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miR-24 Is Elevated in Ulcerative Colitis Patients and Regulates Intestinal Epithelial Barrier Function

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Inflammatory bowel disease is characterized by high levels of inflammation and loss of barrier integrity in the colon. The intestinal barrier is a dynamic network of proteins that encircle intestinal epithelial cells. miRNAs regulate protein-coding genes. In this study, miR-24 was found to be elevated in colonic biopsies and blood samples from ulcerative colitis (UC) patients compared with healthy controls. In the colon of UC patients, miR-24 is localized to intestinal epithelial cells, which prompted an investigation of intestinal epithelial barrier function. Two intestinal epithelial cell lines were used to study the effect of miR-24 overexpression on barrier integrity. Overexpression of miR-24 in both cell lines led to diminished transepithelial electrical resistance and increased dextran flux, suggesting an effect on barrier integrity. Overexpression of miR-24 did not induce apoptosis or affect cell proliferation, suggesting that the effect of miR-24 on barrier function was due to an effect on cell—cell junctions. Although the tight junctions in cells overexpressing miR-24 appeared normal, miR-24 overexpression led to a decrease in the tight junction—associated protein cingulin. Loss of cingulin compromised barrier formation; cingulin levels negatively correlated with disease severity in UC patients. Together, these data suggest that miR-24 is a significant regulator of intestinal barrier that may be important in the pathogenesis of UC. *(Am J Pathol 2019, 189: 1763—1774; https://doi.org/10.1016/j.ajpath.2019.05.018)*

Inflammatory bowel disease (IBD) is comprised broadly of two subcategories: Crohn disease (CD) and ulcerative colitis (UC). CD and UC are chronic inflammatory diseases of the gastrointestinal tract, currently affecting >1.6 million Americans.[1] With a complex etiology and limited lasting medical treatments, IBD patients may experience a lifetime of significant symptoms, which include severe diarrhea, bleeding, and vomiting. One pathophysiological feature of IBD patients is a loss of the intestinal barrier, even in areas that contain an intact epithelium.[2,3] The intestinal barrier comprises a single layer of epithelial cells bound together by proteins that traverse the plasma membrane. This selectively permeable barrier compartmentalizes bacteria and other toxins to the lumen while allowing ions, nutrients, and water to be absorbed. Loss of the intestinal barrier leads to the exposure of luminal components to the underlying tissue. A dysfunctional barrier combined with an aberrant immune response are thought to be major risk factors for IBD.[4]

The protein networks enabling intestinal barrier function are known as cell-cell junctions, which include the tight junction and adherens junction. Cell-cell junctions can confer strength or pore-forming abilities to the barrier.[5]

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A major transmembrane protein in the adherens junction is E-cadherin, whereas tight junction transmembrane proteins include the junctional adhesion molecule family and claudin family of proteins.[5] These transmembrane proteins are connected to cytosolic adaptor proteins, which include the zona occludens protein family and cingulin, which, in turn, connect to the actin cytoskeleton of the cell.[6] This connection between cells to the underlying cellular cytoskeleton confers rigidity and strength to the junction.[7] However, junctional complexes are also highly dynamic.[8] To remove damaged proteins, these complexes are continually internalized and recycled back to the membrane or degraded.[9] To replace degraded proteins, junctional proteins are constantly synthesized by intestinal epithelial cells. When any of these aspects of the junctional dynamics are dysregulated, barrier dysfunction is likely to occur.

One of the pathways the intestinal epithelium uses to regulate the levels of tight junction proteins is by using miRNAs.[10] miRNAs are small noncoding RNAs that repress gene expression by binding to complementary sequences on mRNAs. Multiple studies have assayed alterations in miRNAs in IBD patients using unbiased methods.[11] A subset of these miRNAs, including miR-223 and miR-301a, regulate intestinal epithelial barrier function.[12,13] One miRNA consistently shown to be elevated in IBD patients is miR-24, yet the possible role for miR-24 in IBD is unclear. Two studies have demonstrated 2- and 17-fold changes in miR-24 in colonic biopsies from actively inflamed UC patients compared with controls.[14,15] In addition, two studies have observed a twofold increase in blood-associated miR-24 in UC active patients compared with controls.[16,17] In the results presented herein, using semi-quantitative real-time PCR, miR-24 levels were found to be elevated in biopsies and whole blood from UC actively inflamed patients. To better define a possible role for miR-24 in the pathogenesis IBD, it was determined that miR-24 was expressed by intestinal epithelial cells in UC patients. When overexpressed *in vitro*, intestinal barriers in target epithelial cells failed to establish. Although cells overexpressing miR-24 grew normally, the protein levels of the tight junction adaptor protein cingulin were significantly reduced. When cingulin was down-regulated in intestinal epithelial cells, barrier formation was impaired. Therefore, targeting the miR-24–cingulin axis may represent one pathway to strengthen the intestinal barrier and reduce inflammation in UC patients.

Materials and Methods

Clinical Samples

Colonic tissue biopsies and blood used in the miRNA analyses were obtained from the University of California Los Angeles Center for Inflammatory Bowel Diseases and the G Oppenheimer Center for Neurobiology of Stress and Resilience under the following institutional review board–approved protocols: numbers 12-00420, 13-000537, and 11-000199. Colon tissue biopsies from patients who met diagnostic Rome III criteria[18] for IBS and healthy control subjects were obtained during flexible sigmoidoscopy (at 30 cm) after tap water enemas. Colonic tissue used for the mild and severe inflammation, on the basis of pathology report analysis, was obtained from the University of California Los Angeles Center for Inflammatory Bowel Diseases under institutional review board–approved protocol 18-000209. Specimens were flash frozen in liquid nitrogen, and RNA was extracted with TRIzol reagent (Thermo Fisher Scientific, Waltham, MA). A Ficoll gradient (Roche, Basel, Switzerland) was used to isolate peripheral blood mononuclear cells from whole blood, according to the manufacturer's instructions. All University of California Los Angeles samples were obtained after a written informed consent was provided. Human UC patient colonic tissue used for the microarray analysis was obtained from Origene (Rockville, MD). Diagnoses were confirmed by an independent set of pathologists associated with Origene. Microarray Origene samples were analyzed, as previously described.[19] Samples were obtained through institutional review board protocols and with documented patient consent, all from accredited US-based medical institutions. The microarray data are accessible through the Gene Expression Omnibus (*https://www.ncbi.nlm.nih.gov/geo*; accession number GSE77013).

Cell Culture

Caco-2 and T84 cells (ATCC, Manassas, VA) were grown at 37°C with 5% CO_2. The cells were grown in Dulbecco's modified Eagle's medium (Corning, Corning, NY) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO) and 1% penicillin-streptomycin (Corning). To subculture cells, confluent monolayers were washed with phosphate-buffered saline (PBS) and incubated with 0.25% trypsin with 1 mmol/L EDTA (Thermo Fisher Scientific). Suspended cells were centrifuged at 233 × *g* for 5 minutes. Both Caco-2 and T84 cells were split at a 1:5 ratio.

