Commensal microbiota-induced microRNA modulates intestinal epithelial permeability through the small GTPase ARF4

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The intestinal tract contains many commensal bacteria that modulate various physiological host functions. Dysbiosis of commensal bacteria triggers dysfunction of the intestinal epithelial barrier, leading to the induction or aggravation of intestinal inflammation. To elucidate whether microRNA plays a role in commensal microbiome-dependent intestinal epithelial barrier regulation, we compared transcripts in intestinal epithelial cells (IECs) from conventional and germ-free mice and found that commensal bacteria induced the expression of miR-21-5p in IECs. miR-21-5p increased intestinal epithelial permeability and up-regulated ADP ribosylation factor 4 (ARF4), a small GTPase, in the IEC line Caco-2. We also found that ARF4 expression was up-regulated upon suppression of phosphatase and tensin homolog (*PTEN*) and programmed cell death 4 (*PDCD4*), which are known miR-21-5p targets, by RNAi. Furthermore, ARF4 expression in epithelial cells of the large intestine was higher in conventional mice than in germ-free mice. ARF4 suppression in the IEC line increased the expression of tight junction proteins and decreased intestinal epithelial permeability. These results indicate that commensal microbiome-dependent miR-21-5p expression in IECs regulates intestinal epithelial permeability via ARF4, which may therefore represent a target for preventing or managing dysfunction of the intestinal epithelial barrier.

Large numbers of commensal bacteria inhabit the intestinal tract and modulate various host physiological functions, as well as help digestion and the absorption of dietary components (1). Dysbiosis of the intestinal microbiota is involved in diverse diseases, such as inflammatory bowel disease (IBD)[2] and food allergies (2). These diseases are accompanied by excessive immune activation and disruption of mucosal barrier function, leading to the induction or aggravation of inflammation.

IECs, which play an important role in the absorption of nutrients, separate the intestinal lumen from the intestinal contents. IECs act as a physical barrier to prevent invasion of the intestinal contents, including pathogenic microorganisms. Intestinal epithelial permeability is controlled by tight junctions, which consist of firm contacts between neighboring IECs through cell surface proteins.

Disruption of the epithelial barrier enhances inflammatory responses via activation of the mucosal immune system, increasing the risk of IBD and autoimmune diseases (3). For instance, it has been suggested that the degradation of tight junction proteins on IECs by proteases from commensal bacteria is associated with Crohn's disease (4, 5). Thus, it is required to clarify the mechanism by which commensal bacteria affect and regulate the function of host IECs.

MicroRNAs (miRNAs) are small non-coding RNAs that are first synthesized from non-coding sequences of genomic DNA by RNA polymerase II or III as pri-miRNA. They are then processed by the ribonuclease Drosha into 70-nt-long pre-miRNAs and transported to the cytoplasm. The enzyme Dicer cleaves the pre-miRNAs to produce miRNA/miRNA duplexes. Then, the mature miRNAs are incorporated into RNA-induced silencing complexes that bind to complementary seed sequences in the 3′ untranslated regions of target mRNAs and either facilitate direct degradation of the mRNAs or inhibit their translation into proteins. Posttranscriptional suppression of target genes by RNA interference is involved in various biological processes and diseases. Previous reports have shown that miRNAs modulate the function of IECs. Studies using mice with IEC-specific deletion of Dicer 1 revealed that miRNAs in IECs play a critical role in the induction of the antiparasitic Th2 response (6). Although commensal bacteria have been reported to affect miRNA expression (7, 8), the molecular mechanisms underlying the regulation of IEC function by commensal bacteria through miRNAs have not been fully elucidated. In this study, we evaluated how commensal bacteria affect miRNA expression in IECs and regulate epithelial permeability, which may provide a novel target for the prevention and treatment of diseases related to epithelial barrier dysfunction.

