Supplementary Methods

Generation of miR-29ab Knockout Mice

Homozygous floxed miR29ab1 mice (C57BL6 strain) were generated using the methods described here. For the targeting construct, 2 homologous recombination arms were amplified by polymerase chain reaction (PCR), on 129 SvJ/X1 genomic DNA, a 5′ one of 4171 bp and a 3′ one of 3857 bp (Supplementary Figure 1*A*). Also, the genomic fragment to be deleted, of 600 bp, containing the miR29a and miR29b1 regions, was amplified the same way and cloned in between 2 loxP sites, in a pFlox vector. The recombination arms together with the floxed genes were all cloned into Gateway vectors and then assembled together into a destination vector that represented the targeting vector. 129SvJ/X1 embryonic stem cells were electroporated with the targeting vector and clones were screened by Southern blot. DNA was digested with SacI, and labeled with a 3′ probe. Recombinant clones exhibited 2 bands: a WT one of 5.8 kb and a mutant one of 7.4 kb. One positive clone was identified out of 336 screened. This one was expanded and the targeting event was confirmed by Southern blot. The mutant embryonic stem cell clone was injected into C57BL/6 blastocysts and agouti pups were screened by PCR to verify the generation of homozygous floxed miR-29ab1 mice. EIIacre mice (C57BL/6 strain) were obtained from Jackson Laboratories (Bar Harbor, ME). Homozygous floxed miR-29ab1 mice were bred to EIIacre mice to induce general deletion of the cluster. The mice with the deletion were identified and interbred to obtain the germ line deletion. Mice were kept as heterozygous couples. Genotyping was performed by PCR in DNA extracted from the tail to identify the genotype of the first generation of recombinant mice with floxed miR-29a/b (Supplementary Figure 1*B*).

2,4,6-Trinitrobenzenesulfonic Acid Animal Model

Intracolonic TNBS in 50% ethanol was used to produce colitis in male miR-29a/b$^{-/-}$ and WT mice (10–13 weeks).[11] There were no changes in body weight, body condition, physical appearance, and behavior during the 12 weeks after colonic TNBS infusion.

Water Avoidance Stress Animal Model

The WA stress model was also used to induce visceral hyperalgesia.[12] A Plexiglas tank measuring 25 cm in width × 25 cm in height × 45 cm in length was used. A glass platform 10 cm × 8 cm × 8 cm was placed in the tank of water at 25°C such that the water level was 1 cm below the top of the platform. The mice were placed on the platform for 1 hour at the same time each day for 10 consecutive days. Control mice were placed on the same platform without water for the same time intervals.

Visceral Hypersensitivity Testing

Electrodes (Teflon-coated stainless steel wire, 5- to 10-mm tip separation; Cooner Wire Sales, Chatsworth, CA) were sewn into the external oblique abdominal muscles in order to perform the electromyographic recordings to measure the visceromotor response after colorectal distension.[13] Electromyographic activity was quantified 10 seconds before colonic distension; 20 seconds during colonic distension; and 10 seconds after colonic distension.

Ex Vivo Intestinal Permeability Testing

Approximately 2–3 cm of the distal colon was removed for in vitro permeability studies. The distal colon (2–3 cm) was mounted in an Ussing chamber exposing 0.725 cm^2 of tissue. Tissues were bathed on serosal and mucosal sides by Krebs buffer containing (in mmol/L) 121 NaCl, 5.95 KCl, 1.20 $MgCl_2$, 1.35 NaH_2PO_4, 14.3 $NaHCO_3$, 12.7 glucose, and 11.58 $CaCl_2$. Solutions were oxygenated (95% O_2 to 5% CO_2) and circulated in a volume of 10 mL in water reservoirs maintained at 37°C. Then, short-circuit currents (I_{sc}) were measured every 15 minutes for 30 minutes (EVC4000; World Precision Instruments, Sarasota, FL). I_{sc} values were measured using Krebs-agar bridges connected to silver chloride electrodes. Transepithelial electrical resistance (TER; Ω cm^2) was calculated from the spontaneous or clamped I_{sc} using Ohms' law as a measure of intestinal permeability.[10,13]

In Vivo Intestinal Permeability Testing

Urine was collected after an overnight fast at 2-hour intervals for a total of 24 hours and was analyzed for lactulose and mannitol, as described previously, to determine small intestinal and colonic permeability, respectively.[3,7,10] A lactulose/mannitol excretion ratio $\geq$0.07 indicated increased paracellular intestinal permeability.