Barrier Assays

Transepithelial electrical resistance (TEER) was used as a measurement of barrier integrity. A total of 100,000 cells were plated on 6.5-mm transwell inserts that had 0.4-μm pores (Thermo Fisher Scientific). A TC20 Automated cell counter was used to determine cell numbers (Bio-Rad Laboratories, Hercules, CA). Each subsequent day, TEER was measured using a dual electrode connected to an epithelial volt/ohm meter (World Precision Instruments, Sarasota, FL). On the last day of the experiment, dextran flux was measured. A solution containing 50 μg fluorescein isothiocyanate–labeled 4-kDa dextran and 50 μg of AlexaFluor 555–labeled 10-kDa dextran (Thermo Fisher Scientific), diluted in Hanks' balanced salt solution, was used

(Corning). The dextran solution (100 μL) was added to the top of the transwell; and every 2 hours, for 6 total hours, 50 μL of the media in the bottom of the transwell was sampled. A Synergy HT plate reader was used to measure fluorescence (BioTek Instruments, Winooski, VT). To obtain absolute amount of dextran flux, a standard curve was used.

Transfections

Cells were transfected during plating, and all transfection reactions were performed in Opti-MEM media (Gibco). Seventy micromolar lipofectamine RNAiMax reagent was used, according to the manufacturer (Invitrogen, Carlsbad, CA). For overexpression experiments, both an miR-24 precursor and an miRNA precursor negative control were used at the concentration of 50 nmol/L (PM10737 and AM17110, respectively; Ambion, Austin, TX). For inhibition experiments, a chemically modified[20] miR-24 antisense oligonucleotide and negative control were used at the concentration of 50 nmol/L (YC10201383-FZA and YC10202119-FZA; Qiagen, Hilden, Germany). For siRNA experiments, both the cingulin siRNA and negative control were used at the concentration of 50 nmol/L (S33237 and 4390843, respectively; Ambion). To produce miR-24 overexpression of 15-fold, 50 pmol/L control precursor and miR-24 precursors were used.

RNA Extraction, cDNA Synthesis, and Real-Time Quantitative PCR

An miRNeasy kit (Qiagen) was used to extract and purify mRNA and miRNA from tissue or cells grown on transwells, according to the manufacturer's instructions. To harvest, transwell cells were first washed twice with PBS and then the membrane was excised. The membrane was then submerged in Qiazol before storing lysed cells at −80°C (Qiagen). All other purification steps were performed according to the manufacturer's instructions. To generate cDNA from mRNA, an iScript cDNA synthesis kit was used (Bio-Rad Laboratories). mRNA (500 ng) was reverse transcribed into cDNA, according to the manufacturer's instructions. A miRCURY LNA RT kit (Qiagen) was used to generate cDNA from 100 ng of miRNA, according to the manufacturer's instructions. A CFX384 real-time PCR system was used to amplify and detect SYBR Green—mediated signal (Bio-Rad Laboratories). To measure miR-24, a miRCURY LNA miRNA PCR assay primer was used (YP00204260; Qiagen). The housekeeping primers for miRNA experiments were U6 small nuclear RNA and 5S rRNA (YP00203907 and YP00203906, respectively; Qiagen). Cingulin (*CGN*), E-cadherin (*CDH1*), and claudin-7 (*CLDN7*) primers were purchased from Integrated DNA Technologies (Coralville, IA). β-Actin (*ACTB*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were used as housekeeping genes for mRNA analysis. The primer sequences are as follows: *CGN*, 5′-GCAACAAGGAGCTCCAGAAC-3′ (forward) and 5′-CCCTGGACATGTTTCAGCTT-3′ (reverse); *CDH1*, 5′-GGATTGCAAATTCCTGCCATTC-3′ (forward) and 5′-AACGTTGTCCCGGGTGTCA-3′ (reverse); *CLDN7*, 5′-GGATGATGAGCTGCAAAATG-3′ (forward) and 5′-CACCAGGGAGACCACCATTA-3′ (reverse); *ACTB*, 5′-CCCAGCACAATGAAGATCAA-3′ (forward) and 5′-ACATCTGCTGGAAGGTGGAC-3′ (reverse); *GAPDH*, 5′-ATGTTCGTCATGGGTGTGAA-3′ (forward) and 5′-GGTGCTAAGCAGTTGGTGGT-3′ (reverse); *PLEKHA7*, 5′-TAAAGACAGCCGAGAAGAAG-3′ (forward) and 5′-TGTCGGCACTGAAGTAGTAG-3′ (reverse); *CLDN2*, 5′-TGGCCTCTCTTGGCCTCCAACTTGT-3′ (forward) and 5′-TTGACCAGGCCTTGGAGAGCTC-3′ (reverse); *CLDN3*, 5′-CATCACGTCGCAGAACATCT-3′ (forward) and 5′-AGCAGCGAGTCGTACACCTT-3′ (reverse); and *KRT8*, 5′-CGAGGATATTGCCAACCGCAG-3′ (forward) and 5′-CCTCAATCTCAGCCTGGAGCC-3′ (reverse). The comparative Ct method was used to calculate fold change relative to the housekeeping genes.[21]

Western Blot Analysis

Confluent monolayers were washed twice with PBS and then the membrane was excised. Membranes were then submerged in 1× Lammeli sample buffer (Bioland Scientific, Paramount, CA) supplemented with 10% 2-mercaptoethanol (Sigma). Samples were left on ice for 30 minutes before slowly passing lysates through a 25-gauge needle. Lysates were then incubated at 95°C for 5 minutes, and then 10 μL of each sample was added to a 4% to 20% SDS—containing polyacrylamide gel (Bio-Rad Laboratories). A Trans-Blot Turbo system (Bio-Rad Laboratories) was used to transfer proteins onto a polyvinylidene difluoride membrane. Membranes were then blocked for 1 hour at room temperature in 5% milk in PBS with 0.01% Tween-20. Membranes were then incubated with primary antibody diluted in 5% bovine serum albumin overnight at 4°C. After five 10-minute washes with PBS with 0.01% Tween-20, the membrane was then incubated in secondary antibody diluted in 5% milk in PBS with 0.01% Tween-20 for 1 hour at room temperature. After five 10-minute washes with PBS with 0.01% Tween-20, protein was visualized using a Clarity enhanced chemiluminescence kit (Bio-Rad Laboratories) and ChemiDoc Touch Imager (Bio-Rad Laboratories). The cingulin (117796) and claudin-2 (53032) antibodies were from Abcam (Cambridge, UK). The claudin-7 (349100) and E-cadherin (clone 4A2C7) antibodies were from Invitrogen. The claudin-3 (SAB4500435) and PLEKHA7 (HPA038610) antibodies were from Sigma-Aldrich (St. Louis, MO). The GAPDH antibody was from Cell Signaling Technologies (Danvers, MA; clone 14C10). Horseradish peroxidase—conjugated secondary antibodies were obtained from Jackson Immunoresearch (West Grove, PA).