[2] The abbreviations used are: IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; miRNA, microRNA; SIEC, small intestinal epithelial cell; LIEC, large intestinal epithelial cell; CV, conventional; GF, germ-free; P3CSK4, Pam3CSK4; BA, *Bacteroides acidifaciens* type A43; LJ, *Lactobacillus johnsonii* 129; TLR, Toll-like receptor; LNA, locked nucleic acid; TER, transepithelial electrical resistance; PTEN, phosphatase and tensin homolog; SPRY, Sprouty; ARF, ADP ribosylation factor.
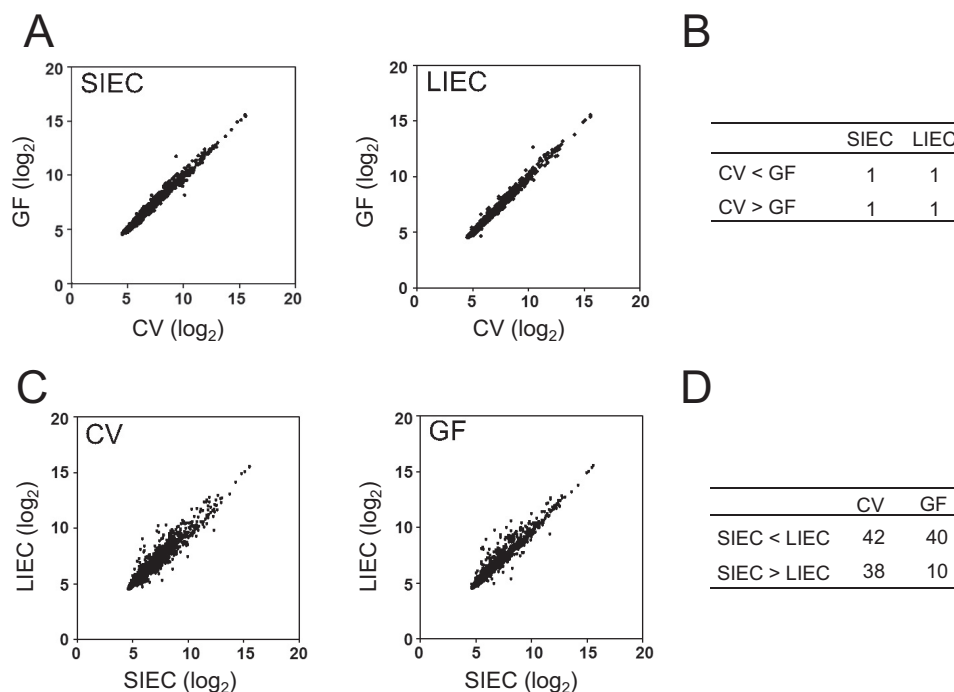
Figure 1. Commensal bacteria regulate miRNA expression in IECs. *A–D*, total RNA, including low-molecular-weight RNA, was prepared from the SIECs and LIECs of CV and GF mice. The expression of miRNA was analyzed by microarray testing. The plots compare the expression of miRNAs between CV and GF mice (*A*) or between SIECs and LIECs (*C*). The numbers of miRNAs with a more than 2-fold difference in expression are shown in *B* and *D*.

Results

Commensal bacteria regulate miRNA expression in IECs

First, we analyzed whether commensal bacteria affect miRNA expression in IECs. We comprehensively analyzed the expression patterns of miRNAs in small and large intestinal epithelial cells (SIECs and LIECs, respectively) from CV and GF mice by microarray. We found differentially expressed miRNAs in both the SIECs and the LIECs of CV *versus* GF mice (Fig. 1*A*). The number of miRNAs with a more than 2-fold change in expression in CV *versus* GF mice is shown in Fig. 1*B*. These results indicate that commensal bacteria modulate miRNA expression in IECs.

In addition, miRNA expression differed in the SIECs *versus* LIECs of all mice (Fig. 1*C*). The number of miRNAs with a more than 2-fold change in expression between SIECs and LIECs of the same mice was larger than that between CV and GF mice, indicating that miRNA expression was more dramatically affected by anatomical location than by the presence of commensal bacteria (Fig. 1*D*).

Commensal bacteria induce miR-21-5p expression in IECs

We next investigated the expression of miR-21-5p, which was identified as a potential microbiota-dependent miRNA by microarray analysis, in SIECs and LIECs of CV and GF mice by qRT-PCR. We found that miR-21-5p expression was higher in both SIECs and LIECs of CV mice compared with those of GF mice (Fig. 2*A*). These results confirmed that miR-21-5p expression in IECs was induced by commensal bacteria.

Furthermore, we investigated the regulatory mechanism of miR-21-5p expression in the human IEC lines HT-29, SW480, and Caco-2. We stimulated the cells with LPS, Pam3CSK4 (P3CSK4), and the heat-killed intestinal bacteria *Bacteroides*

acidifaciens type A43 (BA) and *Lactobacillus johnsonii* 129 (LJ). We found that the expression of miR-21-5p in HT-29 and SW480 cells was increased by all bacterial stimuli used in the experiment; neither the LPS nor the P3CSK4 responses were dose-dependent (Fig. 2, *B* and *C*). In contrast, miR-21-5p expression in Caco-2 cells, which show low responsiveness to bacterial stimulation because of minimal Toll-like receptor (TLR) expression (9), was unaffected by the bacterial stimuli (Fig. 2*D*). Treatment with anti-TLR4 antibody inhibited the LPS-induced expression of miR-21-5p ($p = 0.130$). In addition, the anti-TLR2 antibody partially inhibited the induction of miR-21-5p expression by P3CSK4 (supplemental Fig. S1). The partial effect of the anti-TLR2 antibody may be due to the concentration and/or affinity of the antibody. These results suggest that the induction of miR-21-5p expression by bacterial stimulation is mediated by TLRs.

miR-21-5p affects intestinal epithelial permeability but not IL-8 production from IECs

To clarify the role of miR-21-5p in IECs, we analyzed the effect of the miR-21-5p locked nucleic acid (LNA) inhibitor on intestinal epithelial permeability using filter-grown Caco-2 cells as an intestinal epithelial monolayer model. The transepithelial electrical resistance (TER) of Caco-2 cells treated with miR-21-5p LNA inhibitor was significantly greater than the TER of cells treated with the control LNA (Fig. 3*A*).