In Vitro Epithelial Permeability Testing

FHC, FHs74Int, and mouse colonic epithelial cell lines were used to perform in vitro permeability studies in order to further clarify the mechanisms by which enhanced expression of miR-29a/b increases intestinal permeability.[10] CLDN1 and NKRF were silenced to determine if these factors modulated the permeability of monolayers of our intestinal cell lines. FHC, FHs74Int, and mouse colonic epithelial cells were transfected with 200 nmol/L CLDN1 small interfering (si)RNA, NKRF siRNA, control siRNA, or a negative control.

In vitro epithelial permeability was assessed after 48 hours and repeated 4 times using an In Vitro Permeability Assay Kit (Millipore, Billerica, MA). FHC, FHs74Int, and mouse colonic epithelial cell lines were seeded onto collagen-coated inserts. An epithelial monolayer formed in 7 days, which occludes the membrane pores. The cell monolayer was then transfected with miR-29a, miR-29b, or control precursors for 72 hours. After treatment, fluorescein isothiocyanate-dextran was added on top of the cells, allowing it to permeate through the cell monolayer. The extent of permeability was determined by measuring the fluorescence of the plate well solution using SpectraMax M2e Microplate Reader (Molecular Devices, Sunnyvale, CA).

Functional Analysis of Predicted Messenger RNA Targets of miR-29 Family Members

Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Inc., Redwood City, CA) was done to identify, based on published literature, the molecular pathways and functional groupings to which predicted targets of 3 miR-29s belonged. The targets were uploaded into IPA software and overlaid onto a global molecular network developed from information contained in the application. Networks of these genes were generated by IPA based on their connectivity, each ranked by a score based on the hypergeometric distribution, calculated with the right-tailed Fisher's exact test that corresponds to the negative log of the P value. Networks with a score $\geq$20 were selected for further analysis. Functional analysis in IPA identified the published biologic functions that were most significantly associated with the genes in the network. Genes or gene products are represented as nodes, and the biologic relationship between 2 nodes is represented as an edge (line). All lines are supported by at least one reference in the literature, in a textbook, or from canonical information stored in the Ingenuity Pathways knowledge database.

The GOStat application (http://gostat.wehi.edu.au/cgibin/goStat.pl) was used to determine the biologic processes annotated to the putative miRNAs targets in the GO database (http://www. geneontology.org). The GOA human database was used, only considering biologic processes, GO hierarchy, and GO paths with length $\geq$3. As a cut-off for significance, Fisher's exact test ($P \leq 0.01$) with Benjamini and Hochberg false discovery rate assessment were used to estimate the proportion of GO terms that would be identified by chance alone.

Quantitative Real-Time Polymerase Chain Reaction

Reverse transcription was done using 5 μg total RNA, and TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The primers and probe for real-time PCR were designed using Primer Express software and synthesized at Applied Biosystems (Foster City, CA).

Immunoblotting

Protein extraction was done from the same small intestinal and colon tissues obtained to detect CLDN1 and NKRF. Each sample was homogenized in 0.6 mL PROPRE solution and then centrifuged and the supernatant was transferred to a fresh 1.5-mL tube. Equivalent amounts of protein were resolved and mixed with 4$\times$ sodium dodecyl sulfate polyacrylamide gel electrophoresis sample buffer, after the transfer and specific primary antibody processing, the proteins were detected by the LI-COR Odyssey Infrared Imaging System (LI-COR Bioscience, Lincoln, NE).

Transfections

Pre-miRNA precursors and specific inhibitors of miR-29a, miR-29b, and negative controls were purchased from Ambion (Austin, TX). The FHC and FHs74Int cell were transected using Lipofectamine 2000 system. A 100-nM microRNA concentration was also used.[10]

Luciferase Assay

The genomic region within the 3′-UTR site of CLDN1 or NKRF was amplified using the specific designed primers (for CLDN1: 5′ ATGCCTCGAGCATGTGGTTCAGTGCCT 3′ [forward] and 5′ATGCGCGGCCGCACAACATCACTGT 3′ [reverse]; and for NKRF: 5′ ATGCCTCGAGGCAGAGAGACTCACAGC 3′ [forward] and 5′ ATGCGCGGCCGCTT CTGATCAACT 3′ [reverse]) and the Platinum Taq DNA Polymerase High Fidelity method (Invitrogen, Carlsbad, CA). The resulting fragments containing intact putative miR-29a and miR-29b recognition sequences from the 3′-UTR of CLDN1 or NKRF, or with random mutations, were cloned in the psiCHECK-2 vector (Promega, Madison, WI), which contains both Renilla and firefly reporter genes. Both FHC and FHs74Int cells were cotransfected using Lipofectamine 2000 (Invitrogen) with either the CLDN1 or NKRF WT or mutant vector. Luciferase assays were done 48 hours post transfection following the manufacturer's protocol (Dual-Luciferase Reporter Assay System; Promega). Renilla luciferase activity was normalized to firefly luciferase activity for each sample.