Cell Growth and Apoptosis Assays

To measure cell growth, 50,000 cells were plated in 24-well plates in duplicate. Each day after seeding, cells were

trypsinized and a TC20 Automated Cell Counter (Bio-Rad Laboratories) was used to count the cells. To measure cell proliferation, 10,000 cells were plated in 96-well plates and a bromodeoxyuridine assay kit was performed according to the manufacturer (Cell Signaling). To measure apoptosis, a Caspase GLO 3/7 assay (Promega, Madison, WI) was used using the manufacturer's instructions. Briefly, cells grown on transwells were washed twice with PBS, and the membrane was excised and submerged in the buffer provided by the kit. After 15 minutes of incubation with shaking, a BioTek plate reader was used to measure luminescence from 50 μL of lysate. As a positive control, staurosporine (Tocris, Bristol, UK) was added to the bottom of the transwell at a concentration of 2 μmol/L for 8 hours before conducting the assay. Lysis buffer was used to measure background luminescence. As an alternate method to measure apoptosis, a Click-it terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) kit (Invitrogen) was performed on cells at the end point of TEER experiments, according to the manufacturer. DAPI (Invitrogen) was used as the nuclei stain. Staurosporine was used as a positive control for TUNEL assays and was used at the concentration of 2 μmol/L for a total of 3 hours for Caco-2 cells or 16 hours for T84 cells.

Immunofluorescence

Cells grown on transwells were washed twice with PBS and then fixed in 100% cold methanol for 20 minutes at −20°C. After two subsequent washes with PBS, transwells were blocked in 5% bovine serum albumin in PBS for 1 hour at room temperature. The transwell membranes were then excised from the transwell and incubated with mouse anti−zona occludens protein 1 (339100; Invitrogen) overnight at 4°C in a humidified container. Claudin-2, claudin-3, and claudin-7 antibodies were the same as used for Western blot analyses. The following day, after three washes in PBS, the transwell was incubated with a goat anti-mouse fluorescently labeled 488 antibody for 1 hour at room temperature (Invitrogen). After three washes with PBS, nuclei were stained with DAPI for 1 minute (Invitrogen), and Prolong Gold (Invitrogen) was used to mount membranes onto slides. Slides were imaged using an upright microscope (Zeiss, Oberkochen, Germany).

In Situ Hybridization

A double-DIG labeled miRCURY detection probe for miR-24 (YD00617308-BCG; Qiagen) was used, as previously described.[22]

Statistical Analysis

Statistical differences between groups were evaluated using an unpaired two-tailed *t*-test. Graphing was completed using GraphPad Prism version 6 (GraphPad Software, Inc., San Diego, CA). $P < 0.05$ was considered statistically significant.

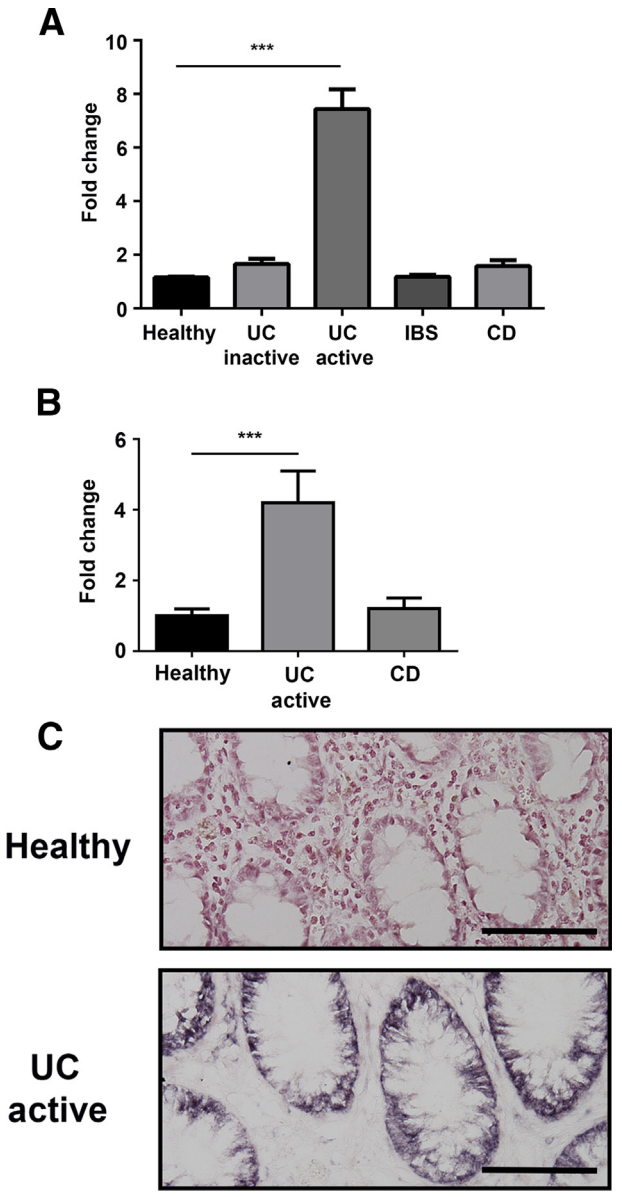

Figure 1 miR-24 is elevated in biopsies and blood from ulcerative colitis patients. **A:** Real-time PCR was used to assess miR-24 levels in colonic biopsies from healthy controls, actively inflamed ulcerative colitis (UC) patients, noninflamed (inactive) UC patients, irritable bowel syndrome (IBS) patients, or Crohn disease (CD) patients. **B:** Real-time quantitative PCR measurements of miR-24 from human blood collected from patients with active UC, CD, or healthy controls. **C:** *In situ* hybridization of miR-24 in colon tissue from an actively inflamed UC patient or healthy control. Data are expressed as means ± SEM. $n = 26$ (**A**, healthy controls); $n = 48$ (**A**, actively inflamed UC patients); $n = 9$ [**A**, noninflamed (inactive) UC patients]; $n = 18$ (**A**, IBS patients); $n = 22$ (**A**, CD patients); $n = 8$ (**B**, patients with active UC); $n = 6$ (**B**, patients with active CD); $n = 7$ (**B**, healthy controls). $^{***}P < 0.001$. Scale bars = 50 μm (**C**).