We next evaluated the effect of the miR-21-5p inhibitor on IL-8 production by SW480 cells upon stimulation with LPS. IL-8 production in the culture supernatants of miR-21-5p inhibitor- and control LNA-transduced cells was similar (Fig. 3*B*). These results indicate that miR-21-5p in IECs is involved in
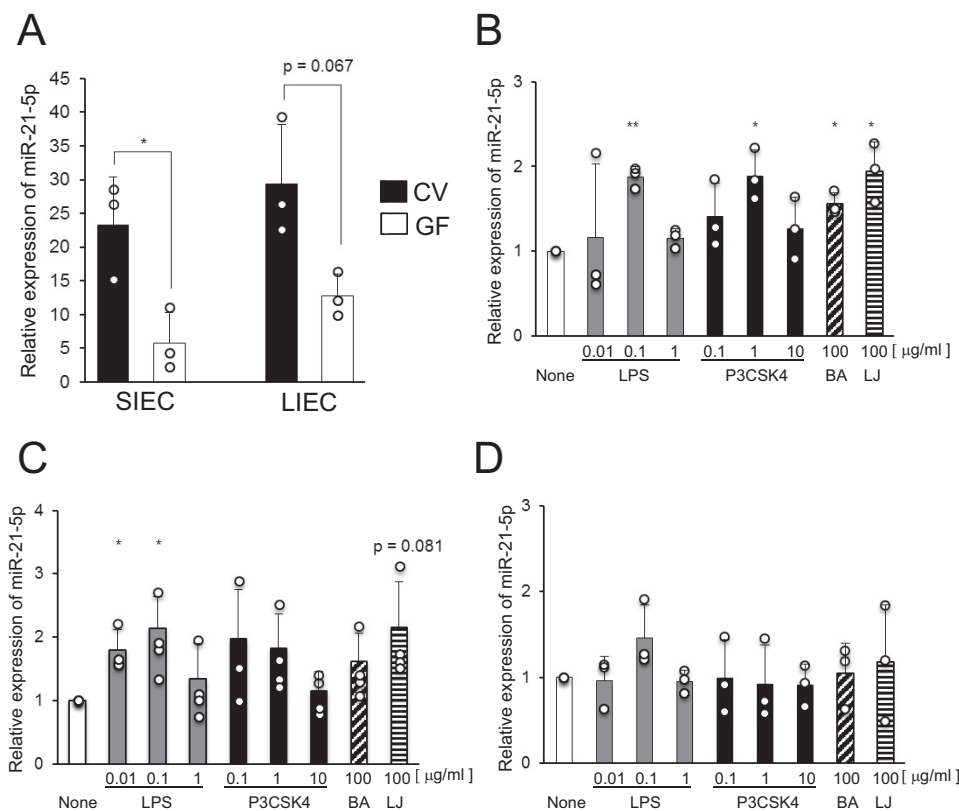
Figure 2. miR-21-5p expression in IECs is induced by bacterial components. *A*, miR-21-5p expression in SIECs and LIECs of CV and GF mice was analyzed by qRT-PCR. Data are expressed as mean ± S.D. of three independent experiments. *B–D*, IEC lines, HT-29 (*B*), SW480 (*C*), and Caco-2 (*D*), were stimulated with LPS, P3CSK4, and heat-killed BA and LJ for 24 h. miR-21-5p expression was analyzed by qRT-PCR. Relative values compared with cells without bacterial stimulation are shown as mean ± S.D. of three (HT-29 and Caco-2) or four (SW480) independent experiments. *, $p < 0.05$; **, $p < 0.01$ (*versus* cells without stimulation).

the regulation of intestinal epithelial permeability, but not IL-8 production upon bacterial stimulation.

miR-21-5p increases intestinal epithelial permeability via ARF4

To identify the mechanisms underlying the modulation of intestinal epithelial permeability by miR-21-5p, a proteome analysis was performed on cells treated with miR-21-5p LNA inhibitor or control LNA. Several spots showed markedly different expression in the two groups, indicating that miR-21-5p regulates the expression of specific proteins. One of these spots was identified by MS-MS analysis as ARF4, which was deemed a candidate target molecule of miR-21-5p in IECs as it had 9-fold higher expression in the presence of miR-21-5p inhibitor than in the presence of the control (Fig. 4*A*). *ARF4*-specific and control siRNAs were then introduced into Caco-2 cells to analyze the role of ARF4 in controlling intestinal epithelial permeability. We confirmed that the expression of ARF4 was decreased by siRNA (Fig. 4*B*). Unexpectedly, the TER of Caco-2 monolayers treated with *ARF4* siRNA was significantly higher than the TER of cells treated with control siRNA (Fig. 4*C*). Because the expression of ARF4 and miR-21-5p appears to increase intestinal epithelial permeability, it is possible that ARF4 is under the control of miR-21-5p but not directly down-regulated by this miRNA. In addition, the tight junction–related proteins claudin-4 and occludin were up-regulated in cells treated with *ARF4* siRNA compared with those treated with control siRNA (Fig. 4*D*). These results indicate that ARF4

increases intestinal epithelial permeability via suppression of tight junction–related proteins.