Confocal Fluorescence Microscopy

FHCs (human colonic epithelial cells) and FHs 74 Ints (small intestinal epithelial cells) were plated onto fibronectin-coated chamber slides (BD Biosciences, San Jose, CA) and transfected with miRNA precursors. Cells were fixed and probed with anti-CLDN1 and anti-NKRF antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). The primary antibody was detected using Alexa Fluor dyes conjugated antibodies (Invitrogen), while the nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen). Cells were observed on a Zeiss LSM 510 META laser scanning confocal microscope (Zeiss, Thornwood, NY). Images were analyzed with LSM Image Browser software (version 4.2, Zeiss).

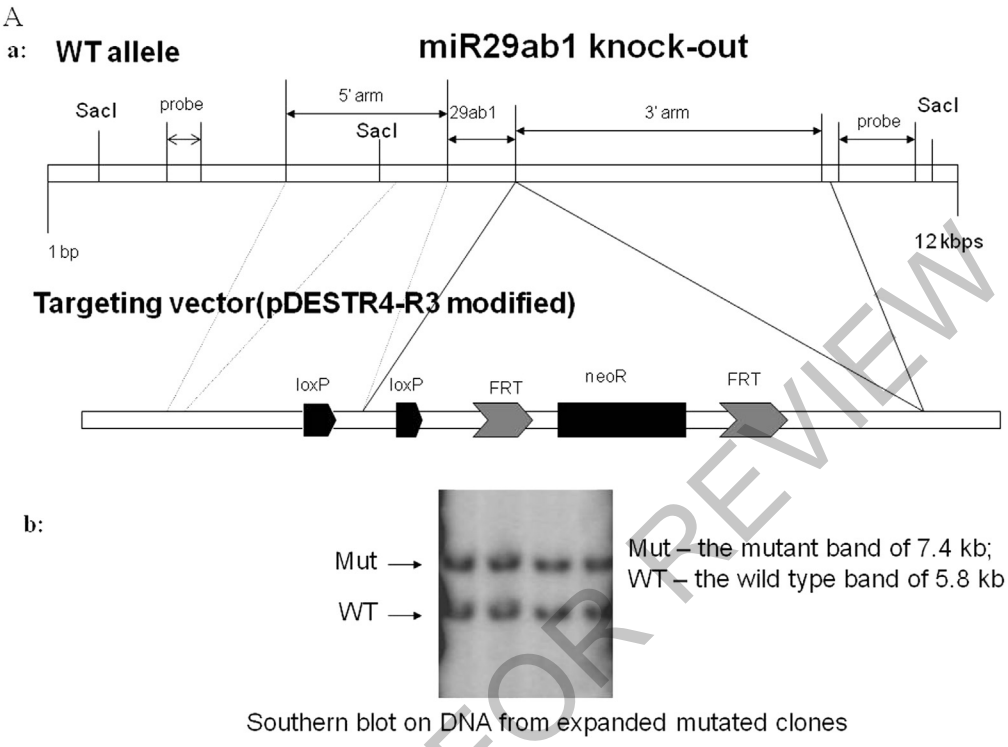
Fluorescence In Situ RNA hybridization

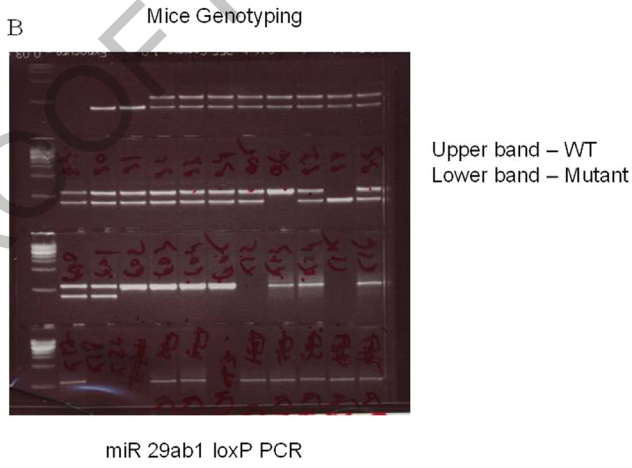
A locked nucleic acid probe with complementarity to miR-29a was labeled with 5′ and 3′-digoxigenin and synthesized by Exiqon (Woburn, MA). A scrambled locked nucleic acid probe was used as negative control. The slides were prehybridized for 30 minutes at 52°C and then 10 pmol of the probe in 60–100 mL of hybridization mixture was added to each slide and incubated for 1 hour at 52°C. Slides were incubated with 3% (vol/vol) H_2O_2 for 10 minutes at room temperature to block endogenous peroxidases before applying horseradish peroxidase–conjugated antibodies; after washing, slides were incubated in blocking buffer for 30 minutes at room temperature, then 100–150 mL antibody was added and incubated for 30 minutes at room temperature. The slides were washed again at room temperature on the shaker; TSA Plus Fluorescein System (PerkinElmer, Waltham, MA) was used for direct fluorescence detection according to manufactory's protocol; finally 25 μL Prolong Gold containing DAPI was added to

each section and the whole slide was covered with glass coverslip. The slides were imaged using an epifluorescence microscope equipped with charge-coupled device camera and image analysis.

Northern blots

Samples containing 20–40 μg total RNA (in a volume $\leq$10 μL) were mixed with Bartel Lab 8 M Urea Loading Buffer. The membrane was prehybridized in hybridization buffer at 55°C for 5 hours with rotation. A total of 1–20 nM biotin-labeled miRNA probe was added to hybridization buffer, incubated at 55°C overnight with rotation. The streptavidin-IR Dye 680 was diluted at 1:10,000 in blocking buffer; the samples were then incubated for 30 minutes at room temperature with gentle shaking, and scanned on LI-COR Odyssey infrared imaging system.





Supplementary Figure 1. (*A*) miR-29a/b knockout mouse (C57BL/6) that was developed by serial breeding of miR-29a/b1loxP/loxP and albumin-Cre þ/þ (Alb-Cre þ/þ) mice. (*B*) Recombinant clones exhibited 2 bands: a WT one of 5.8 kb and a mutant one of 7.4 kb. Genotyping performed by PCR in DNA extracted from the tail to identify the genotype of the first generation of recombinant mice with floxed miR-29a/b.