Results

miR-24 Is Elevated in Biopsies and Blood from Ulcerative Colitis Patients

Multiple studies have seen that miR-24 is elevated in the serum and colon of active inflamed ulcerative colitis

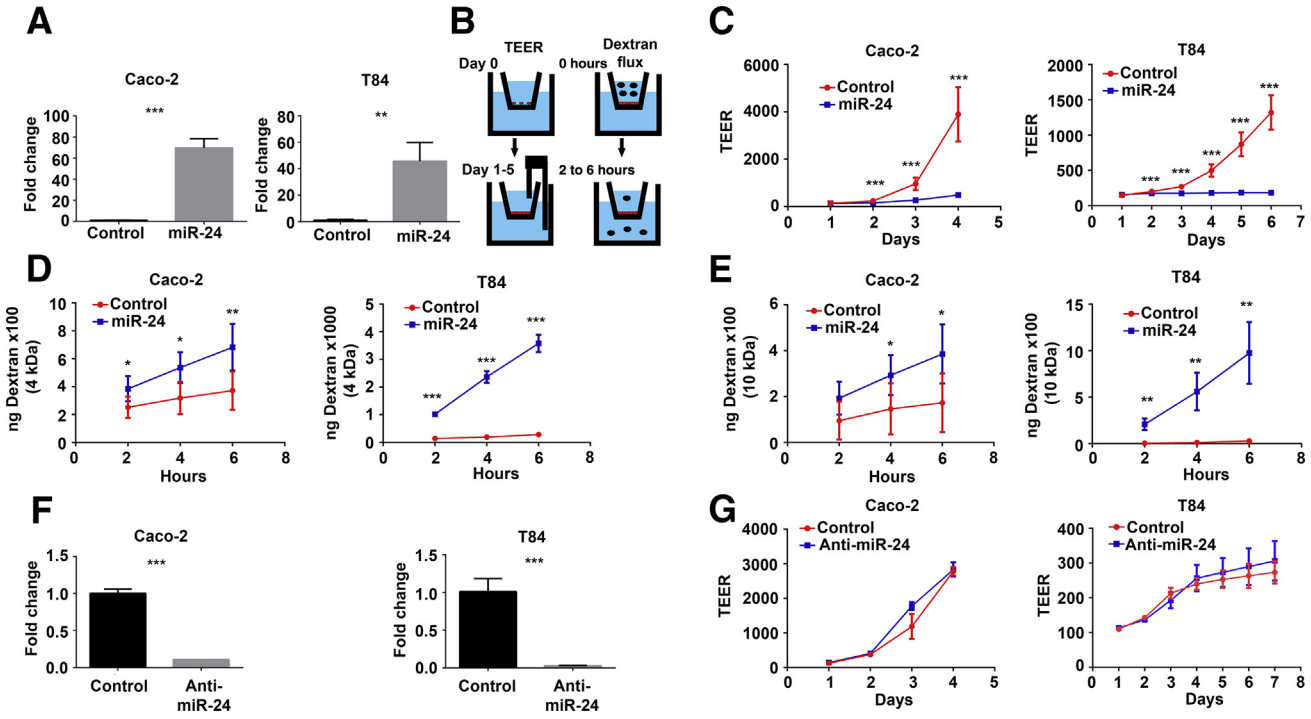

Figure 2 miR-24 overexpression inhibits barrier formation. **A:** Real-time PCR was used to determine miR-24—overexpressing Caco-2 and T84 cells after transfection. **B:** Illustration showing two different methods to measure barrier function *in vitro*. **C:** Measurements of transepithelial electrical resistance (TEER) over time in Caco-2 or T84 cells treated with a control miRNA or miR-24 miRNA. **D** and **E:** A 4-kDa (**D**) or a 10-kDa (**E**) dextran flux in Caco-2 or T84 cells was measured at the end point of the experiment. **F:** At the end point of the experiment, real-time quantitative PCR was used to measure miR-24 expression in control miRNA inhibitor—treated cells or miR-24 inhibitor—treated cells. **G:** TEER was measured in control or miR-24 inhibitor—treated cells during barrier establishment. Each graph is representative of three separate experiments with significant results conducted in experimental triplicate. Data are expressed as means ± SD. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

(UC-active) patients.[14–17] Although many studies have analyzed the effects of miR-24 on oncogenesis, few studies have profiled the function of miR-24 in the context of IBD. To validate these microarrays and RNA-sequencing studies, human colonic biopsies were obtained from patients undergoing colonoscopy procedures and real-time PCR was performed. Healthy controls were compared with UC-active patients, UC-inactive patients, IBS patients, and CD patients. It was observed that miR-24 levels were elevated sevenfold in UC-active patients ($P < 0.0001$) compared with the other groups, who had similar levels of miR-24 (Figure 1A). In UC-active patients, the elevated levels of miR-24 do not appear to be exclusive to colonic tissue as miR-24 was also elevated fourfold in the blood of UC-active patients compared with healthy controls and CD patients (Figure 1B). To determine the subset of cells that express miR-24, *in situ* hybridization was performed on colonic sections from UC patients and on healthy controls. It was observed that miR-24 localized to intestinal epithelial crypts in the colon (Figure 1C). These results indicate that in UC patients, miR-24 is specifically elevated in the intestinal epithelium and this elevation persists in the bloodstream.

miR-24 Overexpression Significantly Disrupts Intestinal Barrier Function

Given the localization of miR-24 to the colonic epithelium, which functions to generate a selectively permeable barrier that lines the gut, the effects of miR-24 on barrier formation were tested. If miR-24 regulates the barrier and miR-24 is altered in IBD, this alteration could contribute to the pathogenesis of IBD. Two barrier-forming human cell lines of intestinal epithelial origin were used, Caco-2 and T84 cells. As miR-24 is elevated in UC-active patients (Figure 1, A and B), an miR-24 mimic was transfected into Caco-2 and T84 cells. A 40-fold increase or greater in miR-24 was observed in both cell lines compared with control mimic—treated cells (Figure 2A). After plating both control or miR-24—overexpressing cells in transwells, two methods to test barrier function were then performed. As tight junctions form a barrier against ion flow, an electrode can be used to measure TEER (Figure 2B). Another method to test barrier function is a dextran flux assay. In this assay, fluorescently labeled dextran is added to the upper chamber of the transwell and the amount of dextran in the lower chamber is measured bihourly (Figure 2B). On every experiment performed, control cells gained TEER at an