Next, we analyzed the expression of ARF4 in SIECs from the proximal, medial, and distal portions of the small intestine and LIECs of CV and GF mice by Western blotting. ARF4 expression was higher in LIECs of CV mice than in those of GF mice, but it was comparable in SIECs of CV *versus* GF mice, indicating that ARF4 may be induced in LIECs by the microbiota (Fig. 4*E*). The finding that both ARF4 and miR-21-5p were induced by the microbiota in LIECs also supports the hypothesis that ARF4 is not a direct target of miR-21-5p. Furthermore, we did not find target sequences in the 3′ region of the *ARF4* gene using TargetScan (http://www.targetscan.org/vert_71/)[3] (33). Taken together, these data indicate that ARF4 in IECs increases intestinal epithelial permeability in a miR-21-5p-dependent manner, although it is not likely to be a direct target of this miRNA.

ARF4 is regulated by known targets of miR-21-5p: PTEN and PDCD4

Previous reports have shown that miR-21 targets *PTEN*, *PDCD4*, and *Sprouty* (SPRY) 1 and 2 (10–13), which are known to be negative regulators of the PI3K–Akt, JNK–AP-1, and Erk signaling pathways, respectively (14–16). These pathways regulate intestinal epithelial permeability (17–19). We hypothe-

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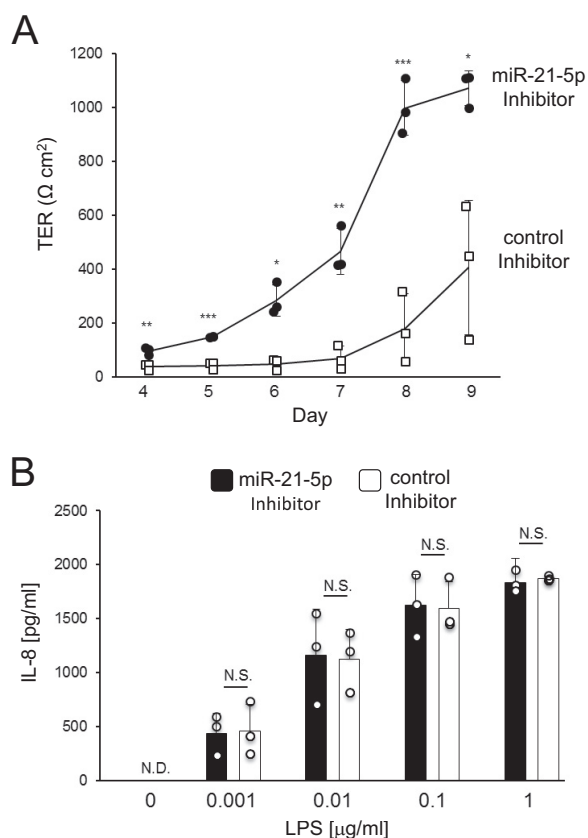
Figure 3. miR-21-5p affects intestinal epithelial permeability but not IL-8 production by IECs. *A*, Caco-2 cells were seeded on transwell inserts on day 0 and treated with miR-21-5p inhibitor on day 1. TER values were measured on days 4 through 9. The results are expressed as mean ± S.D. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$ (*versus* control). *B*, SW480 cells were transduced with the miR-21-5p inhibitor. Cells were then stimulated with 0.001–1 μg/ml LPS for 24 h, and the IL-8 concentration in the culture supernatant was measured by ELISA. The results are expressed as mean ± S.D. of three independent experiments. *N.D.*, not detected; *N.S.*, not significant (*versus* control).

sized that miR-21-5p targets *PTEN*, *PDCD4*, *SPRY1*, and *SPRY2* and activates these pathways to induce ARF4 in IECs, increasing intestinal epithelial permeability. To test this hypothesis, we examined the effects of *PTEN*, *PDCD4*, *SPRY1*, and *SPRY2* siRNAs on ARF4 expression in Caco-2 cells. We found that expression of the mRNA for *PTEN*, *PDCD4*, *SPRY1*, and *SPRY2* was decreased by the relevant siRNA (Fig. 5*A*). The expression of ARF4 was significantly increased in cells treated with *PTEN* and *PDCD4* siRNAs compared with those treated with control siRNA (Fig. 5*B*). *ARF4* expression was only slightly increased in cells treated with *SPRY1* and *SPRY2* siRNAs. This may be due to the reduced efficacy of knockdown by these siRNA constructs compared with the *PTEN* and *PDCD4* siRNA (Fig. 5*A*). Furthermore, the expression of ARF4 was significantly lower in cells treated with an Akt or JNK inhibitor than in control cells (Fig. 5*C*). These results suggest that down-regulation of PTEN and PDCD4 by miR-21-5p induces ARF4 expression through the Akt and JNK pathways, which results in increased intestinal epithelial permeability.