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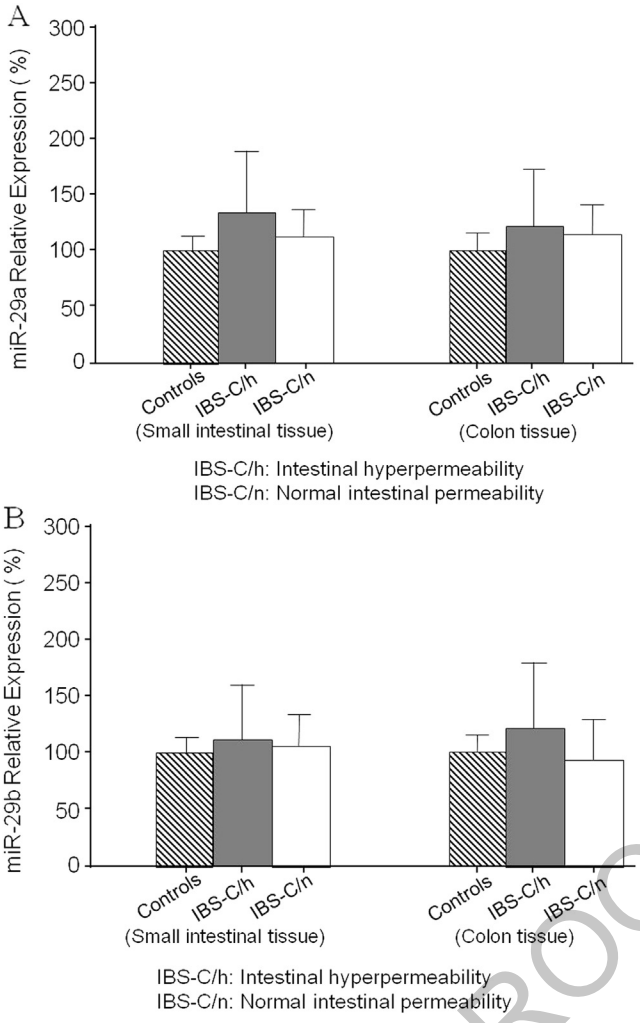