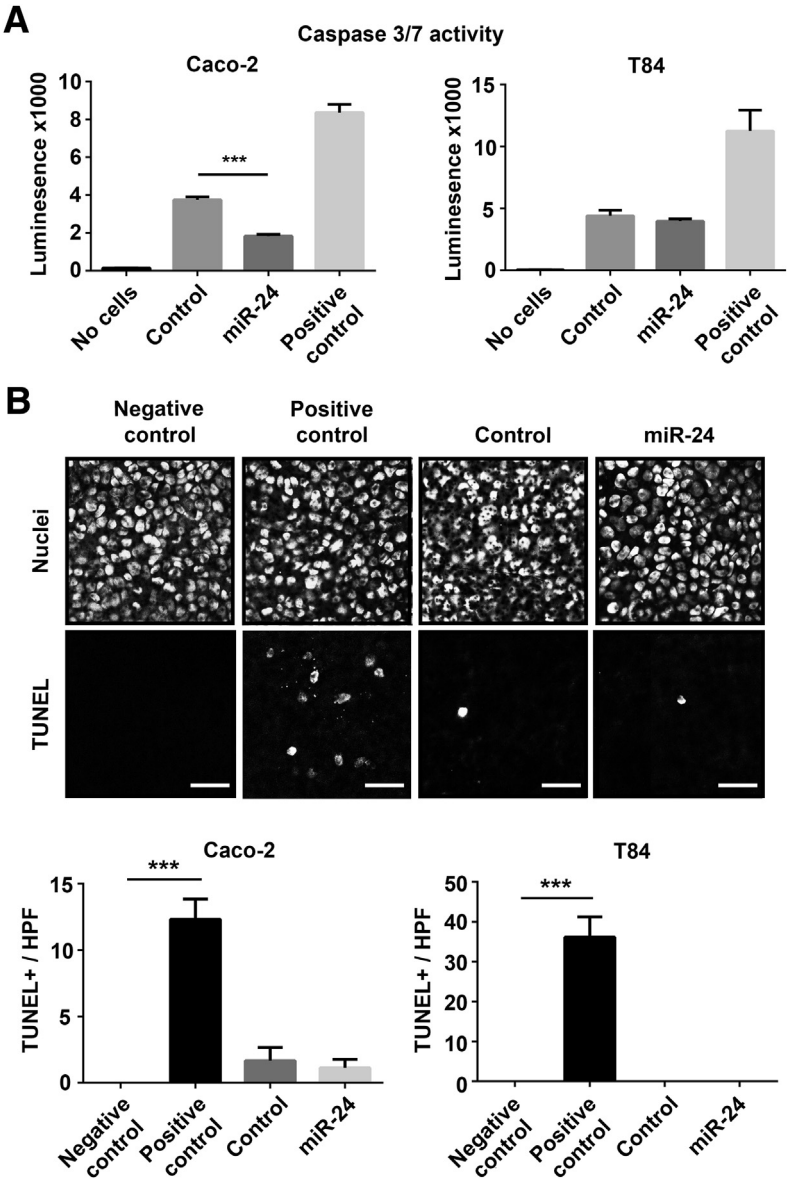

Figure 3 miR-24 overexpression does not increase intestinal epithelial cell apoptosis. **A:** Caspase 3/7 activity, a measurement of apoptosis, on control or miR-24–treated Caco-2 or T84 cells at the end point of the transepithelial electrical resistance experiment. **B:** A terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay was used to determine the presence of cells undergoing apoptosis after control miRNA or miR-24 miRNA treatment. Staurosporine was used as a positive control for apoptosis experiments. Each graph is representative of two (T84) or three (Caco-2) separate experiments conducted in experimental triplicate. Data are expressed as means ± SD. $***P < 0.001$. Scale bar = 50 μm (**B**). HPF, high-power field.

exponential rate. However, cells overexpressing miR-24 had diminished TEER at all time points (Figure 2C). To test if these alterations in barrier function occur at lower levels of overexpression, oligonucleotides were diluted to obtain a 15-fold overexpression of miR-24. Overexpression was still sufficient to impair barrier function (Supplemental Figure S1, A and B). For both T84 and Caco-2 cells, miR-24 overexpression significantly increased the amount of 4- and 10-kDa dextran flux at all time points (Figure 2, D and E). In control Caco-2 cells, some dextran fluxed over time, whereas T84 cells were almost completely impermeable to dextran flux when a high TEER was established (Figure 2, D and E). These data suggest that miR-24 regulates the formation of the intestinal epithelial barrier. To test if miR-24 inhibition could accelerate barrier formation, antisense miR-24 oligonucleotides were added to the cells during barrier formation. Although antisense miR-24 oligonucleotides caused a dramatic decrease in miR-24 in both Caco-2 and T84 cells, no alterations were observed in the establishment of barrier function, as measured by TEER (Figure 2, F and G).

miR-24 Overexpression Does Not Increase Intestinal Epithelial Cell Apoptosis

To understand the potential mechanism for the disruption of barrier function in cells with elevated miR-24, assays for apoptosis were performed. It would be detrimental to the establishment of a barrier if apoptosis is increased. As a first measurement of apoptosis, a caspase 3/7 activity assay was performed on Caco-2 and T84 cells with altered TEERs. Overexpression of miR-24 in Caco-2 cells reduced apoptosis, as demonstrated by decreased caspase 3/7 activity. In T84 cells, no difference in caspase 3/7 activity was

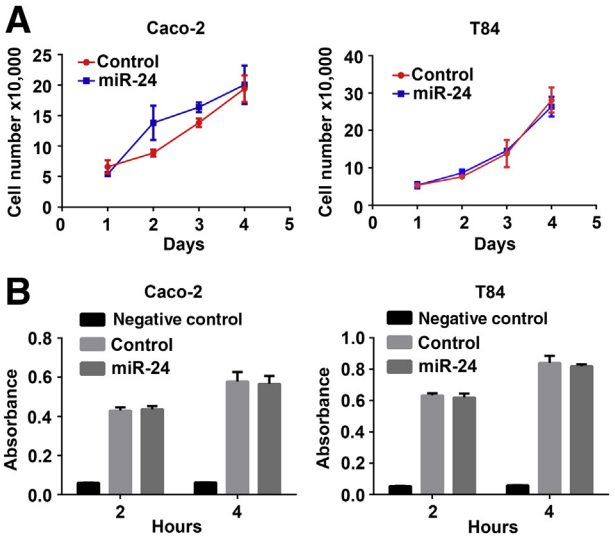

Figure 4 miR-24 overexpression does not reduce intestinal epithelial cell proliferation. **A:** A quantification of Caco-2 or T84 cell numbers over the period of 4 days after transfection with either a control miRNA or an miR-24 miRNA. **B:** To measure cell proliferation, cells were pulsed with bromodeoxyuridine (BrdU) for either 2 or 4 hours before measurement of BrdU incorporation with anti-BrdU antibodies. Cells plated in the absence of BrdU were used as a negative control. Each graph is representative of three separate experiments conducted in experimental triplicate. Data are expressed as means ± SD.

observed between the two groups (Figure 3A). As a positive control, cells were treated with staurosporine, which induces apoptosis in caspase-independent and caspase-dependent mechanisms,[23] as demonstrated by a dramatic increase in caspase 3/7 activity (Figure 3A). As an alternate method to measure apoptosis, a TUNEL assay was performed on transwell inserts from Caco-2 and T84 cells. Both control miRNA− and miR-24 miRNA−treated cells displayed low levels of TUNEL positivity that are indicative of a healthy barrier (Figure 3B). Staurosporine treatment, similar to increased caspase activity, markedly increased TUNEL positivity (Figure 3B).