Discussion

Intestinal commensal microbiota play an important role in the maintenance of host intestinal epithelial barrier functions.

In contrast, dysfunction of the intestinal epithelial barrier is often associated with the development of various diseases. In this study, we demonstrated the molecular mechanisms underlying the regulation of the intestinal epithelial barrier by commensals via miR-21-5p and ARF4.

We found that miRNA expression in IECs was affected by the presence of the commensal microbiota. The effects of commensal bacteria on intestinal miRNA expression have also been shown by Singh *et al.* (8) using cecal tissue sections from CV and GF mice and by Dalmasso *et al.* (7) using ilea and colons from GF and microbiota-transferred mice. Singh *et al.* (8) found 16 miRNAs, whereas Dalmasso *et al.* (7) found nine miRNAs that were differentially expressed depending on commensal bacteria. Our data clearly showed that the expression of miRNAs in murine IECs is affected by the commensal microbiota, using purified primary IECs. We also found that miRNA expression in IECs was influenced not only by the presence of commensal bacteria but also by location. Lee *et al.* (20) similarly observed differential miRNA expression in SIECs and LIECs in a mouse colitis model. The physiological significance of this difference is not fully understood, but it may reflect the differences in the structures and functions of the small and large intestinal epithelia.

The expression of miR-21, one of the most highly conserved miRNAs in mammals, was induced by commensal microbiota. Its microbiota-induced expression may be mediated by TLRs, although the effects of the anti-TLR antibodies were not statistically significant. However, a previous report has shown that miR-21 is induced by LPS in a MyD88-dependent manner (21). Recently, it has been reported that miR-21 is highly expressed in inflamed tissues in diseases such as cancer and IBD (22, 23). Because these diseases are known to be associated with dysbiosis of intestinal microbiota, our results suggest that miR-21-5p may become a useful marker for inflammatory diseases associated with intestinal dysbiosis.

Although miR-21-5p is expressed at a physiological level in IECs of CV mice, it does not induce severe inflammation under steady-state conditions. Inflammation is likely to be induced when this miRNA is excessively expressed upon disruption of intestinal symbiosis or infection with pathogenic microbes. In fact, miR-21 was reportedly induced by pathogenic microbes such as *Helicobacter pylori*, *Salmonella* Typhimurium, and *Mycobacterium* species, leading to excessive immune responses (24, 25). Neither LPS nor P3CSK4 acted in a dose-dependent manner, suggesting that cellular mechanisms suppress the excessive induction of miR-21-5p. It is possible that lower concentrations of the ligands induce miR-21-5p in a dose-dependent manner. Microbiota-dependent miRNAs, including miR-21-5p, will become targets for the maintenance of intestinal homeostasis or its restoration following dysbiosis.

