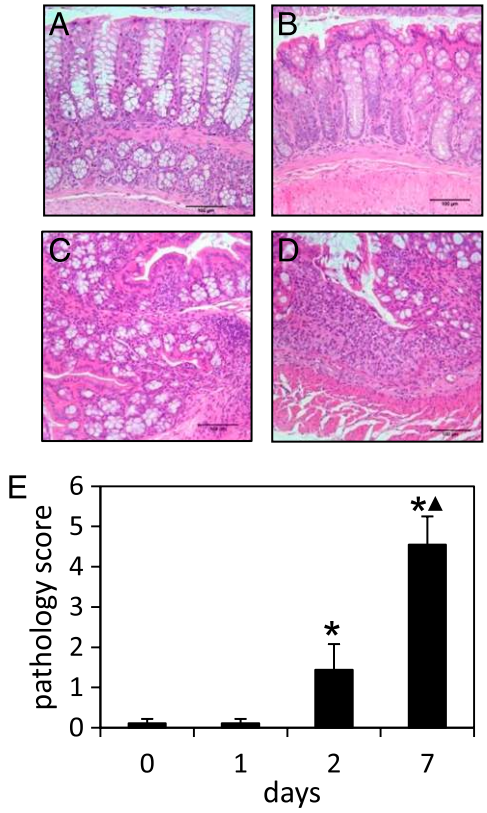

FIGURE 5. Mice were given DSS in the drinking water as described in the *Materials and Methods*. Representative colonic tissues from day 0, pathology score 0 (*A*); day 1, pathology score 1 (*B*); day 2, pathology score 3 (*C*); and day 7, pathology score 6 (*D*). Original magnification ×200 (*A–D*). *E*, Average pathology scores from three mice per group. *Statistically significant difference ($p < 0.01$) compared with days 0 and 1. ▲Statistically significant difference ($p < 0.01$) compared with day 2.

(miR-31, miR-223, miR-142-3p, miR-146a, and miR-155) were significantly elevated in the colon of DSS-treated mice at day 7, a time when inflammation was most severe (Fig. 6*B*). Of these, it was particularly interesting that miR-223 expression was elevated in the colon of both IL-$10^{-/-}$ mice and DSS-treated mice given the relationship of that miRNA to Roquin and IL-17 expression as described above.

Discussion

The findings reported in this study refine our understanding of the molecular pathway by which IL-17 is controlled, and they identify an approach for predicting the development of chronic colonic inflammation based on the temporal appearance of miRNAs in circulating leukocytes. Our data suggest a system in which IL-10 exerts negative regulatory effects on IL-17 and miR-223 expression and positive effects on Roquin expression. A key and early component of this pathway appears to be miR-223, as seen by its potential to target and regulate Roquin expression by binding to the 3′ UTR of the Roquin gene. Thus, in the presence of IL-10, miR-223 would be suppressed and Roquin would be maintained at sufficient levels to hold IL-17 in check. In the absence of adequate levels of IL-10, however, high levels of miR-223 would suppress Roquin and increase IL-17A synthesis. Accordingly, there are multiple situations that could independently or collectively lead to chronic IL-17–driven intestinal pathology, including suppression of IL-10 synthesis, overexpression of miR-223, or suppression of Roquin.