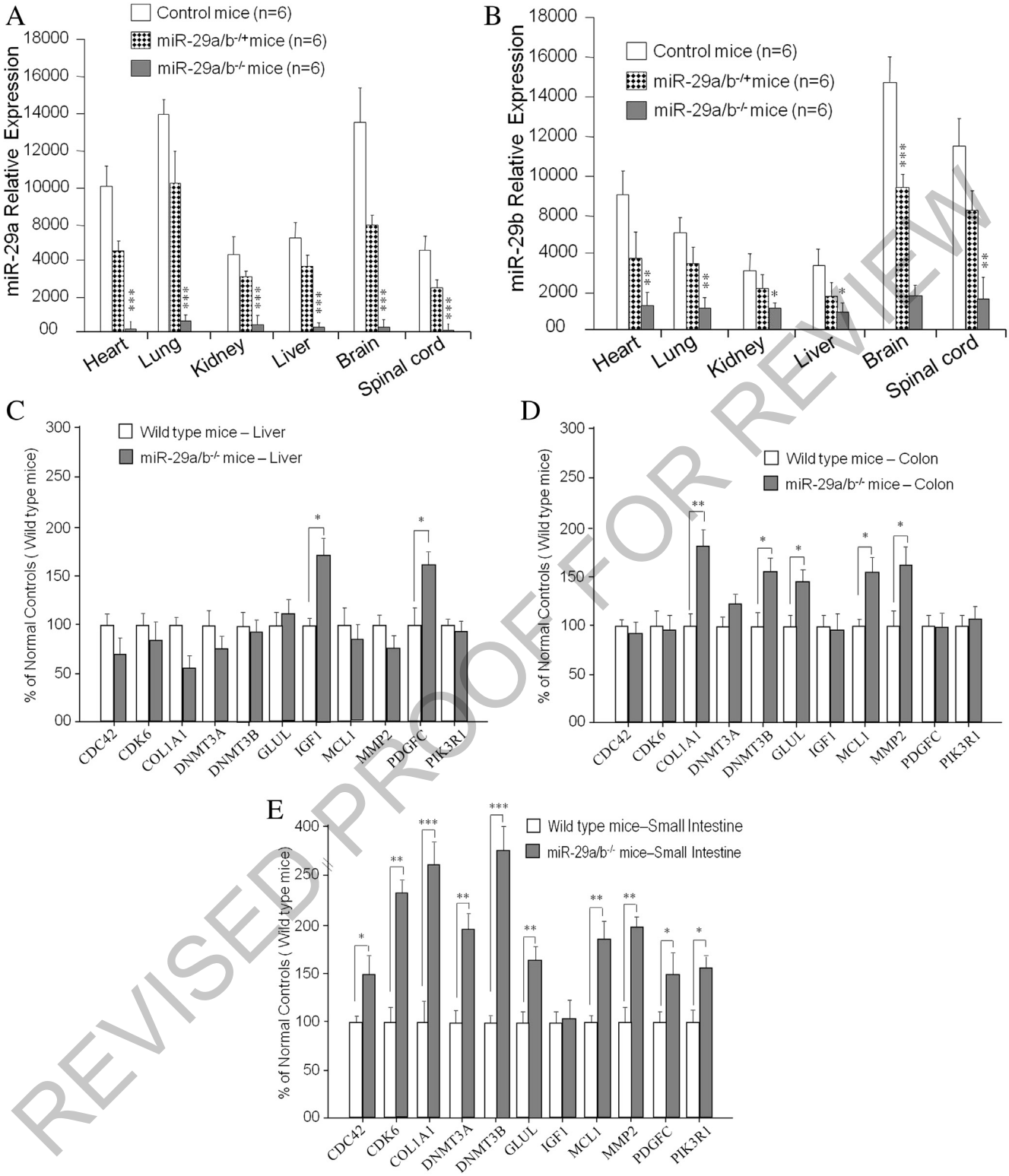
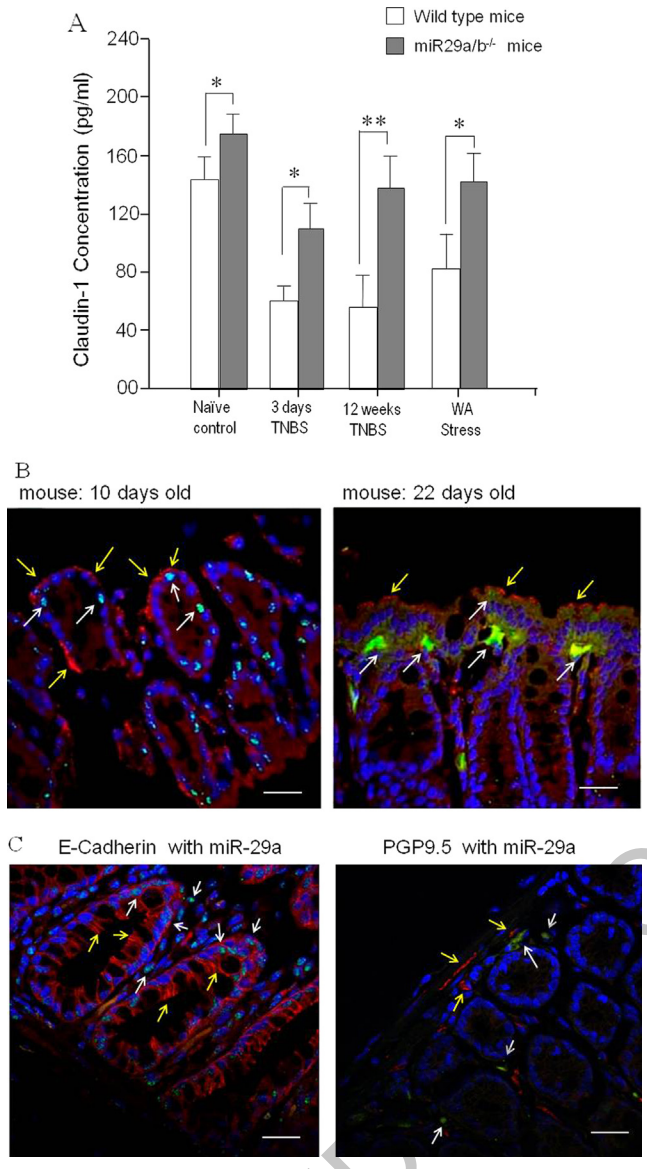