We demonstrated that intestinal microbiota-dependent miR-21-5p expression modulates intestinal epithelial permeability. Similarly, miR-21 enhances TNF-α–mediated increases in intestinal epithelial permeability (26). Recently, miR-122 and miR-29 were shown to increase intestinal epithelial permeability by targeting the transcripts for occludin and claudin-1, respectively (27, 28). Our results revealed that a microbiota-induced specific miRNA increases intestinal epithelial perme-
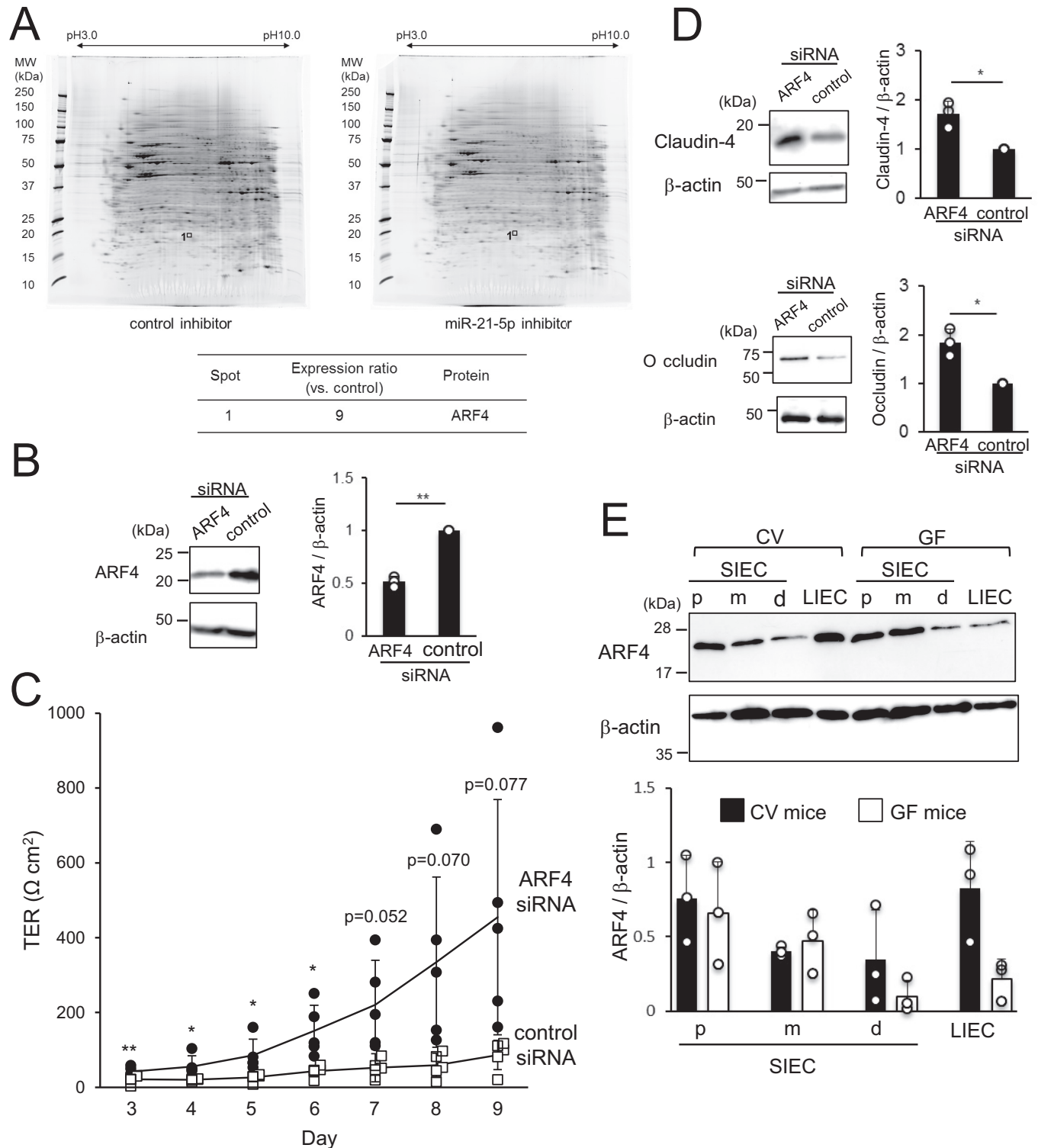
Figure 4. miR-21-5p increases intestinal epithelial permeability via ARF4. *A*, protein expression was compared in Caco-2 cells treated with miR-21-5p LNA inhibitor and control LNA by 2D gel electrophoresis. The spots that were differentially expressed in the two groups were subjected to MS-MS analysis. The spots corresponding to ARF4 are indicated. *B* and *D*, ARF4 siRNA was introduced into Caco-2 cells. After culture for 72 or 120 h, the expression of ARF4 (72 h), claudin-4 (120 h), occludin (120 h), and β-actin (72 and 120 h) was analyzed by Western blotting. Representative blots (*left panel*) and the mean ± S.D. of the relative band intensities normalized to β-actin (*right panel*) of three independent experiments are shown. *, $p < 0.05$; **, $p < 0.005$ (*versus* control). *C*, Caco-2 cells were seeded on transwell inserts on day 0, and ARF4 siRNA was introduced into the cells on day 1. TER values were measured on days 3 through 9. The results are expressed as mean ± S.D. of five independent experiments. *, $p < 0.05$; ** $p < 0.005$ (*versus* control). *E*, ARF4 expression in proximal (*p*), medial (*m*), and distal (*d*) SIECs and LIECs from CV and GF mice was analyzed by Western blotting. Representative blots (*top panel*) and mean ± S.D. of the relative band intensities normalized to those of β-actin (*bottom panel*) of three independent experiments are shown. *, $p < 0.05$.
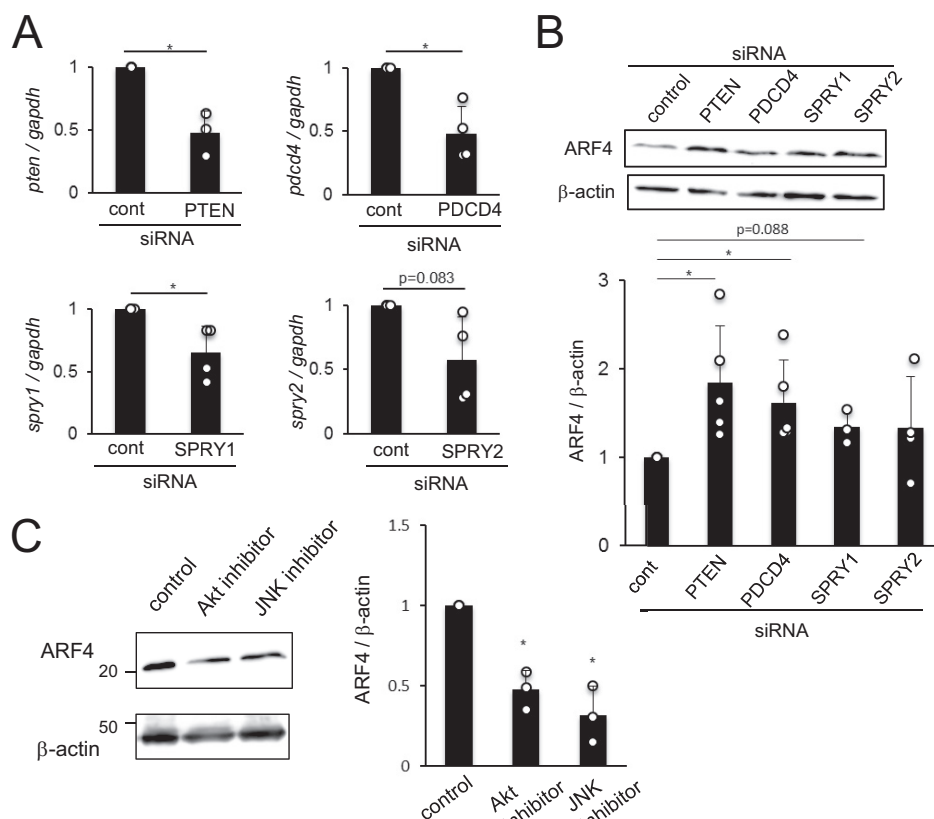
Figure 5. ARF4 is regulated by PTEN and PDCD4, targets of miR-21-5p. *A*, Caco-2 cells were treated with siRNAs to *PTEN*, *PDCD4*, *SPRY1*, or *SPRY2*. After culture for 48 h, the expression of *PTEN*, *PDCD4*, *SPRY1*, and *SPRY2* mRNA was analyzed by qRT-PCR. Expression relative to *GAPDH* is expressed as the mean ± S.D. of three (*PTEN*) and four (*PDCD4*, *SPRY1*, and *SPRY2*) independent experiments. *cont*, control. *B*, Caco-2 cells were treated with siRNAs to *PTEN*, *PDCD4*, *SPRY1*, or *SPRY2* and cultured for 72 h. ARF4 expression was analyzed by Western blotting. Representative blots (*top panel*) and mean ± S.D. of the relative band intensities normalized to those of β-actin (*bottom panel*) of three (*SPRY1* siRNA), four (*SPRY2* siRNA), or five (*PTEN* and *PDCD4* siRNA) independent experiments are shown. *C*, Caco-2 cells were treated with Akt or JNK inhibitor. After 48 h of culture, ARF4 expression was analyzed by Western blotting. Representative blots (*left panel*) and relative band intensities normalized to those of β-actin (*right panel*) of three independent experiments are shown. *, *p* < 0.05 (*versus* control).