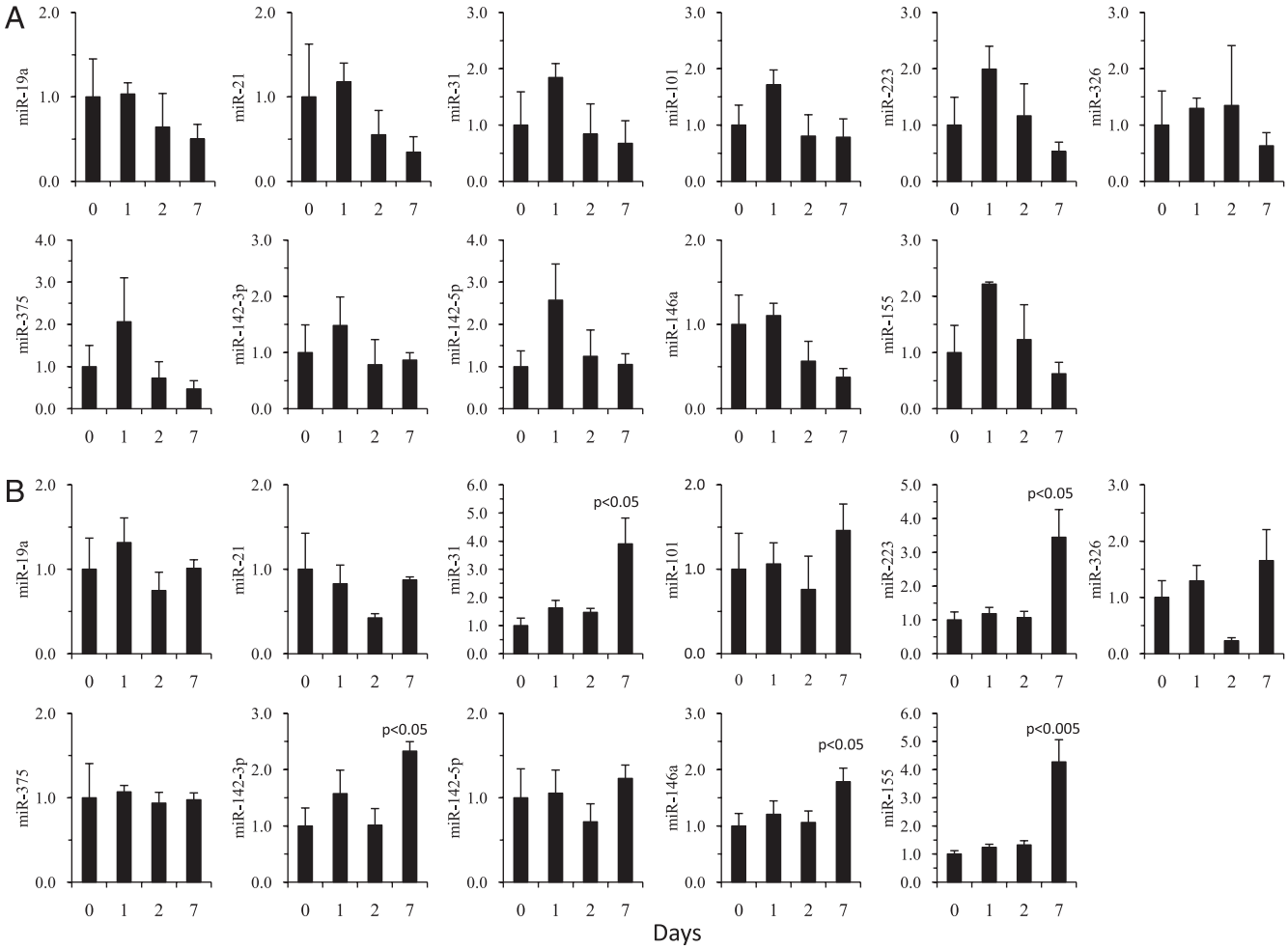

FIGURE 6. qRT-PCR analysis of the same 11 miRNAs studied for IL-10$^{-/-}$ mice using PBL (*A*) and colonic tissues (*B*) of DSS-treated animals on days 0, 1, 2, and 7. Although some miRNAs were elevated in PBLs early during the treatment period (day 1), these were not statistically significant differences. Five miRNAs (miR-31, miR-223, miR-142-3p, miR-146a, and miR-155) were significantly elevated in the colon when inflammation was severe. Expression values were calculated by normalizing to U6 snRNA levels in a sample and recorded as values relative to the expression of day 0 (non-DSS–treated) mice, which was arbitrarily designated as 1.0.

Elevated levels of miR-223 also are significant due to its link to myelopoiesis, erythropoiesis, and lymphopoiesis (23–26). miR-223, which has been shown to be upregulated in patients with CD (17), is involved in progenitor cell and granulocyte differentiation and function (27). Ectopic expression of miR-223 was shown to cause a 30–40% increase in T cells (25) and to reduce LMO2, an essential protein of erythropoiesis (28). However, the complexity of the functional involvement of miR-223 is witnessed by its ability to negatively regulate miR-142 through LMO2 and CEBP-β and attenuate hematopoietic cell proliferation (26). Thus, elevated levels of miR-223 in IL-10$^{-/-}$ mice with severe colonic pathology would promote and sustain the expansion of colonic leukocytes, leading to a chronic inflammatory condition. miR-101 has been linked to the regulation of ICOS expression (29), ICOS^{+} cells being a primary source of IL-17 (2, 30). miR-146a, miR-155, and mir-326 have been linked to Th17 differentiation (16, 18, 21). That multiple genes may be regulated by the same miRNA is not surprising and indeed is predictable given the disproportionately few number of miRNAs available for gene regulation, considering that as much as 30–90% of human genes are believed to be regulated by miRNAs (19).

It is noteworthy that several other miRNAs were elevated in IL-10$^{-/-}$ mice having severe intestinal pathology. Elevated expression levels of miR-19a, miR-21, and miR-31 are associated with cancer, including gastric and colon cancers (31–36). This may occur by a failure to activate tumor suppressor and antiapoptotic responses via programmed cell death protein 4, phosphatase and tensin homolog, and/or tropomyosin 1 (37–40). Elevated levels of miR-19a, miR-21, and miR-31 in IL-10$^{-/-}$ mice may have relevance to the increased incidence of colorectal cancer in patients with IBD (41–43) and in IL-10–deficient mice (44–46).

Although our data reveal a temporal pattern of miRNA regulation in IL-10–deficient mice that was first evident in circulating leukocytes, this was not observed in the DSS model of colonic inflammation. Those differences may be reflective of variations in the pathophysiological basis of inflammation in IL-10$^{-/-}$ versus DSS-treated mice. Development of inflammation in IL-10$^{-/-}$ mice occurs gradually and thus may require the continual seeding of leukocytes from the circulation to the colonic mucosa during the early phase of disease for pathology to be manifest. Colonic pathology induced by DSS treatment occurs rapidly, reaching peak levels within a few days. IL-10$^{-/-}$ mice also differ from DSS-treated mice in that pathology in the former is principally the consequence of cytokine dysregulation, resulting in the synthesis of powerful proinflammatory cytokines, whereas inflammation in DSS-treated mice occurs once the integrity of the intestinal mucosa is disrupted. Therefore, changes in miRNA expression in PBLs may have value for predicting the underlying basis of in-

flammation and may aid in the design of therapeutic protocols in that regard. Finally, it is significant that miR-223, the miRNA that from our study was most definitively associated with IL-17A regulation via Roquin, was dysregulated in the colon of both IL-$10^{-/-}$ and DSS-treated mice, suggesting that miR-223 may serve as a common marker of local intestinal inflammation.

In summary, these findings point to the potential use of monitoring miRNA expression levels in circulating leukocytes as predictive indicators of the development or recurrence of colonic inflammation in IBD.

Disclosures

The authors have no financial conflicts of interest.

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