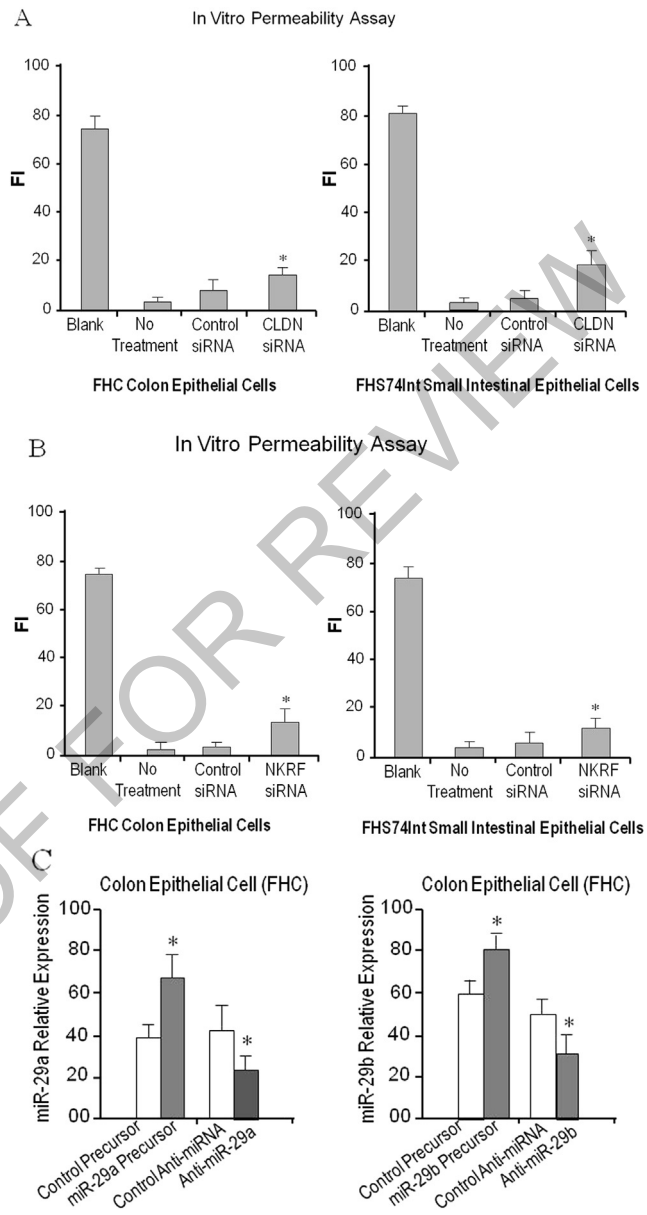
Supplementary Figure 2. (*A*) miR-29a expression in small intestine and colon from IBS-C patients and normal controls. (*B*) illustrates miR-29b expression in small intestine and colon from IBS-C patients and normal controls. There were no significant between-group differences.