ability. Thus, microbiota-targeted intervention may help to recover or control intestinal epithelial permeability. Probiotic bacteria contribute to the regulation of intestinal barrier function (29), which could be mediated by miRNA.

Our findings demonstrated that ARF4 was controlled by miR-21-5p and modulated intestinal epithelial permeability through the regulation of tight junction proteins such as claudin-4 and occludin. Because proteome analysis revealed that ARF4 expression was increased by the miR-21-5p inhibitor, we hypothesized that ARF4 is a direct target of miR-21-5p, which decreases intestinal epithelial permeability. However, we found that ARF4 was an indirect target; its expression was controlled by the direct targets of miR-21-5p, PTEN and PDCD4, and it increased intestinal epithelial permeability. We cannot explain this discrepancy at present, but it may involve the side effects of other miR-21-5p targets. We finally concluded that miR-21-5p targets PTEN and PDCD4 to suppress their expression, up-regulating ARF4 via the Akt and JNK pathways, and, as a result, increasing epithelial permeability. Because it was difficult to directly show the ARF4-dependence for miR-21-5p–mediated control of epithelial permeability in our experimental system, we cannot exclude the possibility that there is another mechanism independent of ARF4 by which miR-21-5p increases epithelial permeability in addition to the ARF4-dependent mechanism.

ARF belongs to the Ras superfamily of small GTPases, which play important roles in various biological and cellular processes (30). Some small GTPases are known to control intestinal function and homeostasis. For instance, knockdown of ARF6 suppresses *Escherichia coli* internalization by the human IEC line T84 (31). On the other hand, PTEN, PDCD4, SPRY1, and SPRY2 are repressors of the Ras signaling pathway (14–16), which is involved in the regulation of intestinal epithelial permeability (17–19). This is the first report to clarify the role of ARF4 in the control of intestinal epithelial permeability as well as the regulation of ARF4 expression by PTEN and PDCD4. Regulation of intestinal epithelial permeability by tight junction proteins is a major mechanism to prevent excessive inflammatory responses and maintain intestinal homeostasis. The growing literature on the mechanisms underlying the regulation of intestinal epithelial permeability may help identify novel targets to treat inflammatory diseases related to intestinal epithelial barrier dysfunction. Further studies *in vivo* and *in vitro* will elucidate the interactions between commensal microbiota and their hosts.