miR-24 Overexpression Does Not Reduce Intestinal Epithelial Cell Proliferation

If cells overexpressing miR-24 were not able to proliferate, then the establishment of barrier would be hindered. To measure cell proliferation, Caco-2 and T84 intestinal epithelial cells were plated sparsely and counted over the course of 4 days or were pulsed with bromodeoxyuridine. It was observed that miR-24−overexpressing cells had similar growth trajectories as control cells over the period of 4 days (Figure 4A). Both control miRNA and miR24 miRNA cells also had similar levels of bromodeoxyuridine uptake at 2 hours, and these levels increased at 4 hours; however, no differences were seen between the groups (Figure 4B).

miR-24 Overexpression Does Not Alter Junctional Morphology

As apoptosis levels and proliferation rates were similar between control and miR-24 miRNA−treated cells, but barrier function was hindered in miR-24−overexpressing cells, it was next sought to visualize the gross architecture of cell-cell junctions. On the final day of the transwell experiment, cells were fixed and junctions were labeled with fluorescently labeled antibodies. Similar to control cells, Caco-2 cells overexpressing miR-24 had intact tight junctions, as observed by zona occludens protein 1, claudin-3, and claudin-7 localization and levels (Figure 5). In T84 cells, claudin-2 immunofluorescence labeling was similar between control and miR-24−overexpressing cells (Supplemental Figure S2). Thus, the effect of miR-24 negatively regulating the intestinal barrier appears to occur through specific barrier-promoting proteins.

miR-24 Regulates the mRNA and Protein Levels of the Tight Junction−Associated Protein, Cingulin

As miR-24−overexpressing cells grew normally and overall junctional appearance seemed normal, Western blot analyses were performed on a panel of established junctional proteins to determine how miR-24 regulates the intestinal barrier. The transmembrane proteins claudin-2, claudin-3, and claudin-7 were unaltered in both Caco-2 and T84 cells overexpressing miR-24 compared with controls (Figure 6A). Although the levels of the cell-cell adhesion protein E-cadherin were unaltered in Caco-2 cells overexpressing miR-24, in T84 cells overexpressing miR-24, a 30% loss in E-cadherin was observed compared with controls (Figure 6A). However, the tight junction−associated protein and known direct target of miR-24,[24] cingulin, was significantly decreased: 60% to 70% in miR-24−overexpressing Caco-2 cells and 80% to 90% in T84 cells compared with controls (Figure 6A). The protein levels of a binding partner of cingulin, pleckstrin homology domain-containing family A member 7 (PLEKHA7),[25] were not altered in miR-24−overexpressing Caco-2 cells compared with controls (Figure 6A). In T84 cells, PLEKHA7 protein levels were reduced significantly by 20%. In both cell lines, claudin-2, claudin-3, and claudin-7 protein levels were not altered by miR-24 overexpression. To better understand how miR-24 might regulate cingulin protein levels, the mRNA levels in transfected cells were determined by real-time PCR. Similar to the protein levels, cingulin mRNA was significantly decreased in miR-24−overexpressing cells (Figure 6B). The mRNA levels of PLEKHA7, claudin-2, claudin-3, and claudin-7 were not altered by miR-24 overexpression, whereas the levels of E-cadherin mRNA were significantly decreased in Caco-2 and T84 cells (Figure 6B). These results suggest that the effect of miR-24 on the intestinal barrier could be due to effects on cingulin mRNA.

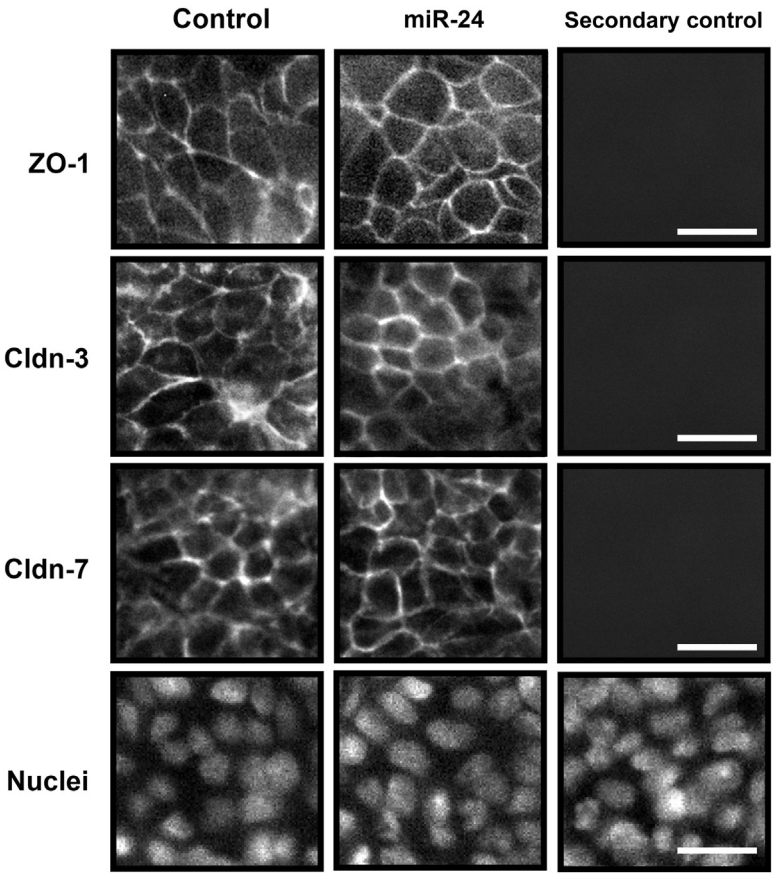

Figure 5 miR-24 overexpression does not alter junctional morphology. Caco-2 cells were fixed at the end point of the experiment, and junctions were labeled with zona occludens protein 1 (ZO-1), claudin-3 (Cldn-3), and claudin-7 (Cldn-7) antibodies. Nuclei were labeled with a DNA dye. To control for nonspecific secondary antibody binding a control was included that only contained the secondary antibody (secondary control). Scale bar = 25 μm.

Cingulin Is Necessary for Barrier Integrity and Is Down-Regulated in Ulcerative Colitis

To test if cingulin is necessary for the establishment of an intestinal epithelial barrier, an siRNA was used to down-regulate cingulin during barrier establishment in both Caco-2 and T84 cells (Figure 7A). Of interest, cingulin delayed the formation of an intestinal barrier as knockdown of cingulin in both cell lines resulted in an attenuated TEER (Figure 7B). Dextran flux was also altered in these cells. Both the 4- and 10-kDa dextran assays revealed increased paracellular permeability that almost doubles over the course of the measurement (Figure 7, C and D). Given the role of cingulin in barrier function and the alteration of barrier function in IBD patients, a compelling question is whether cingulin is altered in IBD patients. Microarray analysis of human colonic biopsies from patients with active UC and healthy controls revealed a significant 3.2-fold decrease in cingulin levels in UC, even when normalized to an epithelial cell marker, cytokeratin 8 ($P = 0.05$) (Figure 7E). However, the microarray analysis did not reveal a difference in cingulin levels in CD compared with controls (data not shown). Furthermore, cingulin expression was decreased in severely inflamed UC patient colonic biopsies compared with mildly inflamed UC patient colonic biopsies (Figure 7F). Therefore, miR-24–mediated regulation of cingulin is likely important for intestinal epithelial barrier function, and this relationship is correlated with disease severity in UC patients.