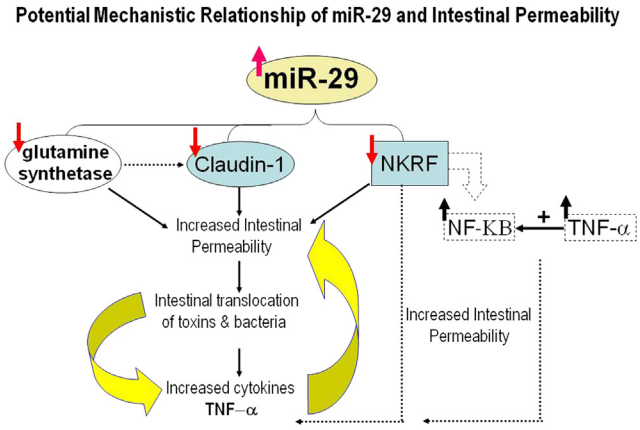
Supplementary Figure 3. (*A*, *B*) Changes in gene expression of miR-29a and miR-29b in different organs of miR-29a/b$^{-/-}$ mice. Target genes were investigated from 3 gastrointestinal organs of miR-29a/b$^{-/-}$ mice (*$P < .05$; **$P < .01$; ***$P < .001$). There are 2 of these 11 targets genes that are significantly increased in liver (*C*); 5 of 11 in colon (*D*); and 10 of 11 in small intestine (*E*).



Supplementary Figure 4. (*A*) Depicts CLDN1 expression in WT vs miR-29a/b$^{-/-}$ mice colon after TNBS or WA stress. There was a significant decrease in CLDN1 in WT mice after WA stress or 3 days and 12 weeks post TNBS (*$P < .05$; **$P < .01$) compared with miR-29a/b$^{-/-}$ mice. (*B*) Depict fluorescence in situ hybridization (FISH) in a 10-day-old mouse colon and a 22-day-old colon. *Yellow arrows* indicate CLDN1 expression and *white arrows* shows miR-29a expression. (*C*) FISH double-labeling with an epithelial marker, E-caderin (*left panel*) and a neuronal marker, PGP9.5 (*right panel*). *Yellow arrows* indicate E-cadherin and PGP9.5 expression and *white arrows* indicate miR-29a expression.



Supplementary Figure 5. In vitro permeability studies in which down-regulation of CLDN (*A*) and NKRF (*B*) increased epithelial cell permeability (*$P < .05$). Fluorescent intensities for negative control (baseline) and untreated epithelial cell permeability are shown along with siRNA control and siRNA CLDN and NKRF silencing group. (*C*) Real-time PCR data that show the constitutive expression of miR-29a (*left panel*) and miR-29b (*right panel*) in colon epithelial cells (FHC) (*$P < .05$).



Supplementary Figure 6. Working model for potential mechanistic relationship(s) between miR-29, NKRF, CLDN1, and glutamine synthetase. The hypothesized relationship between miR29, glutamine synthetase, and CLDN1 is depicted by *dashed arrows/line* (*left side*). Increased miR-29a causes decreased glutamine synthetase that may lead to decreased CLDN1 signaling, which may subsequently directly or indirectly lead to increased intestinal permeability through several potential mechanistic pathways. CLDN1 and NKRF are key elements that regulate intestinal permeability. The increased miR-29a/b cluster down-regulates both CLDN1 and NKRF (*solid line*) that leads to increased intestinal permeability. The increased intestinal permeability caused by tumor necrosis factor$-\alpha$ is due to activation of NF-κB expression and disassembling CLDN1 (*right side, dashed arrow/line*).