Experimental procedures

Mice

BALB/c mice were purchased from CLEA Japan (Tokyo, Japan) and bred under CV conditions. Autogenous GF mice

were bred and housed in our GF facilities at the College of Bioresource Sciences, Nihon University. Mice were maintained in a temperature-controlled room with a 12-h light/dark cycle with free access to food and water. Female mice were used at 10–12 weeks of age. All experiments were approved by the Nihon University Animal Care and Use Committee and conducted in accordance with their guidelines.

Preparation of murine IECs

IECs from the small and large intestines of mice were prepared as described previously (32).

Human IEC lines

The human epithelial colonic adenocarcinoma cell lines HT-29 and Caco-2 were purchased from DS Pharma Biomedical (Osaka, Japan). The human epithelial colonic adenocarcinoma cell line SW480 was provided by the Cell Resource Center for Biomedical Research (Institute of Development, Aging, and Cancer, Tohoku University, Miyagi, Japan). Cells were cultured as described in a previous report (9).

Microarray analysis

Total RNA, including low-molecular-weight RNA, was isolated from IECs using the High Pure miRNA isolation kit (Roche). The expression of miRNA was evaluated by miRCURY LNA™ microRNA array (Exiqon, Vedbaek, Denmark) after analysis on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

Preparation of heat-killed bacteria

BA and LJ were isolated from murine intestinal commensal bacteria and kindly provided by Dr. Itoh (University of Tokyo). BA and LJ were cultured in EG broth supplemented with 0.01% menadione and 0.1% hemin or de Man, Rogosa, and Sharpe (MRS) broth, respectively, for 48 h at 37 °C under anaerobic conditions. The bacteria were collected by centrifugation, washed three times with sterilized water, heated at 80 °C for 50 min, and lyophilized.

Stimulation of IEC lines with bacterial ligands

The human IEC lines Caco-2, HT-29, and SW480 were stimulated with 0.01–1 μg/ml ultra-pure *E. coli* K12 LPS (Invivogen, San Diego, CA), 0.1–10 μg/ml P3CSK4 (Invivogen), and 100 μg/ml heat-killed BA and LJ. After 24 h of culture, cells were collected to extract total RNA, including low-molecular-weight RNA, to quantify miR-21-5p expression. Alternatively, for the measurement of IL-8 production, SW480 cells were stimulated with 0.001–1 μg/ml LPS for 24 h.

Introduction of miRNA inhibitor and siRNA

The miR-21-5p LNA inhibitor or control LNA (Exiqon) and ARF4, PTEN, PDCD4, SPRY1, SPRY2, or control siRNA (Ambion) (all 50 nM) were introduced into cells using X-tremeGENE™ HP DNA transfection reagent (Roche).

Treatment with Akt and JNK inhibitors

The Akt inhibitor LY-294002 (Wako Pure Chemical Industries, Osaka, Japan) and JNK inhibitor SP600125 (Wako Pure

Chemical Industries) were added to cells at a concentration of 50 μM for 48 h.

Quantitative RT-PCR (qRT-PCR)

The protocols for reverse transcription and quantitative analysis of mRNA or miRNA expression by qPCR were described in a previous report (32). We normalized mRNA expression to that of *GAPDH*. Although miRNA expression is usually normalized to U6 or RNU1a1, they were not suitable for this experiment because the miRNA expression of the IECs varied markedly depending on their differentiation status. Therefore, we determined relative expression values using equal amounts of RNA. The nucleotide sequences of the primers used for qPCR are shown in supplemental Table S1.

Measurement of TER

Caco-2 cells were seeded on transwell inserts, and their TER was measured using a Millicell ERS voltohmmeter (Millipore, Darmstadt, Germany). To calculate the TER, we subtracted the resistance of a blank well from the resistance of the sample wells and then multiplied the value by the area of the insert membrane.

Measurement of IL-8 production

The IL-8 concentration in the culture supernatants was measured by human CXCL8/IL-8 immunoassay (R&D Systems Biotech, Minneapolis, MN), according to the instructions of the manufacturer.

Western blotting

Cell lysates were prepared as described previously (32). We used primary antibodies against ARF4 (Proteintech), claudin-4 (Santa Cruz Biotechnology, Dallas, TX), occludin (Abcam, Cambridge, UK), and β-actin (Abcam).

Proteome analysis

Proteome analysis was performed by Takara Bio (Shiga, Japan). Briefly, we compared protein expression in Caco-2 cells treated with the miR-21-5p LNA inhibitor and those treated with control LNA by 2D electrophoresis followed by silver staining. Spots with substantial differences in intensities in inhibitor- and control-treated samples were collected to perform in-gel trypsin digestion and MALDI-TOF/TOF analysis using a 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA).

Statistical analysis

Statistical analyses were performed using two-tailed Student's *t* test.

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