Discussion

The intestinal epithelial barrier is vital for normal gut homeostasis and the prevention of inflammation. IBD patients exhibit a faulty epithelial barrier.[3] The establishment and maintenance of the epithelial barrier is regulated in part by miRNAs.[26] Therefore, aberrant expression of miRNAs may contribute to the pathogenesis of IBD. Multiple studies, using unbiased methods, have identified miR-24 as being elevated in the sera and colonic biopsies from IBD.[14–17] In this study, the gold standard for measuring miRNA expression, real-time quantitative PCR, was used to not only validate these large-scale studies but extend an understanding of colonic miR-24 expression in noninflamed UC, IBD, and CD patients. miR-24 was specifically elevated in colonic biopsies from actively inflamed UC patients and not noninflamed UC, CD, or IBD patients compared with healthy controls. As miR-24 localized to the colonic

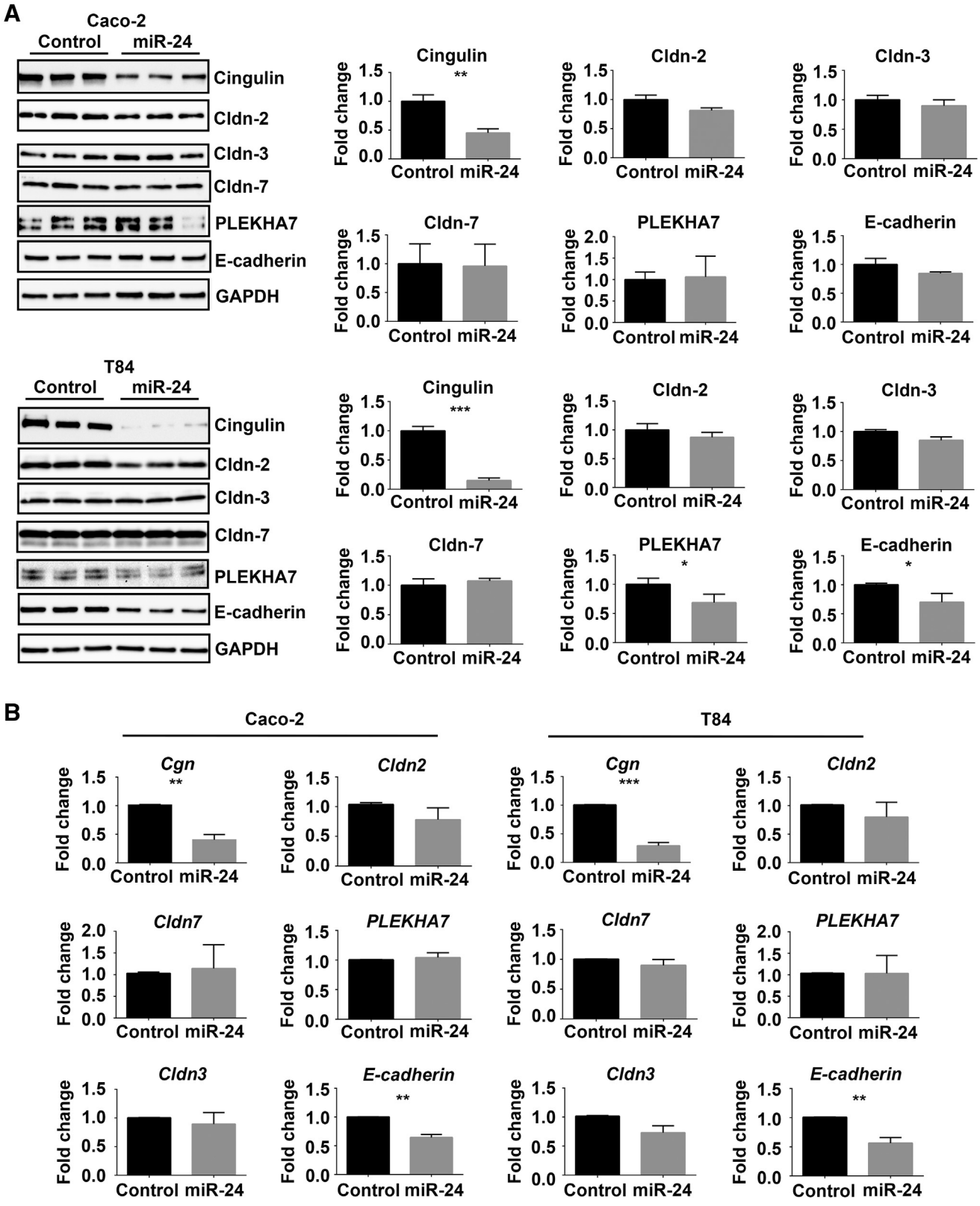

Figure 6 miR-24 regulates the mRNA and protein levels of the tight junction—associated protein cingulin. **A:** After treatment of Caco-2 cells with either a control miRNA or an miR-24 miRNA, Western blot analyses were performed against cingulin, claudin-2 (Cldn-2), claudin-3 (Cldn-3), claudin-7 (Cldn-7), PLEKHA7, and E-cadherin. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the loading control. Each graph is representative of three separate experiments conducted in experimental triplicate. **B:** Real-time PCR was used to quantify *Cgn*, *Cldn2*, *Cldn3*, *Cldn7*, *PLEKHA7*, and *E-cadherin* mRNA levels in control miRNA— or miR-24 miRNA—treated Caco-2 cells. Three independent experiments were performed (**A**). Data are expressed as means ± SD (**A**); data are expressed as means ± SEM (**B**). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

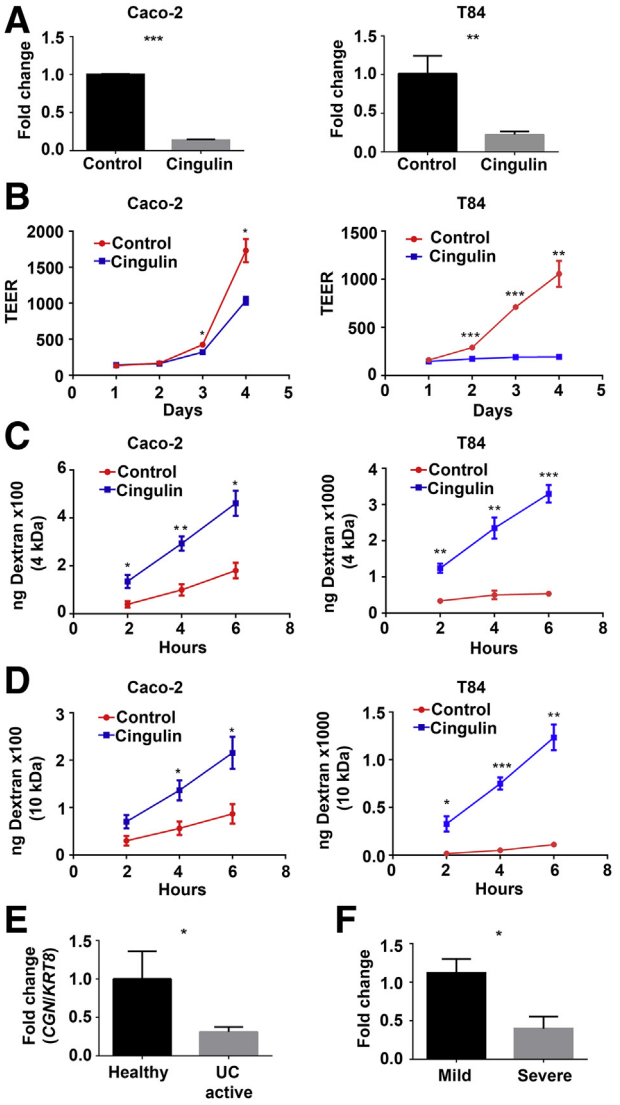

Figure 7 Cingulin is decreased in ulcerative colitis (UC) patients, and down-regulation of cingulin impairs barrier formation. **A:** Real-time quantitative PCR was used to measure cingulin expression in Caco-2 and T84 cells in control or cingulin siRNA–treated cells. Results are representative of three independent experiments. **B–D:** Transepithelial electrical resistance (TEER; **B**) or dextran flux (**C** and **D**) was measured over time in cingulin siRNA–treated Caco-2 and T84 cells. Results are representative of three independent experiments. **E:** Cingulin mRNA levels in healthy controls or UC-active patients, as determined by microarray analysis. To adjust for differences in epithelium, cingulin levels were normalized to cytokeratin 8 (Krt8). **F:** Real-time PCR measurement of cingulin in either mildly inflamed or severely inflamed UC patients. Data are expressed as means ± SEM (**A–D**). $n = 7$ (**E**, healthy controls, and **F**, mildly inflamed UC patients); $n = 8$ (**E**, UC-active patients); $n = 4$ (**F**, severely inflamed UC patients). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

epithelium, the alteration of miR-24 levels in UC appears to be specific to intestinal epithelial cells. The cause for up-regulation of miR-24 in the colon remains unknown as intestinal epithelial cells treated with cytokines or bacterial antigens failed to show an up-regulation in miR-24 levels (data not shown).

Although there is a multitude of evidence for miR-24 alterations in UC patients, information was lacking about the function for miR-24 in intestinal epithelial cells. As a major function of intestinal epithelial cells is to establish a barrier, the role for miR-24 in barrier function was tested and miR-24 was found to have a significant detrimental effect. The intestinal epithelial barrier is a combination of cell growth, death, and junctional dynamics. This study observed that miR-24 overexpression did not alter cell growth, death, or overall junctional architecture. To understand how miR-24 might regulate barrier function even with seemingly normal tight junction architecture, the levels or localization of individual junctional proteins that have been previously shown to be directly or indirectly regulated by miR-24 was assessed.[24,27] Although overexpression did not affect zona occludens protein 1 localization or E-cadherin protein levels, miR-24 overexpression in intestinal epithelial cells resulted in a striking loss of cingulin protein expression. miR-24 regulates cingulin through a single binding site on the 3′ untranslated region of cingulin.[24] Cingulin is a globular protein located in the cytoplasm that can interact with many components of the adherens and tight junctions.[28] A previous study tested if cingulin participated in barrier function; however, those experiments were performed on renal epithelial cells.[29] Therefore, it was tested if cingulin regulates intestinal barrier function, and it was determined that cingulin participates in the formation of a tight barrier.

Although miR-24 inhibits apoptosis, it remains unclear how it regulates apoptosis in intestinal epithelial cells. It has previously been reported in heart, liver, and bladder epithelial cells that miR-24 overexpression also reduces apoptosis.[30–32] The mechanisms for miR-24 regulating apoptosis have been proposed to be direct regulation of Bim and caspase 3/8. Further experimentation testing these known targets of miR-24 in intestinal epithelial cells also represents important questions to be addressed in future studies. As the lack of increased apoptosis was evident when miR-24 was altered, it was clear that miR-24 regulated cell-cell junctions. Previous evidence indicates that TEER measures a pore pathway, whereas dextran flux measures a leak pathway[33]; and it has been shown that there can be alterations to the pore pathway without alterations to the leak pathway.[34] In this study, it was observed that miR-24 and cingulin both regulated TEER formation and dextran flux, suggesting that miR-24 and cingulin are important players in intestinal barrier function. Although these observations are novel for miR-24, cingulin has been linked to both the pore and leak pathways. The pore pathway is established by the binding of the claudin family of proteins between cells and depends on the composition of tight and leaky claudins.[8] The leak pathway occurs when the peripheral actomyosin ring contracts, and this contraction is in part regulated by the small GTPase RhoA. Cingulin has been shown to negatively regulate RhoA and a leaky claudin, claudin-2.[29,35] However, it was observed that increased claudin-2 occurred in the kidney/duodenum and not the ileum or colon of cingulin knockout mice.[36] In this study,

although miR-24 overexpression resulted in a drastic loss of cingulin, there were no alterations in claudin-2 mRNA or protein levels. Further studies on downstream targets for miR-24 and cingulin should include an in-depth characterization of the localization and levels of claudins expressed as well as the role for actomyosin contraction in intestinal epithelial cells.

The elevation of miR-24 levels in both the colon and blood could be a mechanism to open cell-cell junctions, allowing for transmigration of immune cells to sites of inflammation or infection. The increased levels of miR-24 in UC patients appear to be secondary to inflammation as UC patients in remission do not display this increase and genetic alterations in miR-24 have not been observed in UC patients.[37] Regardless of the cause for miR-24 up-regulation, the data presented in this article suggest that reducing miR-24 levels in actively inflamed UC patients could strengthen the intestinal barrier. This strategy could be used to prevent the vicious cycle of immune cell hyperactivation and further barrier breakdown.

Through this work, strong evidence has been provided that a specific miRNA, miR-24, is related to a specific issue in IBD, intestinal barrier dysfunction. miRNAs have been widely studied in IBD, with implications for biomarkers, pathogenesis, and therapeutics. In this study, a role for miR-24 in barrier integrity was established, which lays the foundation for a variety of future studies investigating miR-24 not only in IBD, but also in other barrier-associated diseases.[38] Mechanistically, it was established that in colonic epithelial cells, cingulin is a target for miR-24 and this interaction could be involved in the pathogenesis of UC. Future directions will involve various animal models of colitis and barrier dysfunction, using an miR-24 inhibitor or miR-24 knockout mice to attempt to improve disease outcome.

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Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.ajpath.2019.05.018*.

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